Higher order chromosome organization and recombination dynamics of meiotic prophase I in mouse spermatocytes

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HIGHER ORDER CHROMOSOME ORGANIZATION AND RECOMBINATION DYNAMICS OF MEIOTIC PROPHASE I IN MOUSE SPERMATOCYTES

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HIGHER ORDER CHROMOSOME ORGANIZATION AND RECOMBINATION DYNAMICS OF MEIOTIC PROPHASE I IN MOUSE SPERMATOCYTES

A

DISSERTATION

Presented to the Faculty of

The University of Texas
MD Anderson Cancer Center UTHealth
Graduate School of Biomedical Sciences

in Partial Fulfillment
of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

by

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May, 2019
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my mentor Dr. Francesca Cole for her support, guidance, enthusiasm and scholarship to help me get through the last six years of my graduate career. None of this work would have been possible without her amazing support. Under her mentorship, I learned how to give great presentations, how to write clearly and how to think to solve problems. She also gave me the opportunity to expand my knowledge, meet great scientists and establish productive collaborations with them. She was always available for help and discussion regarding my projects, and she has been an excellent teacher and a mentor during my graduate ventures. Her standards for academic excellence propelled me to become a better version of myself, both as a scientist and as a person. It is truly an honor to have been her first graduate student. I have learned so much from her. I know for a fact that all the valuable trainings I obtained from her will benefit me in whatever I do in the future, and I am forever grateful for everything you have done for me.

I would also like to thank all the past and present members of the Cole laboratory: Maria Sandoval, Lakshmi Paniker, Holly Hamilton-Nguyen, Yunfu Lin, Joanna Baird, April Weiss, Tolkapiyan Premkkumar, Isabella Ferranti, Ericka Humphrey and Melissa Frasca. I would not have enjoyed my Ph.D. training as much as I did without these fun people. Not only were they great to work with but they also taught me what an ideal team work environment is. I can’t thank them enough for being there for me in times of good and bad to support and help me.

My committee members were also a great part of my journey. I’d like to thank Dr. Richard Wood for all the great letters he has written me, Dr. Swathi Arur for being there for me when I nearly broke down during my preparation for candidacy exam, and Dr. Taiping Chen and Dr. Kevin McBride for their additional support and advice. I also want to thank Dr. David Johnson and Dr. Shawn Bratton for their support during my candidacy exam.

I wish to thank Mathilde Biot, an exchange student who I mentored for six months. Thank you for all the experiments you did for me. Not only did you help me with my project, I also learned a lot from your enthusiasm and questions. It was truly fun teaching you.
Additionally, I’d like to thank my Science Park community. Pam Whitney for running my samples on flow cytometry, all the members of our animal facility for taking care of all the mice, Becky Brooks for being a wonderful program coordinator for students, Laura Denton for everything she does, Brian Cunningham for solving all of my computer issues, Dr. Ellen Richie who gave me the opportunity to work under her as a research assistant and helped me grow my desire to pursue Ph.D., and finally, my fellow students Aimee Farria, Sara Martin, Junji Chen, Tolkappiyam Premkumar, Amelie Albrecht and Scott Casey. It was a lot of fun spending time with you guys at the retreats, trivia, movie nights, restaurants and fun events in Austin. I will miss everyone and everything that was Science Park.

Lastly, I’d like to thank my family and friends. My parents, brother, sister-in-law and nephew were a big part of my endeavor and their support was bigger than they can imagine. I would not have been able to go through this without them. I thank my grandfather, who I know would be very happy and proud to know that I finally did it. And I want to thank my friends who were there for me since high school: Joyce Tao, Michelle Kim-Wallace, Joowon Cho and Yoolee Kwon. Joyce, I’d like to take a special thank you for teaching me how to use Illustrator but most importantly, for being there for me to listen to my struggles and problems that I faced during this journey. I love you all.
Meiotic recombination is required for parental chromosomes to find each other (pairing/synapsis) and to exchange genetic information thus allowing faithful segregation of chromosomes and the production of haploid gametes. At the start of meiotic prophase I, meiotic chromosomes organize into loop arrays that extrude out of the chromosome axis. Then, a large number of programmed double-strand breaks (DSBs) are formed at specific chromosomal locations or "hotspots" on parental chromosomes, which are repaired by homologous recombination (HR). HR produces either crossovers, which result in the exchange of flanking markers between homologs, or noncrossovers, which are short regions of gene conversion to the donor genotype. Crossover formation is critical for proper chromosome segregation and crossovers arise from crossover precursors that form at a subset of DSBs that are designated to become future crossovers. Our current understanding of meiotic progression in mammals is largely derived from cytological observation. Many semi-redundant HR pathways can repair meiotic DSBs; however, the time at which different pathways are active, how the pathways interact, and the relative contribution of each pathway towards maintaining germline genomic integrity are poorly understood in vivo at endogenous sites, especially in a mammalian system. More importantly, how germline genome integrity is ensured at both the DNA level by recombination activity and by higher order chromosome structural changes has not been defined. Failure to maintain germline genome integrity can lead to aneuploidy, genetic disorders, birth defects and miscarriages.

To define and dissect the temporal dynamics of different HR pathways and chromosome organization in vivo, I have established a novel and robust system to synchronize mouse spermatogenesis in F1 hybrid mice using the inhibitor WIN 18,446. My
synchronization protocol allows the isolation of cells at specific stages of meiotic prophase I by flow cytometry, allowing me to analyze recombination outcomes at two meiotic hotspots and perform genome-wide Hi-C, a chromosome conformation capture method combined with high-throughput sequencing to investigate changes in higher order chromosome architecture during prophase I.

Here, I provide the first direct molecular evidence that HR pathways that lead to distinct meiotic outcomes are temporally regulated. I have identified two novel classes of noncrossover pathways: 1) one that likely regulates the pairing/synapsis of parental chromosomes during early prophase I; and 2) one that derives from the crossover/noncrossover decision during mid-prophase I. My data show that crossover formation is suppressed until full synapsis is achieved at mid-prophase I, suggesting a previously unknown mechanism that prevents deleterious premature recombination. In addition, I show that alternative repair pathways are not activated until late prophase I, thus preventing designated crossover precursors from inappropriately forming noncrossovers.

Furthermore, the Hi-C data I present provides evidence for dynamic genome reorganization during meiotic prophase I. There is evidence for loop array formation and loop extrusion as chromosomes condense. While topologically associating domains disappear at the onset of meiotic prophase I, chromosome compartments are well maintained. Most meiotic DSBs occur within a gene-dense open compartment A, suggesting that higher order chromosome structure plays an important role in meiotic recombination. Finally, interhomolog interactions and specialized chromosomal architecture in regions of pairing and synapsis could be inferred. Taken together, my data reveals that both chromosome recombination and chromosome structure are highly regulated to ensure chromosome pairing and segregation. These results provide important, novel insights to the field of meiosis and our understanding of germline genomic integrity and mammalian reproductive health.
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Chapter 1: Introduction

1.1 Why is meiosis important?

Meiosis is a specialized cell division program that occurs only in sexually reproducing organisms. Normal diploid cells contain two sets of chromosomes - one set of chromosomes received from the father and another received from the mother. The purpose of meiosis is to produce haploid gametes containing only one set of chromosomes from diploid parental cells. This takes places through two rounds of cell division that ultimately produce sperm in males and eggs in females. Thus, when fertilization occurs, the diploid status is restored, with one set from each parent, allowing a new diploid organism to form. Importantly, the haploid gametes produced during meiosis are genetically distinct from both each other and the diploid parent. As a result, meiosis produces genetic diversity.

As mentioned, normal diploid cells contain two sets of chromosomes, a maternal set and a paternal set. Thus, each chromosome has a complement (homolog) that was derived from the other parent. Different organisms have different numbers of chromosome sets, i.e. humans have 23 sets of chromosomes, whereas mice have 20 sets of chromosomes. When a cell has an abnormal number of chromosomes, it results in a condition called aneuploidy (1).

Fertility and reproductive health rely heavily upon faithful meiosis. Errors in meiosis can result in aneuploid gametes, which can lead to miscarriage or offspring with developmental defects and/or mental retardation. A well-known example is Down syndrome that arises from having an extra copy of chromosome 21 (2). Another example is trisomy 16, a major cause of miscarriage in humans (3).

Meiotic processes and mechanisms show remarkable diversity across species. In this introduction, I will focus primarily on mouse meiosis but will also discuss how meiosis is differentially regulated at the cellular and molecular level in other model organisms and how we can exploit the knowledge gleaned from these models to enhance our understanding of mammalian meiosis, particularly in humans.
1.1.2 Cell division during meiosis

Meiotic cell division shares many similarities with mitotic cell division, but has several striking differences. Unlike mitotic cells, meiotic cells undergo two successive rounds of cell division. The first meiotic division, meiosis I, is a reductional division of the homologs, whereas the second meiotic division, meiosis II is an equational division that produces haploid gametes containing only half of the parental number of chromosomes (4) (Fig. 1). Although meiosis I deviates considerably from mitosis, the division process of meiosis II closely resembles mitotic cell division.

The cell division process during mitosis is relatively short. Both mitosis and meiosis begin from diploid precursors with two homologous copies of chromosomes (homologs). In mammals and many organisms, the chromatid complement is 2C and the cells are diploid or 2N. Prior to entering mitosis, interphase somatic cells undergo one round of DNA replication during S phase to produce sister chromatids. These sister chromatids are attached to each other by cohesion proteins called cohesins, resulting in a total of 4 chromatids per chromosome (4C, 2N). As cells enter prophase, chromosomes condense, duplicated centrioles move to the opposite poles of the cell, and the nuclear envelope disintegrates. Subsequently, spindles form and extend from the opposite poles of the cell and attach to each chromosome via its kinetochore and align chromatid pairs along the metaphase plate. During anaphase, cohesins are cleaved and sister chromatids segregate to the opposite poles, such that each pole will contain a 2N number of chromosomes. At telophase, chromosomes decondense, the nuclear envelope reassembles and two cell nuclei are produced. Following telophase, cytokinesis occurs to produce two interphase daughter cells that are identical to the parental cell (2C, 2N).

Like mitotic cells, meiotic cells undergo one round of DNA replication (4C, 2N). However, as cells enter prophase I of meiosis, a large number of programmed double-strand breaks (DSBs) form throughout the genome. Then, exchange of genetic information between the paternal and the maternal chromosomes occurs, producing crossovers, which are the
Figure 1. Mitosis vs Meiosis.

Left: Mitotic cells undergo one round of cell division to produce two identical daughter cells (2C, 2N). Right: Meiotic cells undergo two rounds of cell division to produce haploid gametes (1C, 1N). Cohesins are lost at crossover sites. At the beginning of meiosis II, cohesins only remain at centromeric regions. Paternal chromosome: blue; maternal chromosome: red; cohesins; gray.
reciprocal exchange of entire chromosome arms. Formation of crossovers between the homologs requires homologous recombination. This physical connection, resulting from crossovers, can be detected during metaphase I as bivalent cruciform structures, called chiasmata. In order for chromosomes to properly segregate, the spindle microtubules must attach to the kinetochore. The spindle assembly checkpoint ensures the attachment by sensing tension at the kinetochore. During mitosis, this tension is provided by sister cohesins that hold the sister chromatids together. In contrast, during meiosis, crossovers formed between the homologs in conjunction with sister chromatid cohesion provide the physical connection necessary to supply sufficient tension. Therefore, crossover formation must occur for accurate chromosome segregation. After prophase I, chromosomes continue to condense and align at the metaphase plate before they divide during anaphase I and telophase I to produce 2C, 1N products. By the end of meiosis I, cohesins remain only at the centromeres. Following meiosis I, the cells enter meiosis II. Meiosis II, like mitosis provides a mechanism for the cells to segregate sister chromatids to the opposite poles; however, in this case, 1C, 1N haploid gametes, rather than diploid cells are the end product (1). My thesis focuses on prophase I during meiosis I, the time when homologous recombination and crossover formation occur.

1.2 Prophase I at the cellular level

1.2.1 Synaptonemal Complex

Changes in chromosome structure and behavior during meiotic prophase I have been extensively studied by cytologic observation in multiple organisms (4). Prophase I is divided into four substages based upon the morphology of the synaptonemal complex: leptonema, zygonema, pachynema and diplonema (1) (Fig. 2). During leptonema, chromosome organization is identical to that observed during mitotic prophase (5). As the chromosomes condense, sister chromatids organize into an array of DNA loops that protrude from a proteinaceous axis formed by proteins known as axial elements. Loops extrude from the axis in all directions, creating a structure resembling a bottle brush. The DNA loops are held by condensins and cohesins, which also participate in chromosome condensation (4, 6, 7).
Figure 2. The structure of the synaptonemal complex.


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Further condensation and axial element assembly occur at zygonema, as do two other major processes: 1) the pairing of homologous chromosomes, defined as an apposition of homologous chromosomes ~400 nm apart, and 2) initiation of synapsis by laying down the transverse element proteins of the synaptonemal complex (8). Synapsis is the formation of a physical connection between homologs via synaptonemal complex proteins. By pachynema, complete synapsis is achieved between the homologs, and at diplonema, chromosomes desynapse by disintegrating synaptonemal complex proteins to allow chromosomes to compact and segregate.

The structure and the role of the synaptonemal complex is highly conserved (4). The synaptonemal complex is created by an array of proteins that form a ~100 nm-wide bridge-like “zipper” structure that physically links homologous chromosomes (9). The synaptonemal complex comprises three components: the lateral element (LE), central element (CE) and transverse element (TE). The proteinaceous axial element that forms at the start of leptonema becomes integrated into the LE of the synaptonemal complex (8) (4). As homologs pair and align in close proximity during zygonema, the LE of the homologs becomes physically connected by TEs that extend perpendicularly from the homolog axis. These TEs overlap and connect at the CE structure. Synapsis continues along the length of the homologs and is complete at pachynema.

Mouse LE proteins include SYCP2 and SYCP3, with the latter being used extensively for cytological delineation of meiosis-specific axis formation. SYCP1 is a notable CE protein that is required for proper assembly of the CE. By observing the localization of SYCP1 and SYCP3 via indirect immunofluorescence (IF) staining of chromosome surface spreads, one can determine how far synapsis has progressed: overlap between SYCP1 and SYCP3 indicates that synapsis has occurred in that region, and complete overlap between SYCP1 and SYCP3 on all chromosomes can be observed during pachynema.

The pivotal role of the synaptonemal complex during meiosis has been studied in multiple organisms by introducing null mutations in synaptonemal complex genes. In the
absence of Sycep1, chromosomes fail to synapse and crossovers do not form in mouse spermatocytes (10). Consequently, no haploid gametes form in these mice. In Caenorhabditis elegans, a transparent, hermaphroditic roundworm, removal of Syp-1 (SYCP1) does not affect pairing but synapsis and crossing over are severely impaired (11). Similarly, in the budding yeast Saccharomyces cerevisiae loss of Sycep1 (Zip1 in yeast) still allows proper pairing but prevents synapsis, and reduces the number of crossovers. As a result, meiotic cells from these mutant yeast strains arrest before the first meiotic division (12-15). Therefore, completion of successful meiosis depends on the faithful assembly of synaptonemal complexes between homologs to promote recombination and DSB repair, as will be discussed later in this chapter.

1.2.2 Pairing vs. synapsis

During prophase I, the distinction between pairing and synapsis is definitive in some organisms but ambiguous in others (4). Pairing, however, precedes synapsis in most organisms. In Sycep1 null spermatocytes, synapsis fails but chromosome axes align homologously, indicating that pairing is not disrupted (10). In C.elegans, pairing is also independent of synapsis and occurs through structures called “pairing centers” (16). However, pairing and synapsis can be difficult to distinguish because both processes occur simultaneously or nearly simultaneously during zygonema. One exception is Sordaria macrospora, in which pairing is distinctively obvious early during zygonema prior to any synapsis (17).

At the initiation of pairing, the chromosomes adapt a unique spatial arrangement within the nucleus known as the “bouquet.” The bouquet forms as a result of chromosome telomeres, which are anchored to the nuclear envelope, clustering together (18). Nuclear envelope protein Sad1/UNC-84 (SUN) is required for chromosome anchoring (19). By forming a bouquet cluster in a confined region of the nuclear periphery, the homolog search is spatially limited so that pairing can be more easily achieved. The chromosomal bouquet can be detected from zygonema to diplonema by cytologically examining chromosome squashes that retain the 3-D architecture of the chromosomes (20).
1.3 Prophase I at higher order structure

Earlier investigation of chromosome behavior and organization at a higher order was accomplished by electron microscopy of chromosome spreads or squashes from multiple organisms (4). There is a direct correlation between chromatin loop size and axis extension: shorter loops result in longer axes; and longer loops result in shorter axes. Because the axis length is proportional to the number and distribution of DSBs per nucleus, shorter loops/longer axes will have a higher frequency of DSBs than longer loops/shorter axes (21) (22). Cytological investigation showed that the meiotic DSB repair and recombination machinery reside on the axis but the sequences that receive DSBs reside within the loops (23) (24). This finding led to a “tethered loop axis model” where the DSB sites on the loop get tethered to the axis, thereby bringing the DSB sequence close to recombination protein complexes. The repair protein complexes on the axis then recruit SPO11, which makes meiotic DSBs (6).

Proper loop organization of meiotic chromosomes plays a critical role during meiotic prophase I. Cohesin proteins, like structural maintenance of chromosome 1 beta (SMC1beta), not only provide cohesion between the sister chromatids during pre-meiotic DNA replication but also hold the DNA loops together and make up the axial element at the start of meiotic prophase I. Smc1beta−/− spermatocytes arrest and apoptose at pachynema, the axis length is shortened by 50% and synapsis is aberrant and incomplete (25). Axis length can be measured using IF images of meiotic chromosomes stained with antibodies against the axial element. Moreover, use of fluorescence in-situ hybridization (FISH) chromosome painting in conjunction with axis staining allows the calculation of minimum and maximum loop sizes by measuring the spread of the FISH signal at particular chromosomes (26). Further investigation using this method revealed that mouse spermatocytes and oocytes from Smc1beta−/− mice had significantly different maximum and minimum loop sizes, suggestive of heterogeneity in loop formation, which can affect global DSB formation dynamics (25) (27). Further, crossovers were absent in Smc1beta−/− spermatocytes and oocytes, suggesting that DSB repair is aberrant in the absence of SMC1beta (25).
Hence, as will be discussed in further detail, both chromosomal organization and structural changes are intricately associated with the DNA recombination activity that occurs during prophase I. DNA loops extruding out of the axis further aggregate to form higher order structures. Advances in biological techniques have enabled investigators to observe eukaryotic genome organization within the nucleus at a resolution beyond that of electron and optical microscopy to envision 3D chromosomal organization within the nuclear space (28). The first attempt to characterize the organization of chromosomes in 3D was accomplished by Chromosome Conformation Capture (3C), developed by Drs. Job Dekker and Nancy Kleckner (29). Although this technique could only probe long-range interactions between a few known loci within limited regions of the genome, it provided the first structural evidence that long-range interactions could be mediated by chromatin looping (29) (30). Dekker’s group advanced the 3C technique by incorporating Next-Generation Sequencing technology to develop Hi-C. Hi-C creates a high-throughput, genome-wide contact map of higher order chromosome architecture in an unbiased way (31). In addition to Hi-C, a haplotype aware Hi-C method was developed by the laboratory of Dr. Bing Ren to study chromosomal structures in an allele-specific manner (32, 33).

These chromosome conformation capture methods enabled the comprehensive investigation of genome organization at a supranucleosomal scale (Fig. 3). At the nucleosomal scale, the DNA double helix wraps around histones to form nucleosomal units. These nucleosomes organize into higher order structures called topologically associating domains (TADs). TADs participate in chromosome organization and packaging so that approximately 2 meters of linear DNA can be packaged into a nucleus that is approximately 6 µm in diameter. Each TAD is essentially a long loop held by a protein known as CCCTC-binding factor (CTCF), which is present in budding yeast, *Drosophila melanogaster* and vertebrates but absent in *C. elegans* (34) (35). CTCF is a critical protein that defines the boundary of each TAD, and null mutations of *Ctf* in mice lead to embryonic lethality (36). Within each TAD, smaller loops form that are held by cohesins and condensins (37) (38) (39).
Figure 3. Higher order chromosome organization.

DNA double-helix wraps around histones to form nucleosome units, which then folds in loops to form TADs. TADs aggregate together to form compartments (A & B). These compartments then aggregate to occupy chromosome territories (‘1pb’ under “Nucleosomal scale” in the figure stands for ‘paire de base’, which translates to ‘base pair’ in French).

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Within the genome, this looping interaction can bring enhancers in close proximity to promoters thereby activating and regulating gene transcription for cell survival and growth (37). Therefore, maintenance of TAD boundaries is important to prevent enhancers of one TAD from promiscuously interacting with gene promoters of another TAD. Aberrant TAD architecture can dysregulate transcription, which can lead to limb malformation or diseases like cancer (40) (41) (42). For example, a gain-of-function mutation of \textit{IDH} gene, which transcribes an enzyme that catalyzes a step in citric acid cycle, is a clinical marker for glioma. Human glioma cells with IDH mutation displayed hypermethylation at CTCF sites, leading to TAD boundary disruption. Loss of TAD boundary caused enhancers of \textit{FIP1L1}, which encodes RNA-processing proteins in neural tissues to aberrantly interact with the promoter region of glioma oncogene \textit{PDGFRA} (43). TADs cluster together to form even higher order structures called compartment A and compartment B (~3 Mb). Compartment A consists of open euchromatic chromatin regions with active genes, whereas compartment B consists of closed heterochromatic chromatin with inactive genes. At a nuclear scale (~100 Mb – 3000 Mb), these compartments organize into chromosome territories within the nucleus (28) (Fig. 3).

Active elucidation of higher order chromosome organization by Hi-C was performed in various mitotic cell types (44) (45). During interphase, chromosomes are decondensed, poorly organized, and form a loose mass. Hi-C contact maps of interphase cells show very distinct TADs and compartment structures. However, at metaphase, these cells lose their TADs and compartment structures (44), because as cells enter mitosis, their chromosomes condense and compact, and as a result, condensins largely replace cohesins (5, 44). Loss of TADs is also consistent with the suppression of transcription observed during mitosis (46). Exit from mitosis must accompany re-establishment of proper TAD and compartment architecture (44).

1.4 Prophase I at the molecular level: meiotic DSB repair by homologous recombination

1.4.1. Meiotic DSB repair by homologous recombination

The current molecular model for meiotic recombination was established from extensive studies in \textit{S. cervisiae} (Fig. 4). Budding yeast is an excellent model organism to study meiotic
Figure 4. Model of meiotic recombination.

Programmed DSB formation initiated by Spo11, is followed by resection to leave a 3’ overhang that invades the homologous substrate for repair. D-loop intermediates form during this homology search and leads to the generation of crossovers or noncrossovers through additional steps. Resolution of double Holliday junctions (dHJs) by the meiosis-specific resolvase MLH1/3 exclusively generates crossovers. By contrast, dHJ resolution by the structure-specific endonucleases (SSEs) generates both crossovers and noncrossovers. Finally, both synthesis-dependent strand annealing (SDSA) and alternative repair of dHJ also exclusively generate noncrossovers. All pathways generate a heteroduplex DNA (hDNA) tract unique to each pathway and will either be converted or restored to the parental genotype. (Unidirectional: gene conversion occurs only on the recipient chromatid; Reciprocal: gene conversion occurs on both the recipient and the donor chromatid).
recombination because of its simple biology, ease of genetic manipulation, and the ability to recover all four haploid spores from a single meiosis by tetrad dissection.

Molecular and structural analysis of an artificial recombination hotspot, introduced into the left arm of chromosome III in budding yeast, helped establish the prevailing model of meiotic recombination (47). Yeast were synchronized to undergo meiosis simultaneously and the subsequent events were examined at the introduced site and at discrete times via DNA-DNA hybridization technology to examine DSB and crossover formation. Careful selection of restriction enzymes and hybridization probes revealed DNA fragments that corresponded to crossover and noncrossover recombination events, and allowed both quantitative and kinetic analyses of crossover and non-crossover events (48, 49). In addition, 2-D gel electrophoresis technology enabled the isolation of DNA repair intermediates produced during meiotic recombination based on size and structure (50). Many of the critical mitotic and meiotic HR proteins and mechanisms initially discovered in budding yeast have been evolutionarily conserved in mammals, including humans, underscoring the value of using budding yeast to study recombination mechanisms (47).

During leptonema in mammals, SPO11 preferentially forms DSBs near the center of ~200 bp regions known as meiotic hotspots. These DSBs can be readily identified by the formation of gamma-H2AX foci that can be observed as bright spots via immunofluorescence microscopy from chromosome surface spreads (Fig. 5). H2AX is a variant of histone H2A, and gamma-H2AX is the phosphorylated form of H2AX that appears in response to DNA damage and recruits the proteins necessary for DNA repair. As SPO11 makes DSBs, leptotene cells also begin to establish the axial element SYCP3. After DSB formation, the broken ends are resected in opposite directions by a 5’-3’ exonuclease to produce 3’ overhangs. The resulting in 3’ overhangs are then coated with replication protein A (RPA), a heterotrimeric, single-stranded DNA binding protein complex, as well as the strand exchange proteins RAD51 and DMC1. RAD51 and DMC1 facilitate strand exchange and homolog invasion (51-53). RAD51 also participates in mitotic DSB repair but DMC1 is expressed exclusively during meiotic HR (54).
Figure 5. Meiotic prophase I based on cytological studies.

Each stage of prophase I can be distinguished by immunofluorescence (IF) using stage-specific markers along with SYCP3, a meiosis-specific axis marker (red): Leptonema is indicated by the appearance of γH2AX foci, Zygonema by the presence of SYCP1, Pachynema by the appearance of MLH1 and Diplonema by observing desynapsing behavior by following the localization of SYCP3. Synaptonemal complex figure adapted from Audrey Lynn, Rachel Soucek, & G. Valentin Borner. "ZMM proteins during meiosis: Crossover artists at work". Chromosome Research (2007) 15:591-605.

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Both RAD51 and DMC1 form distinct foci that peak during zygonema when interhomolog interactions occur. Interhomolog interaction is initiated when the 3’ overhang from one strand invades its homolog to form an intermediate structure called the displacement-loop (D-loop). The formation of a D-loop can be inferred from the presence of RPA2 foci. RPA2 is one of the three subunits of the RPA complex that stabilizes early intermediates (55). Following D-loop formation, meiotic DSB repair bifurcates into either noncrossover or crossover pathway, whose products are the two major outcomes of meiotic recombination (48) (Fig. 4). A number of distinct meiotic recombination pathways contribute to the formation of crossovers and noncrossovers:

1.4.1.1 Synthesis-dependent strand annealing pathway (SDSA)

The D-loop can dissociate after DNA polymerization from the homolog, anneal back to its original chromatid, and generate heteroduplex DNA (hDNA). Heteroduplex DNA contains regions of DNA mismatches that create sequence polymorphisms. Heteroduplex DNA that forms after strand re-annealing contains one chromatid reflecting the donor genotype and the other reflecting the parental genotype. Heteroduplex DNA can then be targeted by the mismatch repair machinery, e.g. the Msh2/6 complex, to either use the parental genotype as a template to restore the parental genotype or use the donor genotype to convert to the donor genotype, thereby producing a noncrossover (56-58). A noncrossover is a short, patch-like repair in which the original sequence has been lost and replaced by the donor genotype. Such a replacement of DNA sequence is called a gene conversion, and results from a repair by the synthesis-dependent strand annealing (SDSA) pathway. In yeast, noncrossover gene conversion tract length averages 1.8 kb (59). SDSA was originally discovered by Paque & Haber in 1999 (60) from studies of mitotic budding yeast cells and was later confirmed to be active during meiotic homologous recombination. It has been shown in vitro that RecQ helicases function in unwinding the D-loop during SDSA (47).

In most organisms, the number of crossovers is much lower than the total number of DSBs that are formed (61). In mice, the approximately 300 DSBs are formed at the initiation of
meiotic recombination. These sites can be quantitated by counting RAD51 or DMC1 foci in chromosome spreads of mouse germ cells. Of the ~300 DSBs, approximately 25-30 will become crossovers, and these are marked with MLH1 foci. MLH1 is part of the protein complex required for crossover formation and can be used to mark the physical location of crossovers on the chromosome axis (62). From this cytological observation, it was inferred that most meiotic DSBs are repaired as noncrossovers. However, this assertion is difficult to confirm experimentally using cytological approaches as there are no antibodies currently available that detect noncrossover events.

Although noncrossover events cannot be studied using immunofluorescence techniques on whole chromosomes, they can be detected at the nucleic acid level. To study noncrossover recombination outcomes at the level of DNA, Dr. Alec Jefferys developed a nested allele-specific PCR assay that uses allele-specific primers to amplify both crossovers and noncrossovers at known human meiotic hotspots (63). Subsequently, it was adapted by the de Massy’s laboratory for use in mice by taking advantage of the presence of specific recombination hotspots in F1 hybrid mice (64). However, contrary to expectations based on cytological observation, their results yielded fewer noncrossover products than crossover products (64), most likely because only selected polymorphisms were probed in their recombination assay.

To enhance the detection of recombination outcomes, my mentor Dr. Francesca Cole, while a postdoctoral fellow in Dr. Maria Jasin’s laboratory, developed a high-resolution molecular method that can detect all recombination outcomes in a non-selective manner at a known hotspot, A3, in mouse spermatocytes (65). The A3 hotspot has a high number of sequence polymorphisms between strains with a frequency of ~1.6% (i.e. 32 polymorphisms over 2 kb). This polymorphic density allows the calculation of frequencies, distributions, and lengths of the gene conversion tracts of all crossover and noncrossover products based on the outcomes of allele-specific PCR. Using this method, she recovered a crossover to noncrossover ratio of 1:10 which mirrors the global crossover and noncrossover ratio inferred
from cytology. More importantly, the average gene conversion tract length of noncrossovers recovered from this hotspot was only ~30 bp (65) (66), which is much shorter than the 1.8 kb observed in yeast. As will be discussed later in this chapter, the shorter gene conversion associated with SDSA-associated noncrossovers enables the identification of noncrossovers derived from alternative pathways in mammals, such as noncrossovers with longer gene conversion. Therefore, this work provided the first molecular evidence supporting the cytological observation that the majority of meiotic DSB repair in mice results in noncrossovers, likely mediated by SDSA.

1.4.1.2 MutLγ pathway

The D-loop can be processed further by a crossover-specific pathway, commonly known as the MutLγ (MLH1/3) -dependent crossover pathway. In this pathway, the D-loop intermediate is extended through DNA synthesis and relies on the homolog as the template for polymerization. This extended D-loop forms a strand exchange intermediate called the single-end invasion (SEI) (67). Then, SEIs are further processed by second-end capture to form a four-stranded double Holliday-junction (dHJ) structure. Each Holliday junction has a cruciform shape, and SEIs and dHJs are collectively known as joint molecules. Joint molecules are formed when DNA DSBs engage with the homologous duplex DNA. These intermediates are known to be specific to the crossover pathway in yeast and are stabilized by the MutSγ (MSH4/5) complex. Stabilization of dHJs by MutSγ can be observed by the formation of MSH4 foci that appear during late zygonema and early pachynema (68). RPA foci, detected early in prophase I, persist until early pachynema prior to the appearance of MLH1 foci in mouse spermatocytes (69) (70) and co-localize with MSH5 during early pachynema in C. elegans (71), indicating that RPA also plays a role in stabilizing joint molecules intermediates. DMC1/RAD51 foci can also be observed during early pachynema, indicating unrepaired or newly formed DSBs (68). The MLH1/3 complex that makes up MutLγ acts as a crossover-specific resolvase of dHJs. MutLγ cleaves dHJs asymmetrically to specifically resolve a dHJ as a crossover
product (Fig. 4). Crossovers can also produce gene conversion tracts that are longer than noncrossover tracts and average 500 bp in mammals and ~2.0 kb in budding yeast (59, 65, 66, 70). These crossovers can be observed cytologically in mid- to late pachynema by staining with MLH1 antibodies. Like the hDNA created during SDSA, dHJ resolved by MutLγ also leads to hDNA tracts that can be restored and/or converted resulting in many possible gene conversion patterns. Approximately 90% of mammalian crossovers are formed by the MutLγ-dependent pathway (72).

The first evidence that noncrossovers are produced by SDSA independent of the MutLγ crossover pathway, came from a temporal analysis of meiosis in the budding yeast strain SK1, which. SK1 readily undergo synchronized meiosis. This allows the study of meiotic DSB repair outcomes over time by isolating DNA products at 30-minute intervals. In this initial study, the authors relied on 2D-gel analysis to distinguish between crossovers and noncrossovers. They found that noncrossover intermediates appeared earlier than crossover intermediates in wild-type cells. When they examined the formation of crossovers and noncrossovers in SK1 cells lacking the meiotic transcription factor Ndt80, which causes the mutants to arrest in pachytene leading to sporulation defects (73, 74), they recovered very few crossovers, but noncrossover formation was unaffected, suggesting that noncrossovers form before pachytene arrest and that noncrossover and crossover pathways are two independent pathways. Notably, joint molecules, believed to arise specifically from the crossover pathway, accumulated but failed to be resolved in these mutants, consistent with a meiotic recombination model in which the majority of noncrossovers and crossovers are derived from distinct intermediates and pathways (48).

In addition to yeast, C. elegans has been used in cytological studies designed to investigate the timing of DNA recombination during meiosis. In the worm gonad, nuclei are organized in a temporo-spatial gradient with early meiotic cells at the distal end of the gonad and late meiotic cells at the proximal end, thus allowing isolation of germ cells at specific stages of prophase I. Although this unique biology of C. elegans is attractive for cytological
investigation, a lack of established recombination assays for detecting both crossovers and noncrossovers at the level of DNA is further hindered by a lack of well-characterized meiotic hotspots (75).

### 1.4.1.3 Structure selective endonuclease (MutLγ-independent) pathway

The dHJ or other recombination intermediates can also be resolved by structure selective endonucleases (SSEs) independent of MutLγ. In humans, SSEs are capable of cleaving not only dHJs but also other DNA structures, such as 3’ flaps, single HJs, nicked dHJs and extended D-loops (47, 76, 77). In addition, SSEs play a critical role in resolving aberrant multichromatid joint molecules that that form when three or more chromatids become interconnected (78, 79). Three SSEs are active during both meiosis and mitosis: MUS81-Mms4 (EME1 in mammals), SLX1-Slx4 (BTBD12 in mammals), and Yen1 (GEN1 in mammals).

Unlike MutLγ, SSEs can cleave dHJs to produce equal numbers of crossovers and noncrossovers (49, 80). The noncrossovers generated by SSEs have distinct phenotypes that distinguish them from noncrossovers derived from SDSA. First, noncrossovers derived from the SSE pathway are predicted to have longer gene conversion tracts than those produced by SDSA. Second, SSE-dependent noncrossovers alter the DNA sequence of the donor strand regardless of the presence of a DSBs (reciprocal) (81), whereas in the case of SDSA, only the recipient DNA strand containing the DSB is altered and the donor strand sequence remains unaltered (unidirectional) (Fig. 4).

SSEs play only a minor role in crossover formation in most organisms; however, the model organism *Drosophila melanogaster* lacks the MutLγ pathway and relies completely on SSE pathways (Slx4 homolog) to form crossovers, and budding yeast use the SSE pathway extensively when the MutLγ-dependent crossover pathway is disturbed, aiding ~50% of the crossover events (82), but null mutation of SSE pathways in budding yeast does not affect crossover formation (49, 78-80). On the other hand, based on estimates derived from analyzing residual chiasmata observed from metaphase cells in *Mlh3* null spermatocytes, only ~10% of
total crossovers in mice rely on the SSE pathway (72). Notably, although MutLγ-dependent crossover pathways are meiosis-specific, SSE pathways are also active in somatic DSB repair (47).

1.4.1.4 Dissolution or alternative repair pathway

Noncrossovers can also be generated by a dHJ dissolution pathway that relies on the BTR complex (STR in budding yeast). The STR/BTR complex is composed of three proteins: Sgs1/BLM, Top3/TOPIIIalpha, and Rmi1/RMI1-RMI2. BLM is a RecQ helicase that unwinds DNA, TOPOIIIalpha is a type I topoisomerase that cuts one strand of double-stranded DNA to remove DNA supercoiling and reanneal the strand, and RMI1-RMI2 are RecQ-mediated genome instability proteins that act as accessory proteins. Together, they dissolve dHJs by moving each single Holliday junction toward the other, during the process of dHJ branch migration. Double Holliday-junction branch migration generates torsional build up due to DNA supercoiling. Ultimately, the torsional stress is relieved by Top3, which decatenates the remaining junction to produce noncrossovers exclusively (47). These noncrossovers would be expected to have longer gene conversion tracts like those produced by SSEs than those derived from SDSA. However, unlike noncrossovers produced by SSEs, only the recipient DNA sequence is converted to the donor genotype, leaving the donor sequence unaltered (unidirectional) (81).

By taking advantage of the assay developed by my mentor Dr. Francesca Cole, our group identified the first in vivo evidence for a noncrossover phenotype that is consistent with the dissolution pathway (70). A postdoctoral fellow in our laboratory characterized recombination outcomes in spermatocytes from mice lacking MLH3, and found a significant number of noncrossovers with longer gene conversion tracts than SDSA-derived noncrossovers (that I will refer to as long noncrossovers) suggesting that they represent the product of either dHJ dissolution or SSE resolution, but not SDSA. This is an important finding because it has been impossible to make a molecular distinction between long noncrossovers derived from SSE resolution or dHJ dissolution vs. short noncrossovers derived from SDSA in
budding yeast, as the gene conversion lengths of noncrossovers and crossovers in yeast are highly variable and too similar to each other to be distinguished – 1.8 kb vs. 2.0 kb, respectively (59).

In addition to the dissolution pathway, long noncrossovers can also be formed by alternative repair pathways. Instead of both HJs being resolved simultaneously, only a single HJ is cleaved, resulting in a nick. Subsequent branch migration of the other HJ could then form an hDNA tract in a trans configuration. Trans hDNA refers to two consecutive hDNA tracts that occur on opposite DNA strands of the same chromatid. Converting these hDNA tracts to the donor genotype by mismatch repair will produce long noncrossovers (58). Another mechanism that could create long noncrossovers is the double SDSA pathway. Following DSB formation, both free 3’ chromatid ends can engage in homolog invasion and DNA synthesis. Subsequent dissociation and reannealing of both ends can produce hDNA in trans, which then can be converted later as long noncrossover (56, 83).

1.4.1.5 Intersister recombination

Mitotic DSB repair by HR favors the use of the identical sister chromatid as a template, leaving the repair outcomes genetically unaltered (84). In contrast, meiotic DSB repair shows strong homolog bias, which can result in gene conversion. Current evidence supports a model in which intersister recombination is actively suppressed during meiotic recombination. It has been proposed that in yeast, Mek1, a meiosis-specific kinase, helps to maintain this homolog bias, as loss of Mek1 function results in significantly more intersister joint molecule structures (85). Plus, intersister recombination occurs during meiosis if the homolog lacks sufficient homology. Early investigation of joint molecules in budding yeast by 2D gel analysis showed that even in wild-type cells, approximately 1 intersister joint molecule was formed for every 10 interhomolog joint molecules (86). In addition, budding yeast lacking Dmc1 very rapidly form intersister joint intermediates, suggesting that Dmc1 also plays a role in homolog bias and that intersister recombination occurs in the absence of Dmc1 (85). Further molecular evidence for intersister recombination was provided by Goldfarb and colleagues (87) who used hemizygous
yeast mutants that lacked homology on the homologous chromosome near a previously identified DSB hotspot, thereby leaving only the sister chromatid as a template for repair. They found that the hemizygous mutants were comparable to wild-type cells with respect to spore viability, nuclear division, and DSB lifespan, indicating that the efficient repair could be achieved using the sister chromatid (87).

Another system where intersister recombination is highly likely to occur is between the X and Y sex chromosomes in male spermatocytes. The sex chromosome in males is heterogametic, and the homology between the X and Y chromosomes is confined to the pseudo-autosomal region (PAR) (88). In addition to limited homology, the Y chromosome is smaller than the X chromosome. In mice, the X chromosome is ~171 Mb, whereas the Y chromosome is ~91.7 Mb (89). Therefore, the prediction is that meiotic DSBs created on the nonhomologous regions of the X and Y chromosomes in male spermatocytes are most likely repaired using the sister chromatid as a repair template. Although it is possible that these DSBs can be repaired by low fidelity non-homologous end joining (NHEJ) mechanism that ligates two DNA break ends together, thus bypassing the need for a homologous template, this is highly unlikely because a large number of NHEJ events would severely compromise the genomic integrity of the sex chromosomes.

Despite the importance of understanding the detailed mechanism of meiotic intersister recombination, there are insufficient experimental approaches available to detect the molecular outcomes of intersister recombination to definitively prove that intersister recombination occurs during meiosis in mammals. However, based on the current literature, we can hypothesize that intersister recombination during meiosis provides rapid and efficient repair of DSBs depending on the availability of substrates. Accordingly, we cannot exclude the possibility that intersister repair can engage in all aforementioned HR pathways during meiosis.

1.4.2. Meiotic DSB landscape

Programmed DSBs are created by a topoisomerase type II-like protein, SPO11, during leptonema. These DSBs mark the initiation of meiotic recombination (90-92). Although DNA
damage severely threatens genomic integrity in most somatic cells, DNA is purposely broken in meiotic cells to generate chiasmata and produce genetic diversity in the germ line. Meiotic DSBs created by SPO11 do not occur at random genomic loci but rather are enriched at defined loci called “hotspots”. On average, there are ~25,000 hotspots located mainly in intergenic regions distal to gene promoters in primate and rodent genomes. During any individual meiosis, however, only ~300 breaks are detected globally per nucleus by cytological assays. Importantly, in both mouse and humans, the actual frequency of hotspot use varies dramatically, resulting in several orders of magnitude difference between hotspots that are used frequently (“hot” hotspots) compared to those that are used rarely (“cold” hotspots) (93-95).

Hotspots display recombination frequencies significantly higher than those of adjacent regions and are often located in areas dense with single nucleotide polymorphisms (SNPs) (96). In most mammals, including humans, the location of meiotic hotspots is determined by PRDM9, PR domain-containing protein 9 (94, 97, 98). Exceptions include birds and Canid family members. In dogs, wolves, and coyotes, meiotic DSBs form near regions containing functional genomic transcription start sites that are associated with CpG islands, and many of these hotspots also overlap with H3K4me3 marks (99) (100, 101). Similar to Canids, mice lacking functional PRDM9 also rely on PRDM9-independent H3K4me3 marks that are mostly associated with gene promoter regions to generate recombination hotspots, and these mice are infertile (95). However, meiotic recombination and crossover formation in Canids occur normally during meiosis (100, 102). Detailed meiotic recombination mechanisms in Canids in the absence of functional PRDM9 are currently not well known. In budding yeast, meiotic DSBs occur largely at nucleosome-depleted and GC-rich sequences adjacent to gene promoter regions (103).

PRDM9 is a rapidly evolving gene, and its expression is limited to mammalian germ cells (104). PRDM9 contains tandem arrays of Cysteine(2), Histidine(2) (C2H2) zinc fingers which bind to specific DNA sequence motifs (105), and a PR/SET domain that is structurally
related to histone lysine methyltransferase SET domains. The PRDM9 zinc finger binding motif displays high promiscuity and flexibility in terms of the DNA sequences it binds (106). Different alleles of PRDM9 contain distinct arrays of zinc fingers with distinct consensus sequence motifs that determine the distribution of meiotic DSBs within the genome, thereby generating a unique landscape of meiotic hotspots. Once bound to DNA, the PR/SET methyltransferase domain of PRDM9 helps generate open chromatin by trimethylating histone H3 on lysine 4 (H3K4me3). This might be further aided by its ability to trimethylate H3K36 (107). SPO11 is then recruited to the hotspot to create DSBs. Approximately ~80% of PRDM9-SPO11 dependent DSBs occur in the central 200 bp of mouse hotspots, and DSBs created by SPO11 lead to an obligate deletion of 2 bp at the DSB site (91).

There are different PRDM9 alleles present in mice and humans that have different consensus sequence binding motifs, likely because the zinc finger array has evolved within the context of its organismal genome of origin or strain genetic background (108). Any genomic rearrangements within the sequences encoding the PRDM9 zing finger array will create new sequence binding motifs, and consequently result in new PRDM9 binding sites (109). When PRDM9 alleles are heterozygous, a novel meiotic hotspot landscape emerges. This can be especially pronounced in F1 hybrid offspring generated by crossing different inbred mouse strains (70, 95, 110). Some meiotic DSB hotspots in F1 hybrid mice display asymmetry, where one parental allele is “hot” and receives significantly more DSBs than the “cold” allele. This asymmetry is due to the higher binding affinity of PRDM9 for the “hot” allele over the “cold” allele. For example, the inbred C57BL/6J (B6) mouse carries the PRDM9\textsuperscript{B6} allele and has a meiotic hotspot distribution determined by the identity of the zinc finger array of PRDM9\textsuperscript{B6} (and vice versa for CAST/EiJ (CAST) mice). Recombination initiated on the “hot” allele will invade into the “cold” allele and use it as a template for DSB repair. As a result, the “hot” allele will convert its DNA sequence to that of the “cold” allele (gene conversion). Therefore, the meiotic hotspots on B6 alleles governed by PRDM9\textsuperscript{B6} will lose “hotness” over time due to gene conversion, a phenomenon known as hotspot erosion. When the CAST allele is introduced into
the B6 background by crossing inbred B6 mice with inbred CAST mice, the PRDM9\textsubscript{B6} zinc finger arrays of the F1 hybrid germ cells will have a strong binding affinity for CAST sequences (and vice versa for PRDM9\textsubscript{CAST} for B6 sequences) because those sites have not been exposed to the eroding action of PRDM9\textsubscript{B6} over time. Consequently, the differential binding affinity of PRDM9 alleles for hotspots on the naïve parental chromosomes will cause allelic bias in DSB formation (111-113).

PRDM9 plays a critical role in meiotic DSB processing but also in facilitating proper pairing. In some inbred mouse crosses, hybrid sterility is observed, for example PWD/PhJ (PWD) x B6 F1 males are infertile. Hybrid sterility is a reproductive isolation mechanism where the offspring produced by two genetically different subspecies are infertile and suppressed from generating a whole new species. The hybrid sterility observed in PWD x B6 F1 hybrid males is due to the failure to fully pair/synapse at the pachytene stage of meiosis. Consequently, the developing germ cells undergo apoptosis, leading to infertility (114). Davies and colleagues (111) found that these hybrid males harbored a large number of asymmetric hotspots. To test whether a new PRDM9 allele can reverse the asymmetry, they replaced the B6 PRDM9 zinc finger array with a human zinc finger array. These “humanized” PRDM9 alleles now recognize a completely different sequence motif, and the number of symmetric hotspots in these animals increased and fertility was rescued. Mathematical modeling revealed a positive correlation between the quantity of symmetric hotspots and the rate of synapsis. Indeed, a high number of asymmetric hotspots was associated with elevated DMC1 heat in these animals (111). DMC1 heat is proportional to the number of cells marked with DMC1 by ChIP-Seq at particular loci. High DMC1 heat suggests that either 1) more DSBs are formed, or 2) there is a delay in DSB repair. Both can arise in delayed homology search/synapsis. Previous findings indicate that more DSBs are created when homologs fail to synapse to promote proper pairing and synapsis; when homologs synapse, further DSBs are prevented from forming (10, 111, 115, 116). This is reminiscent of Prdm9\textsuperscript{-/-} mouse spermatocytes (104) in which pairing and synapsis are defective and gammaH2AX signal is retained until a pachytene-like stage. Alternatively, lack of homology
and delay in pairing/synapsis will lead to a delay in DSB repair, which will be reflected in elevated DMC1 heat as well. This was shown in the X chromosome of mouse spermatocytes, where DMC1 heat indicated a delay in repair (more detail to follow in section 1.4.4. Methods for mapping meiotic hotspots) (117).

A year later, a second group showed that asynapsis observed in hybrid sterile mice with a large number of asymmetric hotspots could be reversed by incorporating 2.7Mb of PWD/PWD mouse sequence into a PWD/B6 interval in the four most frequently asynapsed chromosomes (15, 16, 18 and 19) of hybrid sterile mice (118). That was enough to rescue fertility in these mice, suggesting that asymmetric hotspots that cause unequal DSB formation which leads to compromised or delayed pairing/synapsis, thus delaying/impairing DSB repair. This implies that mouse spermatocytes can tolerate some level of asymmetric hotspots but also require a threshold level of symmetric hotspots to facilitate synapsis.

Intriguingly, in the absence of PRDM9, mouse spermatocytes still form DSBs. However, instead of meiotic DSBs directed at hotspots, SPO11 places an equivalent number of DSBs at naturally occurring H3K4me3 regions of the genome, most of which are located at active gene promoters (95). Despite DSBs forming in equivalent number, molecular processes and chromosome pairing are defective (104), indicating the localization of DSB machinery to defined meiotic hotspots is imperative in ensuring robust meiosis. Recently, it has been discovered that the methyltransferase activity of the PR/SET domain in PRDM9 is important for the precise localization of DSBs to meiotic hotspots (119). Therefore, although PRDM9 is not necessarily required for DSB formation by SPO11, DSBs created at PRDM9-independent H3K4me3 sites are insufficient to complete meiotic recombination. Only DSBs formed by PRDM9-driven SPO11 targeted at meiotic hotspots accomplishes proper pairing, synapsis and downstream DNA repair.

1.4.3. Pairing, synapsis and recombination

In many organisms, programmed meiotic DSBs and the recombination events that follow are required for proper pairing/synapsis, with the exception of C.elegans in which pairing
and synapsis occur independently of recombination (DSBs form on already synapsed chromosomes in *C. elegans*) (1, 6, 120). In the absence of SPO11 in mice, little to no synapsis occurs, which results in pachytene arrest and apoptosis (121) (122). Additionally, lack of functional DMC1 results in pairing and synapsis defects causing spermatocytes to undergo apoptosis during zygonema/pachynema in mice. Any synapsis observed in these mice occurs between nonhomologous chromosomes (123). Apoptosis observed in these animals is likely due to a mid-pachytene checkpoint mechanism that triggers apoptosis in the presence of unrepaired DSB intermediates (124). Similar results were observed in budding yeast lacking SPO11 protein (91).

In contrast, yeast expressing Spo11-Y135F, a mutant protein that lacks the catalytic activity required to induce DNA DSBs, due to the elimination of a specific –OH group, exhibited pairing efficiency comparable to wild-type, but were unable to form synaptonemal complexes, suggesting that meiotic DSBs may not be a prerequisite for pairing (125). Similarly, Boateng and colleagues (126) also found that the catalytic activity of Spo11 could be separated from its role in pairing. They generated FISH probes to three different chromosomes (1, 3 and 7) that allowed them to examine pairing by immunofluorescence. They found that approximately 35% of pre-leptotene (or premeiotic S phase) spermatocytes showed evidence of pairing. When they examined pairing in spermatocytes from a mouse model bearing a mutation analogous to Spo11-Y135F, the mouse spermatocytes retained pre-meiotic pairing whereas spermatocytes from Spo11 null mice did not. However, the Spo11-Y135F spermatocytes, like their yeast counterparts, were devoid of synapsis. Together these results indicate that although the catalytic DSB-inducing activity of SPO11 is not required for pairing, it is required for synapsis. This implies that there may be a DSB-independent mechanism at work during meiosis that facilitates initial pairing, and that SPO11 may have a separate role, unrelated to catalysis, in promoting pre-DSB pairing (126).

However, Ishiguro and colleagues (127) were unable to recapitulate what Boateng and colleagues (126) found because they observed pre-meiotic pairing even in *Spo11* null mutants
and they did not observe any pairing at the preleptotene stage in wild-type spermatocytes. Instead, they observed interhomolog association during early leptotene, likely prior to the formation of DSBs (127).

Despite these discrepancies, two observations are in agreement: 1) at least some meiotic pairing/interhomolog association is independent of SPO11-induced DSB formation; but 2) proper pairing, and consequently proper synapsis, is not observed in the absence of SPO11-dependent DSBs. Collectively, these data suggest that both SPO11-dependent DSBs and the strand invasion that initiates the subsequent homology search are required to stabilize pairing and promote proper synapsis between homologs.

### 1.4.4. Methods for mapping meiotic hotspots

Originally, hotspot identification relied on pedigree analysis methods that lacked high resolution (128). Today, advances in sequencing technology have helped generate genome-wide maps revealing meiotic hotspots not only in several individual mouse backgrounds, but also in humans. The first genome-wide hotspot analysis was accomplished by performing ChIP-Seq for the recombinase DMC1, which is normally recruited to meiotic DSBs, in testes from mice that are unable to repair DNA by HR and fail to undergo homologous chromosome synapsis (Hop2−/−) (110, 129, 130). During meiotic recombination, HOP2 loads onto meiotic chromosomes prior to DSB formation, which then couples with DMC1 and RAD51 on single-stranded DNA (ssDNA) to promote strand invasion. Because Hop2−/− spermatocytes form DSBs normally, but cannot efficiently repair them, Hop2−/− arrest at pachynema with unrepaired DSBs, thus aiding hotspot identification through sequencing (129, 131), in this case single-strand DNA sequencing (SSDS), which takes advantage of ssDNA’s propensity to form hairpin loops. By specifically targeting meiotic DMC1-bound ssDNA, they improved the efficiency, specificity and resolution of mapping meiotic hotspots to levels unattainable by simple DMC1 ChIP-Seq, in which the overall ChIP signal is weak because each spermatocyte only receives ~300 DSBs.
Since the establishment of SSDS, Dr. Galina Petukhova’s group has employed this method to map hotspots in both wild-type mice from various backgrounds and humans (132), indicating that this is a highly reproducible and robust method. However, one caveat is that the heat map produced by SSDS cannot distinguish between the cases that 1) there are more DSBs in a particular region, a true characteristic of meiotic hotspot, or 2) that there was a delay in DSB repair. In other words, the frequency of DSBs cannot be readily distinguished from the lifespan of DSBs. To distinguish between these possibilities (multiple breaks in a particular region or retarded repair kinetics), Lange and colleagues (117) developed a SPO11 oligonucleotide mapping method that takes advantage of the fact that SPO11 remains bound to the 5’-end DNA following its cleavage of the DNA (117). In order for recombination to proceed, SPO11 must detach from the DNA. SPO11 is removed via the action of the endonucleolytic protein MRN (MRE11/RAD51/NBS1) complex that cleaves DNA downstream of the 5’ end. This results in the release of a SPO11-bound DNA fragment that ranges from ~12-34 nucleotides. Sequencing and mapping these fragments provides an accurate heat map of where meiotic DSBs are formed in mice without regard for DSB lifespan (117). A drawback to SPO11-oligonucleotide mapping is that it requires a large number of cells and animals; consequently, SPO11-oligonucleotide mapping is not typically used for mapping meiotic DSB hotspots, and to date, has not been used in humans. Nevertheless, the data from these two methods show significant overlap and are equally valid for the identification of mouse hotspots, and when compared, can provide additional information about DSB formation and repair dynamics at particular hotspots.

Given that the relative “heat” of a particular genomic location revealed by DMC1 ChiP-Seq coupled with SSDS can indicate either a longer DSB lifespan or a higher total number of DSBs (95, 129), normalizing the DMC1 ChiP-Seq signal to the SPO11-oligonucleotide signal will indicate whether the “heat” determined by DMC1 ChiP-Seq coupled with SSDS reflects DSB lifespan or quantity (117). At true meiotic hotspots, the ratio of SSDS heat to SPO11-oligo heat should be equivalent. Using this approach, Lange and colleagues (117) compared SSDS
heat to SPO11-oligo heat on the sex chromosomes in spermatocytes, which remain largely unsynapsed. They found that the ratio of SSDS to SPO11-oligo signal is much higher on the sex chromosomes, especially on the non-homologous regions (non-PAR region), indicating that the DSB lifespan on the sex chromosomes is extended in mouse spermatocytes. Delay in repair is likely due to lack of homology. However, for the most part, SPO11-oligonucleotide mapping correlated well to SSDS hotspots.

1.5 Crossover regulation

As already indicated, crossovers are required to ensure accurate chromosome segregation. Meiotic crossover formation is exquisitely regulated in mammals and other organisms to ensure one crossover per homolog pair. There are five mechanisms that control crossovers: 1) obligate crossover formation; 2) crossover homeostasis; 3) crossover designation; 4) crossover interference; and 5) crossover maturation.

1.5.1. Obligate crossover

Crossover formation has been observed microscopically in many organisms. Regardless of organism, most chromosomes show at least one crossover between any given pair of homologs, and it is rare to find homolog pairs that do not have any crossover formed between them. This observation, referred to as crossover assurance, was codified as the “obligate crossover” rule by Dr. Gareth Jones (133). Such an assurance mechanism makes biological sense because the failure to form any crossovers between homologs can lead to aneuploidy. In fact, sequencing analysis of chromosome 21 from individuals with Down syndrome showed that many mis-segregated chromosomes 21 did not have any crossovers (134).

While almost all organisms manifest obligate crossover regulation, the hermaphroditic nematode C. elegans exhibits a strong obligate crossover mechanism because all of its six chromosomes almost always receive exactly one crossover. C. elegans has holocentric chromosomes that lack physical centromere structures, and the sites where crossovers occur become functional centromeres. This may be why crossover assurance is so strong in this
model organism, which is an especially useful model organism to study meiosis because chromosome pairing and synapsis occur independently of recombination. In addition, failure to form crossovers on the sex chromosomes leads to a phenotype called high incidence of males (Him), which results from progeny lacking X chromosomes and provides a unique opportunity to track both crossover dysregulation and aneuploidy via mutational analysis (16).

1.5.2. Crossover interference

When more than one crossover is formed between a given pair of homologs, they tend to be well-spaced from each other. The non-random distribution of crossovers is termed “crossover interference.” Crossover interference was first observed by Dr. Sturtevant during the construction of the first genetic map of *Drosophila melanogaster* using X-linked genes regulating wing shape, and eye and body color. He hypothesized that if crossover frequency is related to distance, he can use the progeny phenotypes to predict whether a crossover had occurred between two genes on the X chromosome and use those crossover data to predict the genetic distance between the genes. For example, when a fruit fly with long wings and vermillion eyes was crossed to another with rudimentary wings and red eye, the offspring with non-parental phenotype – that is, long wings and red eyes or rudimentary wings and vermillion eyes, indicated a crossover had occurred. When he calculated the frequency of crossovers by examining three traits (yellow body, white eyes and miniature wings), he observed that it was very rare to observe a fly that formed double crossovers between yellow body/white eyes and white eyes/miniature wings in comparison to a single crossover (135, 136), which implied that the presence of one crossover inhibited the formation of another nearby. Later, an identical genetic mapping approach was used in *S. cerevisiae*, using 10 genetic markers residing on the left arm of chromosome VII. This data also showed that the likelihood of two crossovers forming in proximity to each other is much lower than two crossovers forming across a larger distance (137). A current model for crossover interference suggests that a DSB site that has committed to becoming a crossover exerts an inhibitory signal that prevents another crossover from forming nearby.
Unequal spacing between crossovers has been observed by cytology. *C. elegans* exhibits strong crossover interference as it forms only one crossover per chromosome, indicating that the placement of a single crossover inhibits any other crossover from forming nearby (138). In mice, cytological investigation of MLH1 foci (which mark sites of crossovers) in mouse spermatocytes and oocytes in pachynema typically display only one MLH1 focus on shorter chromosomes but more than one on longer chromosomes. Long chromosomes display crossovers that are evenly spaced apart and never occur near each other (70). As a result, crossover distribution along the chromosome is tightly regulated. One mechanism that ensures crossover interference is the class I/ZMM-dependent crossover mechanism that will be discussed in more detail in section 1.5.6., although not all crossovers exert interference.

1.5.3. Crossover homeostasis

If, in most organisms, only a fraction of DSBs form crossovers, then what happens to the number of crossovers when the number of DSBs is altered?

To address this question in yeast, Dr. Scott Keeney’s group used three *spo11* mutant strains they had made previously that show reduced numbers of DSBs in meiotic cells (139, 140). These diploid strains expressed: 1) Spo11 protein tagged with HA at the C-terminus (*spo11-HA/spo11-HA* homozygotes); 2) Spo11 and *spo11Y135F* each tagged with HA at the C-terminus (*spo11-HA/spo11yf-HA* heterozygotes); and 3) *spo11D290A* tagged with HA at the C-terminus (*spo11da-HA/spo11da-HA* homozygotes), which displayed 80%, 30% and 25% of the number of DNA DSBs produced in cells with wild-type *SPO1* genes. Both the Y135F mutation and D290A mutation reside in the catalytic active site of Spo1. Then they (141) measured the crossover frequencies produced by the three strains via analyzing eight genetic intervals with heterozygous markers across three different chromosomes via yeast tetrad analysis of the four haploid meiotic products. They found that crossover frequencies were largely unaltered despite a reduction in the number of DSBs, although some genomic regions displayed varying crossover frequencies. Importantly, a separate experiment focused on a specific meiotic hotspot showed that the proportion of crossovers increased as the number of DSBs decreased.
in these *spo11* mutants, indicating that crossovers are produced at the expense of noncrossovers when DSB numbers are reduced. This phenomenon was named “crossover homeostasis” to indicate the ability of meiotic cells to maintain a fixed number of crossovers even when the total number of DSBs was reduced (141).

Crossover homeostasis has also been observed in mouse spermatocytes heterozygous for null *Spo11* allele. The number of DSBs introduced at the beginning of meiotic prophase I can be estimated by counting the number of RAD51 and/or DMC1 foci, which mark early recombination intermediates, as a proxy for DSBs, and spermatocytes heterozygous for these mutant alleles generated ~15-30% fewer DSBs marked by RAD51/DMC1 foci compared to wild-type. As noted earlier, around 300 DSBs are formed in the beginning of meiosis in mice. In pachynema, the average number of MLH1 foci is 24, indicating that only a tenth of DSBs are repaired as crossovers. In *Spo11* +/- spermatocytes, the number of crossovers marked by MLH1 foci was comparable to that of wild-type spermatocytes (68).

Similar results were found in the presence of more DSBs. Yokoo and colleagues used ionizing radiation (IR) to increase the total number of meiotic DSBs in *C. elegans* (138). Normally, in *C. elegans*, only six DSBs are introduced, one DSB per chromosome, and these sites are where crossovers will form (61). In *spo-11* mutants, endogenous DSBs fail to form, and as a consequence, crossovers fail to form. However, because pairing and synapsis occur independently of DSB formation in *C. elegans* (142), exogenous DSBs created on fully synapsed pachytene nuclei in *spo-11* mutant worms can readily engage in recombination to produce crossovers. When IR dose was increased from 100 to 1,000 rads, the total number of presumptive crossover sites marked by COSA-1 (crossover site-associated-1) also increased. Further, the 1,000 rads dose produced six COSA-1 foci in 90% of the nuclei, indicating that non-spo11 dependent DSBs can satisfy obligate crossover formation. Interestingly, further increases in DSB resulting from IR doses above 1,000 rads and up to 10,000 rads did not increase the number of COSA-1 foci beyond six per nucleus (138, 143). In addition, a similar
experiment using a Spo11 transgenic mouse model that overexpresses SPO11 to produce 25% more DSBs also yields a number of crossover foci comparable to wild-type (68).

Hence, the obligate crossover mechanism sets the lower limit for crossover formation, whereas the crossover interference mechanism sets the upper limit for total numbers of crossovers per nucleus. Together, these two crossover control mechanisms shape crossover homeostasis to ensure that only a limited, optimal number of crossovers form from meiotic DSBs. This was demonstrated in C. elegans by Drs. Hillers and Villeneuve (144) who generated an elongated chromosome mnT12 by fusing chromosome IV with chromosome X. They observed that 51% of these chromosomes had a single focus and the rest formed two crossovers that were placed far from each other (144).

1.5.4. Crossover designation

Crossover designation is a process in which a subset of DSBs become molecularly fated to become crossovers but have not yet fully matured into crossovers (145). This step is proposed to occur at the D-loop to SEI transition and corresponds to leptonema/zygonema of prophase I where the axis and synaptonemal complex are forming (15). Sites associated with DSBs, recombination and crossing over are closely associated with chromosome axis and synaptonemal complex proteins (146). These events can be observed on the chromosome axis as nodules microscopically. Recombination nodules were first discovered in Drosophila melanogaster oocytes using electron microscopy, and they were found to be encased within the synaptonemal complex protein (147). Further studies confirmed that recombination nodules exist in most organisms. Early nodules contain RAD51/DMC1 that mark the ~300 DSB sites per mouse spermatocyte nucleus. As these DSBs are repaired, they form transitional nodules that contain RPA, MutSγ (MSH4/5) and BLM but not RAD51/DMC1. MutSγ promotes crossovers and stabilizes potential crossover precursors. The total number of transitional nodules has now been reduced to ~150 during zygonema to early pachynema. Only a small number of transitional nodules will become recombination nodules marked with MLH1, with ~25 per nuclei by mid-pachynema (Fig. 6) (71, 146).
It is thought that some transitional nodules represent crossover-designated sites. Early evidence for a crossover designation mechanism comes from electron microscopy studies of human oocytes. During zygonema, some of the transitional nodules present in human oocytes were larger than others, and the size of these larger nodules was consistent with the size of the crossover-associated nodules observed during pachynema (148). Similar observations were made in mouse spermatocytes. Moens and colleagues (146) hypothesized that if MLH1-associated recombination nodules are derived from transitional nodules, then nascent MLH1-containing nodules should become associated with RPA-containing transitional nodules. As expected, they found that as MLH1 begin to emerge, both MLH1 and RPA were present in the newly formed recombination nodules. From this observation, they suggested that only one or two out of 15-20 transitional nodules progress to become associated with the crossover marker MLH1. Importantly, transitional nodules are distributed evenly along the length of the chromosome and display interference. Therefore, crossover designation is closely tied to interference and homeostasis. Collectively, these results established the current model of crossover designation as an event in which a subset of DSBs are selected to mature into potential crossovers (Fig. 6).

The current model for crossover designation interference posits that DSB sites chosen for designation spread a strong inhibitory signal around the site that prevents designation of another nearby site (149). This signal decreases with distance, which allows a distal DSB precursor to become designated. By selecting a number of DSBs as future crossovers early on, it guarantees the formation of crossovers, especially obligate crossovers.

Like crossover interference, crossover designation is also genetically regulated. Studies in budding yeast identified Zip3 as a crossover designation marker (145). Zip3 is an E3 SUMO (small ubiquitin-related modifier) ligase that modifies ubiquitin-like molecules and is involved in SUMOylation, a reversible post-translational modification (150). A mammalian ortholog of Zip3
Figure 6. Crossover designation to maturation.

Left: L= leptonema; Z= zygonema; P=pachynema. Some DSB foci reduce to form transitional nodules or intermediate foci (red circles). These nodules are stabilized by MSH4/5 and display interference by preventing formation of other nearby nodules. During zygonema, some of intermediate foci become selected as future crossover sites in a process known as crossover designation. Designated sites are denoted as bigger red circles. At pachynema, designated sites mature into crossovers, which are also display interference, preventing other crossovers from forming nearby. However, crossover maturation inefficiency will cause designated sites to fail to mature (red hollow circle). Black vertical lines between the parental chromosomes indicate interhomolog interactions.

Figure adapted from Audrey Lynn, Rachel Soucek, & G. Valentin Borner. “ZMM proteins during meiosis: Crossover artists at work”. Chromosome Research (2007) 15:591-605.

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is RNF212. In mice, RNF212 can be seen forming foci early during leptonema/zygonema. By early pachynema, ~35% of RNF212 foci co-localize with MSH4 foci but by mid-pachynema, approximately ~92% of RNF212 foci co-localize with MSH4 foci, which is consistent with ~76% of Zip3 foci co-localizing with Msh4 in budding yeast (151). Approximately 82% of RNF212 foci co-localize with MLH1 during pachynema, indicating that RNF212 marks crossover sites (152). In the absence of RNF212, the number of intermediate foci marked with MSH4 dramatically diminishes at early pachynema and no crossovers form. Based on this evidence, RNF212 has been proposed by Reynolds and colleagues (152) to be a crossover designation factor that may SUMOylate MSH4 to stabilize crossover-specific intermediates. Any sites marked with MSH4 that are not stabilized by RNF212 presumably dissociate to form noncrossovers.

Additionally, STUbLs (SUMO-targeted ubiquitin ligases), proteins that ubiquitinate SUMOylated target proteins, are also important for crossover designation and interference (149, 153, 154). HEI10 is a ubiquitin ligase STUbL protein that is required for crossing over (155) that reaches its peak expression levels during mid-pachynema. In the absence of HEI10, RNF212 and MSH4 fail to dissociate from the axis and persist until diplonema in mouse spermatocytes, reflecting an excess of designated sites. Ironically, the stabilized intermediates that persist in the absence of HEI10 fail to form crossovers prompting Qiao and colleagues (154) to propose that HEI10 is likely involved in ubiquitinating RNF212 during crossover designation so that RNF212 may be removed to allow downstream repair activity. Also, they suggested that HEI10 likely plays an important role in crossover maturation but acts upstream of MLH1 because HEI10−/− spermatocytes cannot form MLH1 foci (154). As will be discussed in more detail in the subsequent section, crossover designation is part of the ZMM crossover pathway that also functions in crossover interference.

1.5.5. Crossover maturation

The physical execution of a crossover-designated site becoming a bona fide crossover is called crossover maturation (156) (Fig. 6). Crossover maturation is the step in which a crossover forms between homologs from the designated precursor, and can be identified by the
presence of MLH1 foci. In order for faithful crossover execution to occur, all aforementioned crossover control mechanisms, obligate crossover formation, crossover interference, crossover homeostasis, and crossover designation, need to intricately work together to tightly regulate the final number of crossovers and each of their locations. Theoretically, the maturation of crossovers from designated precursors should be 100%. However, as will be discussed in more detail in a later section, some meiotic products, e.g. juvenile spermatocytes and human oocytes, are prone to maturation errors.

1.5.6. Class I interfering ZMM-dependent vs. Class II non-interfering ZMM-independent crossover pathways

The MLH1/3-dependent crossover pathway produces type I crossovers and is ZMM-dependent. This pathway involves winnowing selected DSB precursors to designation sites, which later become fully mature crossovers. ZMM proteins play a critical role in crossover designation and proper synaptonemal complex formation. Collectively, the yeast proteins Zip1/Zip2/Zip3/Zip4, Msh4/5, and Mer3 make up the ZMM proteins and function to ensure crossover formation that displays interference by promoting synaptonemal complex assembly between the homologs (157). A close ortholog of Zip1 in mice is SYCP1, which makes up the central region of the SC. Zip2 and Zip4 are required for synapsis initiation and crossover formation, and both are implicated in SUMOylation and ubiquitination likely involved in protein interaction and modification (158, 159). Zip3 also has SUMOylation activity and RNF212 and HEI10 are its functional orthologs in mice. Genetic ablation of all ZMM proteins in yeast showed that: 1) DSBs form normally but persist; 2) SEI formation is either delayed and/or formed at severely reduced levels; and 3) there is an almost complete abrogation or significant reduction in the number of crossovers (13, 15). By contrast, noncrossover formation was largely unperturbed in these yeast mutants.

In S. cerevisiae, deletion of any one of ZMM genes lead to defects in synapsis. Zip1 mutants paired but failed to synapse (13). All of ZMM mutants accrued normal levels of SEI and dHJ intermediates without forming normal numbers of crossovers, and the few crossovers
that do form do not display interference (82, 160). The Zip1 mouse ortholog is SYCP1, and mouse spermatocytes lacking SYCP1 align homologous chromosomes but fail to synapse and form crossovers, even though RAD51/DMC1, RPA and MSH4 foci form at frequencies similar to wild-type (10). On the other hand, Rnf212 null mice do not have an apparent synapsis defect (152), even though they fail to form crossovers. Loss of Msh4 and Msh5 in budding yeast leads to more slowly repaired DSBs, impaired synapsis, and a two-to-three-fold reduction in crossover formation, and crossover interference was not observed (15, 161, 162). Loss of MSH4 and MSH5 in mouse spermatocytes results in defective pairing/synapsis and no crossovers, indicating that MSH4/5 promotes pairing/synapsis to eventually form crossovers (163, 164). Mer3 is an ATP-dependent helicase, and its mammalian ortholog is HFM1. Budding yeast lacking Mer3 show delays in DSB repair and a 50-60% reduction in crossovers, whereas noncrossover frequency is unaltered despite extension of gene conversion tracts (165). It has been proposed that Mer3 stabilizes strand invasion and heteroduplex extension after Rad51 has loaded onto ssDNA 3’ overhangs (166-168). Hfm1−/− spermatocytes do not display MLH1 foci and only a few bivalents can be observed at metaphase, likely formed by SSEs. Like spermatocytes with defective RNF212, these spermatocytes do not show any synapsis defects, but DSB repair is delayed. Far fewer MSH4 foci are also observed, indicating aberrant crossover designation (169).

Conversely, crossovers formed from SSE pathways do not show interference and are referred to as ZMM-independent type II crossovers. The non-interfering nature of this pathway was discovered through studies of the residual crossovers observed in ZMM mutants, which did not exhibit crossover interference (15, 82, 160, 166). Investigation of residual crossovers present in Msh4 null yeast by genetic mapping of known intervals showed that interference was disrupted (161). While S. cerevisiae does not necessarily require synapsis to form crossovers (because ZMM-independent crossovers still arise in the presence of impaired synapsis), mice depend on synapsis formation for crossovers to form, as shown by Syct1−/− and Msh4/5−/− mice in which a lack of crossovers is accompanied by defective synapsis. However, failure to form
crossovers in these mice could also be due to a checkpoint that triggers apoptosis before crossovers can form.

1.5.7. Analysis of recombination outcome at mouse meiotic hotspots

Despite the extensive molecular studies of meiotic DSB repair in yeast, worms, and flies, very little detail is known about the recombination pathways in a mammalian system at the DNA level, especially in vivo. I was interested in probing the molecular and structural mechanisms of mammalian meiotic recombination to make better inferences to human meiotic recombination. Moreover, we know little about what happens at the molecular level in meiosis relative to what we know about chromosome behavior based on cytology.

As in budding yeast, some hotspots have been analyzed in mouse and human spermatocytes and oocytes to obtain and amplify meiotic recombination outcomes at the DNA level to study the molecular characteristics, frequency, and distribution of each outcome (65, 70, 170-173). Analysis of mouse hotspots requires high polymorphism density between parental alleles. One way to achieve this is by generating F1 hybrid offspring from crossing two inbred mice of different genetic backgrounds. This allows us to choose meiotic hotspots with high polymorphism density to detect noncrossovers and crossovers at high resolution. One such hotspot, Psmb9, was identified by genetic analysis in R209xB10 F1 hybrid spermatocytes on mouse chromosome 17 and used (64, 170) to amplify recombination outcomes at selected polymorphic regions. My Ph.D. advisor, Dr. Francesca Cole, developed an allele-specific PCR method to amplify crossovers and noncrossovers at another hotspot, A3 located on mouse chromosome 1 from sperm obtained from various F1 hybrid backgrounds. The high polymorphism density enhances the detection of noncrossovers: Crossovers can always be amplified via PCR because they will convert multiple polymorphisms resulting in long tracts; however, noncrossovers cannot always be amplified because gene conversion tracts associated with noncrossovers are short. Also, a gene conversion event that does not incorporate any polymorphisms will result in an outcome indistinguishable from the parental chromatid. By choosing a hotspot that is high in polymorphic density, the detection of
noncrossovers can be maximized. Indeed, recombination analysis at A3 revealed a 10:1 ratio of noncrossovers to crossovers, mirroring the global DSB to crossover conversion ratio of ~10 to 1 (65, 174).

A second hotspot was also characterized, 59.5, located on mouse chromosome 19, the shortest mouse autosome. This hotspot was chosen based upon the high number of DSBs recovered by SSDS sequencing (130) and the high density and even distribution of polymorphisms between inbred mouse strains B6 and DBA. Importantly, B6 and DBA share the same Prdm9 allele, minimizing any potential hybrid incompatibilities between these strain backgrounds that would affect hotspot analysis. Further, the A3 and 59.5 hotspots share a similar polymorphism frequencies and distributions (65, 70). If all chromosomes are equal, recombination analysis at 59.5 should also yield a 10:1 noncrossover to crossover ratio.

However, it has been long hypothesized that meiotic recombination dynamics, mechanisms, and regulation on short chromosomes must differ from long chromosomes based on cytological evidence. Due to the exquisite regulation of crossovers by interference mechanisms, long chromosomes often receive two or more crossover-designated sites, whereas short chromosomes almost always receive only one. Long chromosomes are often guaranteed to mature at least one designated crossover site even if the other designated crossover site fails to mature, satisfying the obligate crossover requirement for proper chromosome segregation (Fig. 6). However, a failure to mature a crossover on short chromosomes, which normally receive only one crossover, can result in a nondisjunction event that can lead to aneuploid gametes. Because the consequences of crossover maturation failure are more deleterious on short chromosomes than long chromosomes, it has been hypothesized that short chromosomes likely have distinct molecular mechanisms that differ from long chromosomes to improve their success rate. Several lines of molecular evidence support this idea. For example, Monte Carlo simulation of random DSB assignment in budding yeast chromosomes yielded 4% of nuclei harboring at least one chromosome that will fail to receive DSBs, and the majority of these were short yeast chromosomes (chr1, 3, and 6). Indeed,
smaller chromosomes recruit more DSBs and retain more DSB machinery at the axis for DSB retention and crossover assurance (103) (291).

Congruent with these data, we found that the 59.5 hotspot in B6 x DBA F1 spermatocytes shows a 1:1 ratio of crossovers to noncrossovers rather than the 1:10 frequency documented at A3 and the expectation based on the number of RAD51 and MLH1 foci seen in cytological experiments (70). This suggest that the 59.5 hotspot is under strong pressure to ensure crossover formation. Although we cannot extrapolate results from a single hotspot to draw conclusions regarding global recombination phenotypes on all short chromosomes, the unique phenotype found at 59.5 provides an excellent tool to analyze the consequence of crossover regulation failure at the DNA level in mutant mice.

Even though advances in sequencing technology have proved to be a useful tool in biological science, application of genome-wide sequencing to detect noncrossovers has been very difficult. A major reason for this is that the error rate in sequencing is roughly equivalent to the recombination frequency at individual hotspots (1~2%) (292). Additionally, Illumina-based sequences are short, ranging from 50 to 250 bp. Therefore, haplotype phasing is difficult because most noncrossovers have short gene conversion tracts that incorporate only a single polymorphism. Recently, the laboratory of Dr. Simon Myers developed a method to sequence noncrossovers genome-wide using F5 hybrid mice. Using this method, they found the average gene conversion tract length for noncrossovers was 30-41 bp (292), consistent with previous reports using the A3 hotspot (65) and human hotspots (55 - 290 bp) (173). However, this genome-wide sequencing method requires the generation of F5 hybrid males, which does not allow infertile mutant animals to be analyzed. Plus, large numbers of samples are required, which is expensive and time consuming. Therefore, performing a fine-scale recombination assay at individual hotspots is a valuable tool to study the frequency, distribution, and characteristics of recombination outcomes.
1.6. Mouse reproductive system

To study the molecular and structural dynamics during meiotic recombination, I used mouse meiosis as my model system. Here, I will discuss the similarities and differences between mouse spermatogenesis and oogenesis, and why I have chosen spermatogenesis for my doctoral research.

1.6.1 Oogenesis

Oogenesis is a process in which an oocyte develops from progenitor oogonia. Both oocytes and spermatocytes derive from precursor cells called primordial germ cells (PGCs). Around mouse embryonic stage E10.5 (embryonic gestation 10.5 days), mPGCs (mouse PGCs) migrate and colonize the gonad and proliferate. Sex differentiation in the gonads occurs at E12.5, and proliferating female mPGCs are now called oogonia. Oogonia continue to proliferate by mitosis in the embryonic ovaries until E13.5. Then at E14, oogonia enter meiotic prophase I to become primary oocytes. Oocytes complete crossover formation and progress to diplonema. Prior to birth, oocytes undergo dictyate arrest at late diplonema and form primordial follicles that remain in the so-called reserve. At and soon after birth, approximately 2/3 of the oocyte pool is eliminated by massive apoptosis known as “female oocyte attrition” (175, 176). This occurs in part by derepression of LINE1-transposons during epigenetic reprogramming (177). During puberty, the remaining primordial follicles of the reserve undergo folliculogenesis to grow in size. Just prior to ovulation, dictyate oocytes progress to metaphase I and arrest again at this stage. Upon ovulation, meiosis I completes and one oocyte and one polar body are released. The oocyte then arrests again during metaphase II. It is not until fertilization that meiosis II completes and releases another polar body (178).

1.6.2. Aneuploidy associated with female (and male) meiotic recombination

It is estimated that approximately 20% and 1-2% of human and mouse eggs, respectively, are aneuploid (179). There are three major possible events that can lead to aneuploidy: 1) cohesion fatigue; 2) crossover maturation inefficiency; and 3) loss of integrity in the spindle attachment and assembly check point in older women.
Prior to entry into meiosis, the cohesins that hold sister chromatids together form during premeiotic DNA replication. These cohesins are positioned during embryogenesis and remain in the oocyte pool until menopause. As women age, the cohesins lose integrity, a phenomenon known as cohesion fatigue. It is postulated that the weakening of the connection between sister chromatids is the major cause of age-related aneuploidy observed in human eggs. Although it has not been shown for humans, it has been shown that oocytes do not replace old cohesins with new cohesins in mice (180), suggesting that the high aneuploidy level observed in human eggs may be due to a lack of replenishment of cohesion in human oocytes. Without robust sister chromatid cohesion, the tension required to pass through the spindle assembly checkpoint will be suboptimal, leading to missegregation of chromosomes (178).

Another factor that may contribute to a suboptimal number of crossovers and aneuploidy, is an aberrant crossover maturation program collectively referred to as crossover maturation inefficiency. Crossover maturation inefficiency is more pronounced in human oocytes than spermatocytes. Compared to male chromosomal loop formation that occurs at the beginning of prophase I, females form relatively shorter DNA loops, thus leading to longer axes. Because the number of DSBs formed on the chromosome is proportional to the length of the axis, females receive more DSBs and as a result, more recombination and crossovers. But proportionally, females actually form fewer crossovers per axis length due to crossover maturation inefficiency (156, 181). Proper crossover maturation ensures an optimal number of crossovers with interference to form.

Shorter chromosomes often receive fewer crossovers than long chromosomes due to interference and consequently have only one crossover designation site. Therefore, failure to mature a designated crossover site, particularly on a short chromosome, can result in no crossovers for that chromosome. Alternatively, chromosomes that receive two designation sites will likely have one on each arm of the chromosome, away from each other and separated by
Figure 7. Crossover maturation inefficiency in females.

Top. Examples of optimal tension vs. reduced tension due to a distal crossover that can mono-orient chromosomes. Bottom. (i) Optimal tension is produced due to proximal placement of crossovers. Crossover maturation inefficiency can lead to a loss of crossovers and minimal tension (ii). (iii) Optimal tension produced with two interfering crossovers can be lost when crossover maturation inefficiency at one of the crossovers results in a single distal crossover (iv) or loss of age dependent cohesion loss can lead to reduced tension (v) or minimal tension (vi). Reprinted from Shunxin Wang, Terry Hassold, Patricia Hunt, Martin A. White, Denise Zickler, Nancy Kleckner, Liangran Zhang. “Inefficient crossover maturation underlies elevated aneuploidy in human female meiosis”. 2017, Cell 168, 977-989

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the centromere. Proper maturation of both designation sites will confer optimal tension. However, failure to mature one of these designation sites will lead to a single crossover that is formed distally from the centromere/kinetochore, and these are more likely to undergo nondisjunction due to the loss of cohesins at the site of crossovers. (Fig. 6 & 7).

Evidence for this comes from an early investigation by Ross and colleagues using yeast artificial chromosomes (YACs) and five auxotrophic genetic markers. A pair of yeast artificial chromosome homologs were derived from a bacteriophage \( \gamma \) plasmid with specific yeast genes with restriction sites inserted at precise locations that act as markers for crossovers. Yeast tetrad analysis performed with these spores indicated that crossovers that formed distally from the centromere had nondisjunction rates of up to 15%, whereas proximal crossovers had nondisjunction rates of no more than 5%. This suggests that distal crossovers are less likely to result in proper chromosome segregation (182), which is supported by the finding that distal crossovers are associated with chromosome trisomies 16 and 21 in human females (134). Furthermore, crossovers form in close proximity to the centromere are equally deleterious as shown by an experiment with yeast artificial chromosomes (YACs) in which gene conversion events that occurred in the YAC pericentromeric region were associated with a higher frequency of chromosome mis-segregation during meiosis I (183).

Therefore, both the presence of a crossover and its physical location are important for ensuring proper chromosome segregation. Crossover formation coupled with sister chromatid cohesion, mediated by cohesion proteins, physically connects the homologs. When the spindles, which originate at opposite poles of the cell, attach to the centromere/kinetochore of the homologs and begin to stretch or pull the chromosome toward the poles, the kinetochore experiences tension. The tension is dependent on the position of the crossover. Crossovers that form near the centromere (proximal) provide the most optimal tension. This was exemplified by a clever experiment designed by Lacefield and Murray (184) using budding yeast lacking Mad2. Mad2 (mitotic arrest deficient 2) functions as a part of spindle assembly checkpoint mechanism that facilitates the biorientation of the chromosomes. However, in the
absence of Mad2, 52% of budding yeast chromosomes still successfully biorient their chromosomes, and most of the mis-segregated chromosomes were long chromosomes (185). Crossover mapping using SNPs in a yeast hybrid strain revealed that the distal (telomere proximal) crossovers are more likely to form on the long chromosomes and that these are more likely to disjoin in the absence of Mad2. This indicates that the spindle assembly checkpoint assists chromosomes with distal crossovers to bi-orient, and that crossovers that form proximally to centromeres likely promote proper chromosome segregation even in the absence of Mad2. To test whether a proximal crossover can rescue the segregation defect, they integrated the tetramerizing Lac repressor/operator system to produce DNA crosslinks at various locations on the long chromosome that showed higher frequency of distal crossovers, and therefore, higher incidence of aneuploidy. The Lac repressor/operator system produce an artificial tether that mimics crossovers. Using this approach, they found out that only the tether near the centromere in this chromosome was able to rescue chromosome segregation (184).

Additionally, despite proper crossover maturation, suboptimal tension can still occur due to cohesion fatigue as oocytes age. Loss of cohesins near proximal crossovers can compromise the spindle’s physical linkage to the kinetochore and therefore reduce tension leading to mis-segregation of chromosomes. Likewise, a single distal crossover that forms as a result of crossover maturation inefficiency, compounded by cohesion fatigue, will lead to compromised tension. Because cohesins are lost where crossovers form, the loss of cohesins near distal crossovers will reduce the physical connection between the homologs, thus reducing tension leading to a higher probability of mis-segregation of these chromosomes (156).

Crossover maturation inefficiency is not limited to females. Sperm from young men display a higher incidence of aneuploidy and young men are more likely to father a child with Down syndrome (186). Our lab showed that juvenile human spermatocytes have longer axes, akin to those of human oocytes, and a 30% reduction in MLH1 foci compared to adult men that may be due to crossover maturation inefficiency (70).
Overall, any event that disrupts spindle attachment and reduces tension on the kinetochore complex can also result in aneuploid gametes. Spindle assembly checkpoint proteins help bi-orient chromosomes under sub-optimal tension, i.e., chromosomes with distal crossovers. If sub-optimal tension is formed, it can also promote an equational configuration of the kinetochores akin to what occurs in mitotic division, resulting in premature segregation of the sister chromatids during metaphase I (Fig. 7). Equational kinetochore configuration can arise from a crossover forming near the centromeric region, which results in the loss of cohesion near the centromere (179). Therefore, maintenance of cohesion near the centromere is imperative to segregate sister chromatids to the same pole during meiosis I and to the opposite pole during meiosis II (187).

Even though studying female meiotic recombination is important and has strong clinical implications, the timing of oogenesis and complex biology of the female reproductive system makes experimental investigation of meiosis difficult. Because female meiosis occurs in a single wave, only a small number of oocytes are produced (178). Therefore, I have chosen male mouse meiosis as a model system to study meiotic recombination.

1.6.3. Mouse spermatogenesis

The onset of spermatogenesis within the seminiferous tubules of the testes coincides with male puberty. Spermatogonia are the precursor cells that will become spermatocytes through the process of spermatogenesis. Spermatogonia undergo three major processes: 1) expansion by mitosis; 2) meiosis; and 3) spermiogenesis (Fig. 8).

Spermatogonia A can either commit to differentiation or continue to proliferate by mitosis to produce additional spermatogonia A with the ability to self-renew, thereby maintaining a stable pool of spermatogonium. The rest commit to irreversible differentiation by six mitotic division (A1, A2, A3, A4, In (Intermediate), and B cells) with the exception of the first round of spermatogenesis where spermatogonia undergo only five mitotic divisions (188). For each spermatogenic wave, approximately 9 - 11 spermatogonial cells proliferate, and but
Figure 8. The cycle of seminiferous epithelium.

Each stage of spermatogenesis, ranging from I to XII, indicates the type of cells present at particular cross sections of the seminiferous tubules. The red arrow indicates the stage when retinoic acid is required for meiotic entry. The numbers of days that cells spend at each stage is indicated at the bottom of the table. Each spermatogenic wave fires every 8.62 days.

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without complete cytokinesis, resulting in a syncytial colony of 500 – 2000 cells that enter synchronized meiosis. After spermatogonia B form, the cells enter the preleptotene stage and initiate meiosis. From preleptotenea, spermatocytes enter meiosis I and progress through the substages of prophase I: leptonema, zygonema, pachynema, and diplonema. Then, they undergo metaphase I to separate the homologs. Successful homolog segregation and cell division lead to the formation of secondary spermatocytes the byproducts of meiosis I. These secondary spermatocytes rapidly enter and complete meiosis II to segregate the sister chromatids, thus generating haploid round spermatids. Round spermatids then begin a series of morphological changes known as spermiogenesis to finally mature into spermatozoa with motile tails and a compacted sperm head. As spermatogenesis initiates in spermatogonia within the seminiferous tubules, the cells migrate slowly from the outer basal lamina toward the inner lumen. By the time spermatozoa are formed, they reach the lumen of the seminiferous tubules and migrate into the epididymis where they reside until future release. In mice, the developmental processes required to form spermatozoa from spermatogonia take ~60 days, over one spermatogenic wave/cycle.

My research interest lies in the substages of prophase I where meiotic recombination occurs. In order for meiosis to occur, retinoic acid must be expressed endogenously within the testes to activate the Stra8 gene in spermatogonia to initiate meiotic entry. Spermatogenesis can be further divided into 12 stages (I to XII) defined by type of cells present at particular location of the seminiferous tubules. Unlike oogenesis, spermatogenesis continuously produces sperm by inducing multiple spermatogenic waves, which guarantees recovery of a sufficient number of spermatocytes for analysis. Importantly, a new spermatogenic wave initiates every 8.6 days, and the amount of time germ cells spend in each stage of spermatogenesis is both stable and predictable (189) (Fig. 8).

1.6.4. Transcription and XY sex body formation in male spermatocytes

In addition to spermatocyte production being different from oocyte production in that male germ cells are continuously produced, male meiosis has unique features not observed in
female meiosis. During male meiotic prophase I, a large amount of transcription occurs during mid pachynema. This contrasts starkly with mitotic prophase in which transcription is largely suppressed due to chromosome compaction and loss of compartment structures (44). This difference has been attributed to the length of mitotic prophase vs. meiotic prophase I; mitosis only lasts for ~2 hours (190), whereas meiotic prophase I lasts ~11 days (189). The time spent in prophase I necessitates transcription of genes required for downstream events (191). In particular, Piwi-interacting RNAs (piRNAs) that are important for silencing retrotransposons are transcribed during mid-pachynema (192).

During germ cell development, a vast amount of DNA demethylation occurs followed by de novo methylation to establish genomic imprinting. Genomic imprinting is a type of epigenetic reprogramming that causes genes to be expressed in a parent-of-origin specific manner. Epigenetic modification typically effect gene expression without altering DNA sequence. DNA methylation generally represses gene transcription and transposon transposition, whereas demethylation generally activates transcription and can activate transposon transposition that can trigger genome-wide translocation events and genomic instability (193). Transposons do not leave structural or chemical marks that are recognized by DNA repair proteins (192); however piRNA contains sequences complementary to transposable elements, and when transcribed, they can bind to PIWI protein, which then enters the nucleus and scans for nascent RNA transcripts that are complementary to transposable element. When transposon RNAs are transcribed, piRNAs recruit histone methyl transferase to deposit repressive histone marks that silence transposon transcription (194).

A unique feature of spermatocytes is the formation of the sex body (195). Females have two X chromosomes that behave like autosomes in that there are no inherent delays or defects in pairing or synapsis. By contrast, males have heteromorphic X and Y sex chromosomes. These have vastly different sizes and share little homology (196). The Y chromosome is ~700 kb in mice and 2.6 Mb in humans, whereas the X chromosome is 171 Mb in mice and 155.3 Mb in humans (89). As a result, synapsis occurs only at the pseudoautosomal region (PAR), a
short region that shares sequence similarity between the X and Y chromosomes, and that must receive at least one DSB to successfully pair and synapse the sex chromosomes, and that DSB must be repaired as a crossover in male spermatocytes.

Given the small size of the PAR, a random distribution of DSBs will generate a high number of sex chromosomes without DSBs in the PAR region. Despite this, early mouse genetic analysis using three genetic intervals (two located on PAR region) on the Y chromosome revealed that the frequency of crossovers formed on the PAR is 7-fold higher than in females (197). This suggests that there is a mechanism that promotes more DSBs to form on the PAR during meiosis. Kauppi and colleagues (26) hypothesized that the PAR can accomplish this by organizing its chromatin in smaller loops so that a longer axis will form, thus increasing DSB potential. Other reports using FISH probes and MLH1 foci to measure the relationship between axis length and crossover frequency revealed that axis length is proportional to crossover frequency in mouse spermatocytes (198). Indeed, axis length measurements using FISH probes specific to sequences on the PAR and mouse chromosomes 18 and 19 revealed that the axis formed on the PAR is at least 10-fold longer than the autosome axis. A longer axis indicates that there is less DNA content per µm of axis length (Mb/µm). Such a difference can presumably result in 10 times more DSBs on the PAR (26).

Outside of the PAR, the X and Y chromosomes remain mostly unsynapsed. During prophase I, a checkpoint mechanism called “meiotic silencing of unsynapsed chromatin” (MSUC) is triggered in pachynema to detect unsynapsed chromosomes. Any unsynapsed chromosomes undergo MSUC-mediated chromatin remodeling to repress the transcription of genes located on unsynapsed regions. MSUC drives “meiotic sex chromosome inactivation” (MSCI) that silences transcription of the largely unsynapsed sex body in male spermatocytes (199). Transcriptional silencing of the sex body is a prerequisite for faithful progression of prophase I. It has been proposed that MSCI may occur in order to prevent transcription of potentially toxic genes on the sex body. In support of this argument, XYY male spermatocytes show a high incidence of Y-Y synapsis. As a result, the Y chromosome fails to undergo
transcriptional silencing, misexpresses lethal sex-linked genes, and the spermatocytes arrest at mid-pachynema (200). Therefore, failure to trigger MSCI is detrimental to meiotic cells. When extensive asynapsis is present, i.e. PWD x B6 F1 hybrid sterile males (refer to section 1.4.2. Meiotic DSB landscape), MSCI fails to occur, leading to prophase I arrest (199).

Sex chromosomes in males are more likely to fail to synapse, resulting in gametes with sex chromosome aneuploidy (201). Offspring formed from these gametes will have a sex chromosome disorder such as Turner syndrome (XO), Kleinfelter syndrome (XXY), trisomy X (XXX), XYY syndrome or XYYY syndrome (202).

1.7. Current gap in knowledge

There are critical questions that currently remain unanswered in the field of meiosis. Mechanistic investigation of model organisms, such as budding yeast and C. elegans has provided important insights into how cells ensure proper chromosome segregation. Our overarching goal is to understand how meiosis is regulated and processed in a mammalian system. Many meiotic events that occur in model organisms are also well conserved in mice, but defining the details of mammalian meiotic recombination beyond those discovered through cytology is largely unknown. It has been difficult to test the ideas gleaned from model organisms in mice due to lack of tools available to study mammalian meiotic recombination, especially the noncrossovers until recently. Ultimately, our work will uncover mechanisms involved in meiotic recombination in mice to make inferences about human meiosis and identify potential clinical applications that will improve human reproductive health, fertility and understanding of hereditary diseases. My thesis project aims to gain insights into the following questions in the field:

1) How do chromosomes avoid non-allelic homologous recombination during meiosis?

Compared to the genome of budding yeast, mammalian genomes are much larger and are replete with repetitive DNA sequences that are largely absent in yeast, with the notable exception of ribosomal DNA (rDNA) repeats that encode ribosomal RNA (203). The sizes of the yeast, mouse and human genomes are 12.1 Mb, 2.8 Gb and 3.3 Gb, respectively. Importantly,
the overall recombination rate of an organism tends to be inversely proportional to its density of repetitive sequences. Indeed, the recombination rate observed in mammals is far lower than in budding yeast (204).

Approximately 70% of the human genome consists of repetitive elements, which include LINEs (long interspersed nucleotide elements), SINEs (short interspersed nucleotide elements), low-copy repeats (LCRs) transposons, retrotransposons and satellite, telomeric, subtelomeric and centromeric repetitive DNA. (205). For example, Alu repeats, a class of SINE, share a high level of sequence similarity to each other and are present in roughly 1 million copies in the human genome making up ~10% of its sequence. This high density of repetitive elements can cause problems when recombination occurs: when DSBs form at repetitive sequences, non-allelic substrates can be used as repair templates during HR, resulting in a process referred to as NAHR (non-allelic homologous recombination). A crossover resulting from such illegitimate recombination will cause gross chromosomal abnormalities, whereas a noncrossover will not lead to the reciprocal exchange of the entire chromosomal arm and is often phenotypically inert (Fig. 9). Germ cells containing chromosomes that formed illegitimate NAHR-crossovers often have certain regions of the genome deleted or duplicated. If these cells are involved in a successful fertilization event, they can produce offspring with genomic disorders. The etiology of many known developmental disorders, such as hereditary neuropathies and mental retardation point to germ line crossovers mediated by NAHR. For example, NAHR between LCRs on chromosome 17 can lead to duplication of the short arm of chromosome 17 in one meiotic product and a deletion of the same region in another. The duplication event can cause Charcot-Marie-Tooth disease, a condition that causes nerve damage in the arms and legs. The deletion event can result in offspring with hereditary neuropathy with liability to pressure palsies (206, 207).

How can such deleterious recombination be prevented? Budding yeast address this problem by altering the chromatin structure of the repetitive sequences. The ~150 rDNA genes
Figure 9. Non-allelic homologous recombination.

DSBs formed on repetitive sequences (black boxes) increase the chance of using the wrong substrate to repair these DSBs, resulting in NAHR. Although a noncrossover event will be relatively inert, a crossover event will lead to gross chromosomal abnormalities. Haploid gametes resulting from these meioses will contain chromosomal deletions and duplications which can lead to an individual with genomic disorders. For example, Charcot-Marie-Tooth disease arises from a haploid gamete with a duplicated short arm of chromosome 17.
that encode ribosomal RNA in budding yeast exist in tandem repeats spanning a ~9-kb region. Recombination in this region is largely suppressed by the product of a gene called silent information regulator 2 (SIR2). Sir2 protein is a histone deacetylase involved in gene silencing and chromatin remodeling. Mieczkowski and colleagues (208) performed chromatin immunoprecipitation experiments in \textit{sir2} mutants using Spo11 antibodies. They took the Spo11-bound DNA fragments and hybridized them to a DNA microarray containing all yeast genes and intergenic regions. They found that the loss of Sir2 increased the number of DSBs at the rDNA locus, as well as at telomeric and subtelomeric regions. This suggests that budding yeast prevent NAHR by making DNA inaccessible in regions of repetitive DNA to prevent DSB formation (208). In alignment with this finding, recombination is largely absent in the pericentromeric regions of mice and humans (209, 210).

Once DSBs are formed on repetitive sequences, how can NAHR be prevented? Any repetitive sequences that are abundant in mammals can become a potential template for DSB repair. Therefore, the choice of template for DSB repair will dictate whether NAHR occurs. In budding yeast, chromosome pairing is likely involved in suppressing the use of non-allelic substrate. This was shown in two ways using a reporter to assess crossover events: 1) by inserting a homologous chromosome with ~15% sequence divergence from \textit{Saccharomyces cerevisiae} into \textit{S. cerevisiae} as a pairing partner thus causing defective pairing; and 2) by creating a null \textit{NDJ1} mutation thus preventing chromosome bouquet formation and delaying pairing (211). Each experiment yielded a 7-fold increase in NAHR (212) suggesting that pairing is crucial in preventing NAHR.

Given the number of repetitive sequences in the mammalian genome, it is unlikely that NAHR is completely suppressed in mammals. It is possible that NAHR occurs frequently during meiosis but with less noticeable effects on copy number variation. Germ cells with non-idiopathic copy number variations generated by NAHR further contributes to genetic diversity, conferring each individual with predispositions and susceptibilities to some diseases but not others. Regardless, the probability of idiopathic chromosomal rearrangements that occur during
prophase I can only be estimated by directly measuring and identifying individuals with genomic disorders known to arise from NAHR, and they are relatively rare compared to seemingly normal individuals. In fact, when Turner and colleagues (213) performed sperm-based assays in humans to analyze four known NAHR hotspots, including the region that causes Charcot-Marie-Tooth/Neuropathy, they found that the prevalence of NAHR is similar to disease-based estimates. For example, their sperm-based estimate of NAHR occurrence linked to Charcot-Marie-Tooth disease is 1/23,000 to 1/79,000, whereas the disease-based estimate is 1/23,000 to 1/41,000 (213). Therefore, NAHR that leads to idiopathic crossovers can potentially be suppressed during meiotic recombination. It is also possible that the fertilization events resulting from germ cells with chromosomes that underwent NAHR are inviable. Regardless it is important to define the mechanisms meiotic cells use in mammals to suppress NAHR, whether they are similar to those used in yeast, and how they are regulated.

2) How do homologous chromosomes pair and synapse properly?

It is well established that meiotic DSBs are required for synapsis, although some pairing is observed prior to DSB formation (126). However, very little is known about how homologous chromosomes pair or how they recognize their homologous partner. It has been postulated that recombination is required for homolog recognition and pairing, and with the exception of C. elegans, in which pairing occurs independently of DSB formation, most organisms fail to show proper homolog pairing and alignment in the absence of meiotic DSBs (see section 1.4.3 Pairing, synapsis and recombination).

On the other hand, temporal analysis performed in synchronized budding yeast strongly supports the idea that synapsis precedes any recombination and is likely required for recombination, as noncrossovers were not observed until the time full synapsis was achieved (15, 48). Likewise, spermatocytes resulting from semi-synchronous spermatogenesis in juvenile mice, had no evidence of either noncrossovers or crossovers until most of spermatocytes were in pachynema, well after synapsis has occurred (64). However, the large genome of mammals requires that the free 3’ ends generated by SPO11-induced DSBs need
to scan a much larger region of the genome compared to organisms with smaller genomes. If
synapsis is required before recombination, then it suggests that meiotic chromosomes have a
mechanism that allows them to find and recognize their homologous partners, perhaps by
detecting sequence homology without strand invasion. However, if this was the case, a large
number of repetitive sequences could complicate pairing and potentially lead to a large number
of misaligned chromosomes which creates a higher probability of NAHR. Another possibility is
that both recombination and synapsis occur simultaneously and are interdependent on each
other.

3) How is it that all DSBs are repaired by diplonema?

Based on microscopy, it is thought that wild-type and mutant spermatocytes that
progress to metaphase I have repaired all SPO11-induced DSBs because very few
chromosome fragments or nicks remain at metaphase I and chromosomes still condense and
compact, which could be hindered by the presence of unrepaired DSBs. Therefore, if
spermatocytes have progressed to metaphase I, it indicates that they have repaired all of their
breaks.

If so, then how does the sex body in spermatocytes repair its DSBs? Only 5% of the
DNA of the human Y chromosome engages in recombination and crossing over (203).
However, even though the PAR regulates its chromatin structure to receive more DSBs, DSBs
are formed along the lengths of both the X and Y chromosomes as evidenced by gamma H2AX,
DMC1 and RAD51 focal staining during early prophase I, making it likely that the mouse X
chromosome repairs the DSBs by intersister recombination.

When Matos and colleagues (214) studied the role of Mus81/Mms4 and Yen1 in yeast,
they found that the deletion of YEN1 had no effect on spore viability whereas mus81 mutants
had a 50% reduction in spore viability with evidence that chromosome segregation failed during
meiosis I, leading to the hypothesis that Yen1 may be involved at a later stage. Indeed, they
performed additional experiments to show that Yen1 cannot resolve HJs prior to the formation
of metaphase II spindles are formed, and that phosphorylation of Yen1 suppresses its activity
on dHJs until anaphase II (214, 215). These results indicate that each recombination pathway is activated at distinct times during meiosis to repair DSBs. Additional evidence supports the notion that dissolution and SSE provide back-up pathways to cope with unrepaired DSB intermediates (80).

Do such back-up DNA repair pathways exist in mammals? If we can examine meiotic events at the DNA level at precise stages, then recombination outcomes that arise later than typically expected could be indicate that these outcomes derive from a backup pathway.

4) How do chromosome movement, organization and behavior relate to recombination outcomes at the molecular level? How are they interdependent with one another?

As noted earlier, there are many semi-redundant meiotic recombination pathways that can repair DSBs. To date, the pathways and the interrelationships between them have not been defined in an in vivo mammalian system. By studying recombination outcomes in knock-out mice, we can infer that individual meiotic recombination pathways are intricately interrelated. For example, there is cytological evidence for crosstalk between class I and II crossover pathways. When BLM is genetically ablated in mice, MLH1 foci remain unperturbed but the total number of chiasmata (i.e. the total crossovers from both pathways) increases compared to wild-type. This indicates that in the absence of BLM, most notable for its activity in dissolution, class II crossover pathway products increase (216). Because recombination and meiotic progression are interrelated, it is important to understand the recombination pathways associated with chromosomal behavior through the progression of prophase I. If we can connect the events that happen at a DNA level to a cytological chromosomal level, then we can also infer the intermediates associated with each pathway. We can also address the following questions: are different pathways interrelated and interdependent on each other? Are these pathways temporally distinct and related to chromosomal behavior at each stage? Do the pathways act contemporaneously? How are noncrossover pathways affected in the absence of another pathway? How do they interact in relation to crossover pathways and crossover intermediates from class I and II crossover pathways?
5) How do higher order chromosome structures change with meiotic progression?

Structural changes in chromatin and higher order chromosome organization affect cellular function, but remain largely unexplored in meiosis, particularly. chromosome organization through progression of prophase I. Meiosis must achieve multiple tasks: loop extrusion and axis formation, DSB formation at meiotic hotspots, homolog pairing, SC formation, crossover formation by recombination, transcription activation, and formation of the XY sex body and transcriptional silencing of unsynapsed chromatin. How do meiotic chromosomes regulate these events while condensing chromosomes during meiosis? What exactly is the nature of higher order of chromosomes structure? How do higher order structural changes correlate with each critical event that needs to occur through the meiotic progression?

To address these big picture questions, the goal of my thesis project was to: 1) isolate meiotic recombination events during specific stages of meiosis to study the temporal control of meiotic recombination by measuring recombination outcomes at the level of DNA; and 2) characterize higher order chromosome organization and the topological changes that occur at specific stages of meiosis.

However, to achieve these goals, I first had to develop a method to overcome the issue of all stages of spermatogenesis taking place in the testis at all times making it difficult, if not impossible to isolate sufficient numbers of cells at the right meiotic stage for molecular analysis. To address this problem, I developed a novel assay to isolate spermatocytes enriched at specific stages of prophase I by synchronizing mouse spermatogenesis.
Chapter 2: Materials and methods

This section is in part adapted from the materials and methods section of a research paper “Dynamic reorganization of the genome shapes the recombination landscape in meiotic prophase” published in *Nature Structural & Molecular Biology* in March 2019 by Lucas Patel, Rhea Kang, Scott C. Rosenberg, Yunjiang Qiu, Ramya Raviram, Sora Chee, Rong Hu, Bing Ren, Francesca Cole and Kevin D. Corbett with permission from the publishing journal (217). Hi-C figures in this section are extracted directly from the publication mentioned above.

2.1. Mice

C57BL/6J x DBA/2J (BxD) F1 hybrid male mice were generated by crossing inbred mouse strains C57BL/6J (B) and DBA/2J (D). *Mlh3*<sup>−/−</sup> BxD male mice were obtained by intercrossing B and D heterozygous mice for the *Mlh3* null allele (a gift of P. Cohen). The *Mlh3*<sup>+/−</sup> allele was backcrossed for up to seven generations into B and D mice (Jackson Laboratories) to render the A3 and 59.5 hotspots homozygous. At least three animals per time point for each genotype were analyzed. For Hi-C analysis, we mated female C57BL/6J and male CAST/EiJ mice (obtained from The Jackson Laboratory).

2.2 Synchronization of spermatogenesis

Synchronization of mouse spermatogenesis was performed as described in (218). One to three days postpartum (dpp) BxD F1 and BxCast F1 male neonates were pipette fed with WIN 18,446 at 100 mg/g of body weight for 7 consecutive days every 22 - 24 hours. On the 8th day of treatment, all mice were intraperitoneally injected with 200mg of retinoic acid suspended in 10ml dimethyl sulfoxide. Mice were allowed to recover 10 to 49 days after injection to synchronize spermatocytes at three distinct stages of prophase I – zygonema, pachynema and diplonema. Days post injection (dpi) were determined based upon published spermatocyte prophase timing and experimentation. Male neonates from *Mlh3* experimental crosses were tailed between 0 to 2 dpp for genotyping to determine the presence of homozygous null alleles prior to pipette feeding.
2.3. Isolation of synchronized spermatocytes by flow cytometry

Spermatocytes were isolated as described previously (66, 70) from approximately 1.5 decapsulated testes in 7.5mg (0.5mg/ml) of collagenase suspended in 15ml Gey’s Balanced Salt Solution (GBSS) for 15 minutes at 33°C at 500 RPM. After collagenase treatment, the seminiferous tubules were washed once in 15 ml of GBSS and digested in 15ml GBSS/7.5mg (0.5mg/ml) trypsin/20 to 50µl DNase I for another 15 minutes. The amount of DNase I was dependent upon the size of the testis. Then, 750ml of newborn calf serum (NCS) was added and cells were mechanically segregated using a transfer pipette for 3 minutes. Segregated cells were filtered through a 70mm cell strainer and spun at 2,000 RPM for 3 minutes. After supernatant removal, the pellet was re-suspended in 25ml of DNase I by tapping and washed once with 10ml GBSS/10ml DNase I/500ml NCS for 3 minutes at 2,000 RPM. Next, the cell pellet was re-suspended in 6ml GBSS/2% NCS/12 µl DNase I and stained with 5µg/ml of Hoechst 33342 (2.5µg/µl in DMSO and stored at 4°C in light-protected tubes) for 45 minutes at 33°C at 500 RPM. Finally, 0.2µg/ml of propidium iodide (PI) was added, filtered once more into 70µm cell strainer and placed under BD Aria or Fusion flow cytometer machines for sorting. The cells were initially separated by side scatter (y-axis) vs. forward scatter (x-axis) plot to disregard doublets followed by side scatter (y-axis) vs. PI(x-axis) plot to gate only on live cells. The spermatocytes were then separated by blue fluorescence (y-axis) vs. red fluorescence (x-axis) emitted by Hoechst 33342. Cells emitting the highest blue fluorescence located on top of cell profile were identified as 4C spermatocytes going through meiotic recombination, and only a seemingly synchronized 4C spermatocytes represented by small regions of densely compacted 4C populations were gated for sorting. Synchronized 4C spermatocytes were sorted into 2ml of GBSS containing 5% NCS. Sorted cells were counted by hemocytometer, and ~5,000 to 24,000 cells were set aside for post-sort chromosome spreads. The remaining cells were spun down in 1.5ml tubes at 3,100 RPM for 6 minutes, snap froze in dry ice, and stored at -80°C until genomic DNA isolation.
2.4 Preparation of pre-sort chromosome spreads

Approximately half of the testis was devoted to surface chromosome spreads to determine synchrony and prepared simultaneously with the sort preparation. Half of the testis was digested in 4mg of collagenase suspended in 2.2ml of TIM (Testes Isolating Medium: 45mM KCl, 104mM NaCl, 1.2mM MgSO₄, 1mM sodium pyruvate, 0.6mM KH₂PO₄, 0.1% (w/v) glucose, 6mM sodium lactate, pH 7.3, filter sterilized) for 55 minutes at 33°C at 500 RPM. The isolated seminiferous tubules were then washed with ~15ml of TIM three times and digested for another 15 minutes at 33°C at 500 RPM with 2ml TIM/1.4mg trypsin/20µl of DNase I. Immediately after trypsin digestion, 10mg trypsin inhibitor/500µl TIM and 50µl DNase I were added, and seminiferous tubules were mechanically segregated with a transfer pipette for 2 minutes. The segregated cells were filtered through a 70µm cell strainer and spun down at 1,000 RPM for 5 minutes. After supernatant removal, the pellet was re-suspended in 15µl of DNase I by tapping, brought to a total volume of 15ml in TIM and spun down at 1,000 RPM for 5 minutes. The washing step followed by DNase I treatment was repeated once more. Subsequently, the pellet was suspended in 4ml of 1xPBS buffer, pipetted up and down for homogenization and transferred to four 1.5ml tubes in equal volume. The tubes were spun down at 1,500 RPM for 5 minutes. After a complete removal of supernatant, the cell pellet was suspended in 80µl 0.1M sucrose and incubated at room temperature for 5 minutes to allow cells to expand. A total of 4-6 slides were prepared in a humidifying chamber. Approximately 30 seconds before the end of sucrose incubation, 65µl of 1% PFA (pH 9.2)/0.1% Triton X-100 solution was placed on the slides. Expanded cells were dropped onto each slide in 20µl aliquots onto the PFA solution. Slides were incubated at room temperature in a humidifying chamber for 2.5 hours, opened ajar for 30 minutes, fully opened for 30 minutes, then washed once with MilliQ water and twice with 0.4% Photo-Flo 200 solution. Slides were left to dry at room temperature and folded into an aluminum foil for storage at -80°C until ready for cytology.
2.5. Crosslinking for Hi-C library prep

At least 250,000 sorted cells were spun down at 500xG for 5 minutes at 4°C. The supernatant was removed and gently re-suspended in 5ml of resuspension buffer (1xPBS + 1% NCS). In order to crosslink the cells, 286µl of 37% Formaldehyde (Fisher Bioreagents Cat# BP531-500) was mixed with the cell suspension gently by inversion to bring the final formaldehyde concentration to 2% and incubated at room temperature for 10 minutes. Then, 470µl of 2.5M Glycine (Sigma Cat# 50046) was added to bring the final glycine concentration to ~200mM, mixed gently by inversion, incubated at room temperature for 5 minutes, then finally incubated on ice for 15 minutes. Cells were pelleted by centrifugation at 500xG for 2 minutes at 4°C, supernatant was discarded, and cells re-suspended in 1ml of ice cold 1xPBS. One last centrifugation was performed for 2 minutes at 4°C, supernatant was removed, pellet was snap frozen in dry ice discharged with 100% ethanol, then stored in -80°C until ready for sequencing.

2.6. Preparation of post-sort chromosome spreads

Around 5,000 to 24,000 sorted cells were used to make 4-6 slides of post-sort chromosome spreads to score the purity of sorted cells. The cells were spun down at 1,500 – 1,850 RPM for 5 minutes. The supernatant was removed and re-suspended in 125µl of 1xPBS. The cell pellet was spun down again at 1,500 – 1,850 RPM for 5 minutes. After a complete removal of supernatant, the cell pellet was resuspended in 80µl sucrose solution, incubated at room temperature for 5 minutes, and dropped on top of 65µl 1% PFA (pH 9.2)/0.1% Triton X-100 on the slides in a humidifying chamber. The slides were incubated overnight. Next morning, the slides were washed once with MilliQ water, then twice with 0.4% Photo-Flo 200. Slides were let dry at room temperature and folded into an aluminum foil for storage at -80°C until ready for cytology.

2.7. Confirmation of synchrony by cytology

Synchrony of spermatogenesis was confirmed by cytology from pre- and post-sort chromosome spreads of synchronized testes sacrificed at specific time points. At the beginning
of immunostaining, slides were thawed and blocked using 100µl of ADB (Antibody Dilution Buffer: 10% 10xPBS/10% goat serum/3% IgG-free BSA/0.05% Triton X-100) at 37°C for 30 minutes with parafilm coverslips. Then, the slides were incubated overnight at room temperature with primary antibodies. Next morning, slides were washed once with 1xPBS/0.4% Photo-Flo 200, then another with 1xPBS/0.4% Photo-Flo 200/0.01% Triton X-100 for 5 minutes each. The slides were blocked again briefly at 37°C for 10 minutes and incubated with fluorescent secondary antibodies at 37°C for 1 hour followed by one wash with 1xPBS/0.4% Photo-Flo 200, another with 1xPBS/0.4% PhotoFlo/0.01% Triton-X, then finally with 0.4% Photo-Flo 200. Stained slides were air dried in the dark and mounted with Prolonged DAPI overnight. Mouse anti-SYCP3 (1:200) and Rabbit anti-SYCP3 (1:200) were used to detect the chromosome axis in conjunction with guinea pig anti-H1t (1:1,000) or mouse anti-gammaH2AX (1:10,000) (STAR key resources table) to score and distinguish zygonema, pachynema, and diplonema to assess their level of synchrony. All secondary antibody dilutions were 1:200. A total of 100 to 160 cells were scored per time point.

2.8. Genomic DNA isolation from sorted spermatocytes

Each sorted cell pellet was thawed briefly, suspended in 500µl of 0.2xSSC (diluted from freshly prepared 1xSSC, pH 7.0), and the following were added sequentially: 60µl of beta-mercaptoethanol, 10µl of 20µg/µl Proteinase K and 50µl of 10% SDS. After 1hr incubation at 55°C at 600 RPM with occasional inversions, two rounds of phenol/chloroform extraction were performed. Afterwards, the aqueous layer was transferred in separate tubes containing 2 to 6µl of linear polyacrylamide (LPA) per sample to better visualize the DNA pellet. Two volumes of ice-cold 100% ethanol was added to the aqueous layer and incubated in dry ice for 10 minutes, then in -20°C for 45 minutes to an hour. Next, the tubes were spun down at 15,000 RPM (full speed) for 15 minutes, then the pellet was washed with ~500ul of 70% ethanol for 3-5 minutes at full speed. After removing all ethanol, DNA pellet was suspended in total of 300µl of milliQ water and 1/10 volume of 3M Sodium Acetate (pH3.2). Then, three volumes of ice-cold 100%
ethanol was added and the precipitation steps were repeated once more. The final DNA pellet was air dried for ~3 minutes, suspended in 20-40\mu l of 0.5mM Tris (pH 7.4) and kept at 4°C overnight for homogenization. Next day, the tubes were transferred to -20°C for long-term storage until ready to recombination assay.

2.9. Quantification of DNA by amplification efficiency

Quantification and amplification efficiency was performed as previously reported in (65, 174) for the A3 and 59.5 hotspots. The isolated 4C DNA samples were quantified by nanodrop, diluted to a rough concentration of <20ng/\mu l. One and 2\mu l aliquots were run on 0.8% agarose gel to compare with control DNA (12.5 ng/\mu l) to confirm the quality and concentration empirically. Next, the amplification efficiency of DNA samples was calculated by seeding 2 molecules of DNA per well and performing two rounds of nested allele-specific PCR (1° PCR Rx: 1x96°C 2 minutes; 26x 96°C 20 seconds, optimized annealing temperature 30 seconds, 65°C extension for 5 minutes; Hold 4°C). The primary amplicons were diluted in 35 \mu l of dilution buffer (10mM Tris-\text{HCl} pH 7.5/5\mu g/ml sonicated salmon sperm DNA) and a total of 1\mu l/well from the primary PCR were seeded into 2° PCR (1x96°C 2 minutes; 27x 96°C 20 seconds, optimized annealing temperature 30 seconds, 65°C extension for 4 minutes; Hold 4°C) using allele-specific forward primers and universal reverse primers in total of 16 to 48 wells at both A3 and 59.5 hotspots. Each well for both 1° & 2° PCR contained 0.72\mu l of 11.1x buffer (10mM each: dATP, dTTP, dGTP, and dCTP, 110mM (NH₄)₂SO₄, 45mM MgCl₂, 450mM Tris-\text{HCl} pH 8.8, 67mM betamercaptoethanol, 44\mu M EDTA, and 1.13mg/ml non-acetylated BSA), 0.05\mu l of 2M Tris, 10\mu M of allele-specific forward primer, 10\mu M of universal reverse primer, 0.048\mu l of KAPA Taq, 0.0216\mu l of Pfu, 30 molecules of DNA and MilliQ water brought up to total volume of 10\mu l. The amplified DNA from every well were then run on 0.8% agarose gel to calculate the \mu_{\text{amp}} by the following equation: Ln (total negative wells/total wells)/2 molecules. The acceptable range of \mu_{\text{amp}} was from 0.2 to 0.8. If the assumed concentration did not meet this range, then
new assumption was made for another round of amplification efficiency test until the $\mu_{\text{amp}}$ satisfied this range.

2.10. Noncrossover assay

Noncrossover assay by PCR was previously described in (174). Once amplifiable DNA concentration was determined for each sample, 30 molecules of DNA per well were seeded in two to four 96-well plates for two rounds of nested allele-specific PCR at both A3 and 59.5 hotspots using allele-specific forward primers and universal reverse primers (1° PCR Rx: 1x96°C 2 minutes; 26x 96°C 20 seconds, optimized annealing temperature 30 seconds, 65°C extension for 5 minutes; Hold 4°C). The primary amplicons were diluted in 35µl of dilution buffer (10mM Tris-HCl pH 7.5/5µg/ml sonicated salmon sperm DNA) and a total of 1.6µl/well from the primary PCR was seeded into 2° PCR (1x96°C 2 minutes; 36x 96°C 20 seconds, optimized annealing temperature 30 seconds, 65°C extension for 4 minutes; Hold 4°C).

Reagents and buffers used for PCR per well were equivalent to amplification efficiency, but adjusted in proportion to a total volume of 8µl/well for primary PCR and 30µl/well for secondary PCR. Allele-specific D forward primer was used at A3, whereas allele-specific B forward primer was used at 59.5 to amplify recombination outcomes on the “hotter” chromatid that received more DSBs (Table 1 and 2). The same combinations of primer sets were used for amplification efficiency as for the noncrossover assay. As a positive control, 8-wells of allele-specific PCR amplifying the other chromatid was also performed simultaneously. To check for successful amplification, a random well of 12 were chosen per plate and run on gel. Each amplified well was then suspended in 260 – 290µl denaturation buffer and loaded in 25µl to 40µl per well onto 6-12 nylon membranes to produce replicate dot-blots followed by one washing with 2xSSC buffer. The blots were then allowed to dry overnight. For genotyping each recombination outcomes, the blots were hybridized using allele-specific oligonucleotides (ASOs) specific to polymorphisms present on the opposite chromatid at both aforementioned hotspots (B ASOs for A3; D ASOs for 59.5; Table 3 and 4) labeled with gammaP$^{32}$ by Southern. The hybridized
Table 1. Allele-specific primers (ASPs) used to amplify recombinants at 59.5. Polymorphisms are indicated in red.

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**Universal Primers**

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Table 2. Allele-specific primers (ASPs) used to amplify recombinants at A3. Polymorphisms are indicated in red.

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Table 4. Allele-specific oligonucleotides (ASOs) used for genotyping A3. Polymorphisms are indicated in red.

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blots were exposed overnight on a phosphorimager, scanned, and stripped using boiling 0.1% SDS approximately 3-4 times until radioactivity detected by Geiger counter was significantly reduced. The stripped blots were then re-hybridized until all ASOs of interest for both hotspots were queried.

2.11. Crossover assay

In order to determine the frequency of MLH1/3 independent crossovers, Mlh3 knockout spermatocytes were seeded into wells at 400 molecules per well for crossover assay at 59.5 hotspot. Two rounds of nested allele-specific PCRs using allele-specific B forward and allele-specific D reverse primers were performed (1° PCR and 2° PCR reaction protocol identical to amplification efficiency test). Then, 2.5µl per well from 2° PCR plates were run on a gel to locate positive wells with crossover molecules amplified. Tertiary PCR was performed by seeding 0.75µl of positive wells from 2° PCR for 30 cycles using nested universal primers. A couple of negative wells were also seeded for 3° PCR as a negative control. The 1µl per well of tertiary amplicons were run on 0.8% agarose gel and then the remaining were suspended in 290 µl of denaturation buffer and loaded onto nylon membranes at 25µl per well to produce replicate dot-blot blots followed by one washing with 2xSSC buffer. The blots were then allowed to dry overnight. For genotyping recombination outcomes, the blots were hybridized using allele-specific oligonucleotides (ASOs) specific to polymorphisms present on the both B and D chromatid at 59.5. as described above. The hybridized blots were exposed ~2 hours on phosphorimager, scanned, and stripped using boiling 0.1% SDS approximately 4-5 times until radioactivity detected by Geiger counter was significantly reduced. The stripped blots were then re-hybridized until all ASOs at 59.5 were queried.

2.12. Statistical analysis

Recombination frequencies of all samples analyzed were calculated as means and standard deviations per 10,000 molecules after Poisson correction and amplification adjustments at each polymorphism across each hotspot using formulas describe in (70). Total
noncrossover frequency was calculated by adding recombination frequency at each polymorphism. Note: the total wells denominator was adjusted to remove any wells where crossover recombination would obscure noncrossovers (this is particularly important for 59.5, which has a high frequency of crossovers). Statistical significance for noncrossover distribution in the center vs distal polymorphisms at both A3 and 59.5 between samples were calculated using Fisher’s exact test, two-tailed by comparing the total raw number of noncrossover event obtained for each sample. The significance of noncrossover frequency between samples in both wild type and \( \text{Mlh3}^{-/-} \) was also calculated by Fisher’s exact test, two-tailed. The central 200nt regions of A3 and 59.5 hotspots were determined by adding and subtracting 100nt from their PRDM9 binding sites (70). All graphs were created in Prism 6/7 software.

2.13. Hi-C library preparation and sequencing

Hi-C experiments were performed largely as previously described (31, 39, 219). Briefly, 600,000-800,000 cells (Table 5) were cross-linked with 2% formaldehyde for 10 min at room temperature, then the reaction was quenched using 200 mM glycine for 5 min at room temperature, then 15 minutes on ice, then samples were frozen in liquid nitrogen. Nuclei were isolated and directly applied for digestion using the 4-base cutter restriction enzyme MboI (NEB) at 37°C overnight. The single strand overhang was filled with biotin-14-dATP (Life Technologies) using Klenow DNA polymerase (New England Biolabs). In contrast to traditional Hi-C, the ligation was performed when the nuclear membrane was still intact (in situ protocol). DNA was ligated for 4 hours at 16°C using T4 ligase (New England Biolabs). Protein was degraded by proteinase K (New England Biolabs) treatment at 55°C for 30 min. The crosslinking was reversed with addition of 500 mM NaCl and incubation at 65°C overnight. DNA was purified by ethanol precipitation, sonicated to 300-700 bp fragments, and size-selected using SPRI magnetic beads as described (39). Biotinylated DNA was selected with Dynabeads MyOne T1 Streptavidin beads (Life Technologies). Sequencing libraries were prepared on beads, checked using an Agilent Bioanalyzer 2100 and quantified using a Qubit
Table 5. Cell preparations for Hi-C.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Days post-RA injection</th>
<th>Pre-sort purity (SYCP3-positive cells)</th>
<th>Post-sort purity</th>
<th>Total sorted cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zygonema #1</td>
<td>25 (third wave) ^a</td>
<td>33% zygonema / 67% diplonema</td>
<td>94% zygonema / 1% round spermatids / 5% DAPI ^b</td>
<td>600,000</td>
</tr>
<tr>
<td>Zygonema #2</td>
<td>33 (fourth wave)</td>
<td>0.6% leptonema / 42% zygonema / 58% diplonema</td>
<td>5% leptonema / 91% zygonema / 1% diplonema, 3% DAPI</td>
<td>743,750</td>
</tr>
<tr>
<td>Pachynema #1</td>
<td>30 (third wave)</td>
<td>99% pachynema / 1% diplonema</td>
<td>77% pachynema / 23% diplonema ^c / 1% round spermatids</td>
<td>807,250</td>
</tr>
<tr>
<td>Pachynema #2</td>
<td>47 (fifth wave)</td>
<td>Not available</td>
<td>85% pachynema / 14% diplonema / 1% round spermatids</td>
<td>600,000</td>
</tr>
<tr>
<td>Pachynema #3</td>
<td>21 (second wave)</td>
<td>100% pachynema</td>
<td>79% pachynema / 21% diplonema</td>
<td>620,000</td>
</tr>
</tbody>
</table>

^a “Second wave”, “third wave,” “fourth wave,” and “fifth wave” refer to synchronized waves of spermatogenesis.

^b DAPI refers to DAPI-positive, SYCP3-negative cells that are likely somatic cells from surrounding tissue.

^c Cells were scored as diplonema if even a single chromosome pair showed evidence of desynapsis.
Libraries were sequenced on an Illumina HiSeq 4000 with 100 cycles of paired-end reads.

2.14. Hi-C data analysis and bioinformatics

Hi-C data pre-processing and analysis was performed largely as previously described (32), with modifications for assignment of haplotype of each read. We aligned each read to the mm10 genome assembly using BWA-MEM (220) with default parameters except the clipping penalty (-L flag) was set to 13. Next, WASP (221) was adapted to identify reads containing one or more SNPs, then the read was re-aligned after flipping each allele to the value in the CAST genome. For SNP identification, we used data from the Wellcome Sanger Institute Mouse Genomes Project (222), accession code ERS076381. Dividing 226,138,14 SNPs by a total genome length of 2,725,521,370 (one copy of each chromosome including X and Y) gives 0.83% SNP density, or one SNP in 120 bp on average. SNP-containing reads were kept for further analysis only if the read mapped to the same genomic location in both mapping steps. Next, the haplotype at each SNP location was identified, and the haplotype of the read classified as either ambiguous (no SNPs), B6 (all SNPs mapping to B6), or CAST (all SNPs mapping to CAST) (Table 6). On average, only 0.5% of reads containing multiple SNPs showed a mixture of B6 and CAST alleles, and these reads were discarded (these reads could arise from multiple sources, including an inter-homolog ligation junction within the read, capture of a meiotic or pre-meiotic recombination event, or sequencing errors). Read pairs corresponding to B6-B6, CAST-CAST, or B6-CAST interactions were then separated for later analysis.

For construction of Hi-C contact maps and contact probability analysis, all read pairs including those without SNPs were used. BAM files were further processed using pairtools (https://github.com/mirnylab/pairtools) to identify ligation junctions and produce Hi-C. pairs files. Hi-C contact maps in .hic format were constructed using the pre function in Juicer (223). Hi-C contact maps were visualized in Juicebox (224) with balanced normalization applied (225).
Table 6. Hi-C read counts.

<table>
<thead>
<tr>
<th></th>
<th>Zygonema #1</th>
<th>Zygonema #2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mapped read</td>
<td>197,614,046</td>
<td>153,901,397</td>
</tr>
<tr>
<td>pairs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6 (% of mapped)(a)</td>
<td>74,259,687 (37.6%)</td>
<td>57,821,970 (37.6%)</td>
</tr>
<tr>
<td>CAST (%)(b)</td>
<td>54,628,271 (27.6%)</td>
<td>42,564,406 (27.7%)</td>
</tr>
<tr>
<td>B6-CAST (%)(c)</td>
<td>6,537,482 (3.3%)</td>
<td>5,124,838 (3.3%)</td>
</tr>
<tr>
<td>Ambiguous (%)(d)</td>
<td>61,065,373 (30.9%)</td>
<td>47,493,793</td>
</tr>
<tr>
<td>Unassignable (%)(e)</td>
<td>1,123,233 (0.6%)</td>
<td>896,390 (0.6%)</td>
</tr>
<tr>
<td>GEO accession number(f)</td>
<td>GSM3476500</td>
<td>GSM3476501</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Pachynema #1</th>
<th>Pachynema #2</th>
<th>Pachynema #3</th>
<th>E14 ES Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total mapped read</td>
<td>176,921,227</td>
<td>155,119,468</td>
<td>155,072,169</td>
<td>522,314,158</td>
</tr>
<tr>
<td>pairs</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6 (% of mapped)(a)</td>
<td>65,861,659 (37.3%)</td>
<td>56,354,326 (36.3%)</td>
<td>56,304,306</td>
<td>N/A</td>
</tr>
<tr>
<td>CAST (%)(b)</td>
<td>48,728,160 (27.5%)</td>
<td>43,068,165 (27.8%)</td>
<td>43,130,548</td>
<td>N/A</td>
</tr>
<tr>
<td>B6-CAST (%)(c)</td>
<td>6,164,297 (3.5%)</td>
<td>5,882,454 (3.8%)</td>
<td>5,645,524 (3.6%)</td>
<td>N/A</td>
</tr>
<tr>
<td>Ambiguous (%)(d)</td>
<td>55,104,704 (31.2%)</td>
<td>49,049,415 (31.6%)</td>
<td>49,251,537</td>
<td>N/A</td>
</tr>
<tr>
<td>Unassignable (%)(e)</td>
<td>1,062,407 (0.6%)</td>
<td>765,108 (0.5%)</td>
<td>740,254 (0.5%)</td>
<td>N/A</td>
</tr>
<tr>
<td>GEO accession number(f)</td>
<td>GSM3476502</td>
<td>GSM3476503</td>
<td>GSM3476504</td>
<td>GSM1908921</td>
</tr>
</tbody>
</table>

\(a\) Read pairs were assigned as B6 if both reads were assigned to B6, or if one read was assigned to B6 and the other was ambiguous (i.e. contained no SNPs).

\(b\) Read pairs were assigned as CAST if both reads were assigned to CAST, or if one read was assigned to CAST and the other was ambiguous (i.e. contained no SNPs).

\(c\) Read pairs were assigned as B6-CAST if one read was assigned to B6, and the other to CAST.

\(d\) Read pairs were assigned as ambiguous if neither read contained SNPs. These read pairs were added to the B6 and CAST read pairs for total Hi-C maps and contact probability graphs.

\(e\) Read pairs were assigned as unassignable if either read contained SNPs mapping to both B6 and CAST. These could represent reads with internal inter-homolog ligation junctions, sequencing errors, or meiotic gene conversion tracts. These read pairs were excluded from downstream analyses.

\(f\) Data for meiotic prophase is publicly available at the NIH Gene Expression Omnibus under series GSE122622 and the listed individual dataset accession codes. E14 ES cell data is available under series GSE74055 and the listed individual dataset accession code.

[76]
For assignment of compartments, we used the eigenvector function in Juicer (223). Eigenvectors were calculated for control E14 cells, zygonema (combined dataset), and pachynema (sample #1). Eigenvectors and additional genomic features were visualized using Integrated Genomics Viewer v. 2.4.10 (226). Custom scripts are available at Github (https://github.com/lucaspatel/nsmb_mousehic). For comparisons of our Hi-C contact maps with interphase Hi-C contact maps, we used a previously-published dataset obtained using the above library preparation and sequencing methodology from mouse E14 embryonic stem cells (strain background 12910la; GEO sample GSM1908921) (45). We re-mapped this dataset to the mm10 genome assembly using the same procedure as above, but without considering haplotype. All figures showing Hi-C contact maps are displayed with a linear white-to-red gradient, and report maximum contrast (red) in terms of CPKB, “Hi-C Contacts Per Kilobase per Billion mapped contacts”, calculated as follows:

\[
\frac{\text{(# of contacts in bin)} \times 10^9}{\text{(total # of mapped contacts in matrix)} \times \text{(bin size in kb)}}
\]

Contrast levels for each panel in a given figure are adjusted to equivalent CPKB values.

For correlation of compartments with other genomic features, we used A/B compartment calls from the control interphase dataset as these were more robust than from zygotene or pachytene, but agreed closely with meiotic datasets across most of the genome. For DSB hotspots, we used hotspot locations and normalized intensity assignments from a previously-published ssDNA map from spermatocytes of a C57BL/6J x CAST/EiJ F1 hybrid mouse (GEO sample GSM1954839) (110).

For crossovers, we used the B6xCAST and CASTxB6 crossover locations from a previously-published multi-species cross (797 crossovers total; re-mapped from mm9 to mm10 reference genome) (227). For PRDM9 binding sites and H3K4me3 sites, we used PRDM9 and H3K3me3 peaks called from ChIP-Seq data on B6xCAST F1 hybrid mice (GEO sample GSE60906) (108). Peak locations (6,955 PRDM9 peaks and 80,940 H3K4me3 peaks) were re-mapped from mm9 to mm10 reference genome.
For DSB hotspot analysis, we first calculated the center of each DSB hotspot and assigned the hotspot to either the A or B compartment. We then plotted the cumulative hotspot intensity distribution for each compartment (genome-wide or per chromosome) in Prism 7, and calculated P-values using a Kolmogorov-Smirnov test (Figure 10e). We next used regioneR (228) to calculate the significance of enrichment of DSB hotspots overlapping the A compartment, compared to a random distribution (Figure 10a). We used a similar analysis to calculate the significance of enrichment of crossovers, PRDM9 binding sites, and H3K4me3 peaks (Figure 10b-d). Other statistical tests were performed using Prism v. 7 (GraphPad Software).

For comparison of Hi-C contacts with transcription data, we used a published RNA Polymerase II ChIP-Seq dataset for 16-dpp C57BL/6J mouse testes (GEO Sample GSM1083638), remapped to mm10 (229). For comparison with piRNA clusters, we used data from the piRNA cluster database (230, 231), specifically 12.5-dpp (SRR772029/GEO GSM1096583) and 14.5-dpp (SRR7720230/GEO GSM1096584) C57BL/6J mouse testes samples (232).

2.15. Contact probability calculation

Contact probability versus genomic distance \((P(s))\) curves were calculated as previously described (46, 233). Briefly, we divided all genomic separations into logarithmically-sized bins, starting at 10 kb and increasing by a factor of 1.12 per bin. We first calculated the number of Hi-C contacts in each dataset that fell into each bin. We next calculated the number of possible Hi-C contacts at each distance across the genome or within an individual chromosome, using a fragment size of 250 bp to approximate the ~256 bp size of MboI-generated restriction fragments. Dividing contact number by potential contacts in each bin yielded contact probability \(P(s)\), which we then normalized by setting the value of \(P(s)\) at a distance of 100 kb to 1. Due to their distinctive organization in meiotic prophase, the X and Y chromosomes were considered separately in this analysis.
2.16. Data and code availability

All custom scripts and code are available at Github:

https://github.com/lucaspatel/nsmb_mousehic

All sequencing data have been deposited in the NCBI Gene Expression Omnibus database under accession number GSE122622:


All previously-published data used in our analysis is available at the links below:

ERS076381: ftp://ftp-mouse.sanger.ac.uk/current_snps/strain_specific_vcfs/CAST_EiJ.mgp.v5.snps.dbSNP142.vcf.gz
SRR772029/SRR7720230 (processed): http://www.smallrnagroup.uni-mainz.de/piRNAclusterDB.htm
Figure 10. Distribution of meiotic DSBs and other chromatin features by compartment

(A) regioneR analysis (228) of the genome-wide overlap between the gene-rich A compartment (as assigned from the control interphase dataset) and 14,951 DSB hotspots in spermatocytes from a B6xCAST F1 hybrid mouse (110).

(B) regioneR analysis as in (A) of 6948 PRDM9 peaks measured by ChIP-Seq on spermatocytes from a B6xCAST F1 hybrid mouse (108).

(C) regioneR analysis as in (A) of 80,856 H3K4me3 ChIP-Seq peaks in spermatocytes from a B6xCAST F1 hybrid mouse (108).

(D) regioneR analysis as in (A) of 784 mapped crossovers between B6 and CAST chromosomes in a multi-species cross.

(E) For each chromosome, the cumulative intensity distribution of B6xCAST hotspots was calculated for hotspots located in A (blue) or B (green) compartments (compartment calls from
the control interphase dataset). P-values were calculated using a Kolmogorov-Smirnov test (227).
Chapter 3: Results

This section is in part adapted from the materials and methods section of a research paper “Dynamic reorganization of the genome shapes the recombination landscape in meiotic prophase” published in Nature Structural & Molecular Biology in March 2019 by Lucas Patel, Rhea Kang, Scott C. Rosenberg, Yunjiang Qiu, Ramya Raviram, Sora Chee, Rong Hu, Bing Ren, Francesca Cole and Kevin D. Corbett with permission from the publishing journal (217). Hi-C figures in this section are extracted directly from the publication mentioned above.

3.1 Isolation of meiotic prophase I cells by synchronizing spermatogenesis

3.1.1 Confirmation of synchronization

To analyze recombination at zygonema, pachynema, and diplonema of meiotic prophase I, we synchronized mouse spermatogenesis in wild type C57BL/6J (B) x DBA/2J (D) F1 hybrid neonates using an inhibitor of retinoic acid production. Retinoic acid is required for spermatogonial differentiation and the eventual initiation of meiosis (189). After inhibitor treatment, retinoic acid injection induces synchronous spermatogonial differentiation and meiotic induction, which is maintained over multiple rounds of spermatogenesis into adulthood (218) (Fig.11B). The duration of meiotic prophase stages is highly stereotypic allowing us to estimate when successive synchronized rounds have reached particular stages of meiosis (189). Spermatocyte spreads and immunofluorescence confirmed synchronization (Fig. 11C). In contrast to an unsynchronized animal, each estimated time point (days post injection, dpi) was enriched for specific stages. Further, as the synchronous rounds of spermatogenesis initiate every 8.6 days and meiotic prophase I lasts 12.3 days, we were able to enrich for zygonema and diplonema from two successive rounds of spermatogenesis simultaneously.

3.1.2 Isolation of cells from specific stages of meiotic prophase I

To isolate synchronized meiotic cells, we used flow cytometry that previously allowed us to isolate late prophase I spermatocytes (late 4C) for recombination analysis (66, 70). 4C cells from synchronized testes showed a different cell population profile with a few densely
Figure 11. Synchronization of mouse spermatogenesis allows isolation of specific stages of meiotic prophase.

(A) The proposed DSB repair pathways of meiotic recombination.
(B) Schema for synchronizing mouse spermatogenesis. RA, retinoic acid.
(C) Distribution of meiotic prophase stages in unsynchronized (left) and synchronized animals at specific days post RA injection (dpi). In gray is a depiction of some of the progressive stages from RA induction. A1, A1 spermatogonia; B, type B spermatogonia; PL, preleptonema; L,
leptonema; Z, zygonema; P, pachynema; D, diplonema; MI, metaphase I; SP, spermiogenesis. Note that zygonema from the next round of spermatogenesis coincides with diplonema from the preceding round (17, 25, and 35 dpi).

(D) FACS profiles of unsynchronized (left) and synchronized testicular cells from wild type animals at the indicated dpi. Cells are separated by Hoechst fluorescence, in which the Y axis (Blue) separates cells based upon DNA content (4C, 2C, or 1C) and X axis (red) separates cells based upon chromosome compaction and condensation. Representative image from the indicated sorted population stained for homolog axis marker SYCP3 and either the DNA damage and sex body marker gamma-H2AX (left) or in the inset histone H1t (a marker of mid-pachynema and later).
populated regions as compared to that of unsynchronized testes (Fig. 11D). Sorting the cells from these regions yielded highly enriched populations of zygonema (99% ± 1.6, N = 2, n = 172), pachynema (77% ± 3.3, N = 3, n = 291) and diplonema (81% ± 3.1, N = 3, n = 300).

3.2 Temporally and spatially distinct noncrossover recombination

3.2.1 Distal noncrossovers during zygonema

Analysis of recombination at the Psmb9 hotspot during the first semi-synchronous round of meiosis in mouse spermatocytes suggested that noncrossovers and crossovers form in mid- to late pachynema with noncrossovers potentially preceding crossovers (64). These results were consistent with that found in budding yeast (48). We previously found that the first juvenile rounds of meiosis have differential use of recombination pathways than adult rounds (70), necessitating a method to analyze specific stages in adult spermatocytes.

We analyzed recombination outcomes in the enriched adult spermatocyte populations at two well-defined hotspots A3 and 59.5, which have markedly different characteristics (65, 66, 70). The A3 hotspot has a noncrossover to crossover ratio of 10:1 reflecting the global DSB to crossover ratio in mouse and human spermatocytes. The 59.5 hotspot has a noncrossover to crossover ratio of 1:1 and DSBs at this site are more likely to form crossovers than at A3. Two types of noncrossovers can be observed at A3: singleton noncrossovers, which convert only a one polymorphism, and co-converted noncrossovers, which convert two or more polymorphisms. At A3, the co-converted noncrossovers are highly enriched in the central 200bp of the hotspot (hereafter, hotspot center). In contrast, the majority of noncrossovers at 59.5 are singletons. Finally, at A3, recombination is initiated relatively evenly between the B and D chromosomes, whereas, at 59.5, recombination is highly enriched on only the B chromosome. We used these two hotspots with different characteristics to study when noncrossover and crossover recombination occurs during meiotic prophase.

Surprisingly, approximately half of total noncrossover recombinants were amplified in zygonema (Table 7; A3 45.0%; 59.5 54.1%), suggesting a large fraction of meiotic
Table 7. Noncrossover and crossover frequencies at A3 and 59.5.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Genotype (N)a</th>
<th>Singleton (molecules tested)</th>
<th>Singleton Frequency x10^4 ± SDb (CI, 95%) c</th>
<th>Co- conversions (molecules tested)</th>
<th>Co- conversion Frequency x10^4 ± SD (CI, 95%)</th>
<th>COs d (molecules tested)</th>
<th>CO Frequency x10^4 ± SD (CI, 95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z</td>
<td>Wild type (3)</td>
<td>52 (8,359)</td>
<td>60.1 ± 16.5 (55.6 to 64.6)</td>
<td>7 (8,359)</td>
<td>8.4 ± 5.9 (4.0 to 12.8)</td>
<td>0 (8,359)</td>
<td>0</td>
</tr>
<tr>
<td>P</td>
<td>Wild type (3)</td>
<td>176 (11,721)</td>
<td>120.7 ± 17.6 (118.1 to 123.3)</td>
<td>82 (11,721)</td>
<td>56.4 ± 12.4 (53.7 to 59.1)</td>
<td>75 (11,721)</td>
<td>45.8 ± 11.5 (43.2 to 48.4)</td>
</tr>
<tr>
<td>D</td>
<td>Wild type (3)</td>
<td>69 (8,022)</td>
<td>88 ± 24.4 (82.2 to 93.8)</td>
<td>50 (8,022)</td>
<td>64.1 ± 20.7 (58.4 to 70)</td>
<td>32 (8,022)</td>
<td>40.2 ± 17.8 (34 to 46.4)</td>
</tr>
<tr>
<td>Z</td>
<td>Wild type (3)</td>
<td>59 (15,288)</td>
<td>40.1 ± 10.3 (37.5 to 42.7)</td>
<td>1 (15,288)</td>
<td>0.7 ± 1 (-1.3 to 2.7)</td>
<td>1 (15,288)</td>
<td>0.7 ± 0.7 (-0.7 to 2.1)</td>
</tr>
<tr>
<td>P</td>
<td>Wild type (3)</td>
<td>91 (13,127)</td>
<td>67.3 ± 13.0 (64.6 to 70)</td>
<td>4 (13,127)</td>
<td>3.5 ± 3.1 (0.5 to 6.5)</td>
<td>297 (13,127)</td>
<td>202.3 ± 30.2 (198.9 to 205.7)</td>
</tr>
<tr>
<td>D</td>
<td>Wild type (3)</td>
<td>92 (15,461)</td>
<td>71.2 ± 21.4 (66.8 to 75.3)</td>
<td>5 (15,461)</td>
<td>4.2 ± 4 (0.7 to 7.7)</td>
<td>190 (15,461)</td>
<td>136.5 ± 42.2 (130.5 to 142.5)</td>
</tr>
<tr>
<td>SPh</td>
<td>Wild type (6)</td>
<td>117 (22,011)</td>
<td>66.3 ± 16.9 (63.2 to 69.4)</td>
<td>15 (22,011)</td>
<td>9.0 ± 5.9 (6 to 12)</td>
<td>306 (22,011)</td>
<td>162.3 ± 38.5 (158 to 166.6)</td>
</tr>
<tr>
<td>P</td>
<td>Mlh3−/− (3)</td>
<td>154 (17,164)</td>
<td>89.1 ± 17.3 (86.4 to 91.8)</td>
<td>9 (17,164)</td>
<td>5.2 ± 4.1 (2.5 to 7.9)</td>
<td>1 (240,969)</td>
<td>0.04 ± 0.11 (-0.2 to 0.3)</td>
</tr>
<tr>
<td>D</td>
<td>Mlh3−/− (3)</td>
<td>142 (11,068)</td>
<td>129.8 ± 22.5 (126.1 to 133.5)</td>
<td>60 (11,068)</td>
<td>55.1 ± 13.5 (51.7 to 58.5)</td>
<td>11 (339,968)</td>
<td>0.33 ± 0.53 (0.02 to 0.6)</td>
</tr>
</tbody>
</table>

A3 (no shading) and 59.5 (shading). a N, number of animals tested. b SD, standard deviation. c CI, confidence interval. d COs, crossovers. e Z, zygonema. f P, pachynema. g D, diplonema. h SP, sperm.
recombination is completed while homologs are synapsing. Moreover, the distribution of noncrossovers was significantly different in zygonema as compared to diplonema ($A3 \, p = 0.0043; 59.5 \, p < 0.00001$, Fisher’s exact test, two-tailed; Fig. 12A and 12B). At all hotspots analyzed, the majority of noncrossovers are found in their centers (64, 65, 171, 173, 234), including $A3$ and 59.5 (Table 8, Fig. 12A and 12B; e.g., central noncrossovers in diplonema: $A3\, 73.2\% ; 59.5\, 66.7\%$). By contrast, noncrossovers found in zygonema were dispersed throughout the hotspots with no enrichment in their centers. Congruently, at $A3$ there were 8-fold fewer co-converted noncrossovers at zygonema as compared to diplonema (Fig. 12C, Table 7). At both hotspots, the distribution of noncrossovers was unchanged between diplonema and sperm (Table 8, Fig. 12A and 12B and (65, 70), indicating that meiotic recombination is likely completed by diplonema and suggesting synchronization does not alter the meiotic recombination profile (Table 7 and 8).

### 3.2.2 Central noncrossovers during pachynema

The remaining central noncrossovers form during pachynema with a distribution similar to that of diplonema, including a discernable peak of recombination activity in the hotspot centers (Fig. 12A, 12B, and Table 8). Intriguingly, the noncrossover frequency at $A3$ was ~1.2-fold higher in pachynema than in diplonema, particularly amongst singletons. These pachytene-stage noncrossovers may reflect heteroduplex strands that have yet to be restored to parental genotypes by mismatch repair (235). Importantly, the frequency of noncrossovers observed in sperm is similar to that found in diplonema, suggesting that most recombination is completed during pachynema.

Taken together, we propose that distal noncrossover recombination begins during zygonema, but ends during pachynema, while central noncrossover recombination begins and ends during pachynema. The disparate temporal and spatial patterns suggest these noncrossover recombinant classes (distal and central) may be regulated independently with distinct functions during meiosis.
Figure 12. Noncrossover frequency and distribution at A3 and 59.5 shows two distinct populations of noncrossovers.

(A) Representative samples of noncrossovers mapped at A3 in zygonema (Z), pachynema (P), and diplonema (D). Here and elsewhere, the number of n_plot shown and corresponding length of the plotted maps is proportional to the noncrossover frequencies at diplonema. n_tot, number of total noncrossovers mapped; n_plot, proportional number of noncrossovers mapped. The central 200bp are indicated by yellow bar. The proportion of central and distal noncrossovers at each stage and in sperm (SP) is shown at the bottom left. Ticks at the top show polymorphisms tested. kb, kilobase pairs; n.s., not significantly different. P value is Fisher’s exact test, two-tailed.

(B) Same as (A) but for 59.5.

(C) Histogram of noncrossover frequency at A3 for singletons (left) and co-conversions (middle) and at 59.5 for total noncrossovers in zygonema (Z, top), pachynema (P, middle), and diplonema (D, bottom). Frequency is mean ± SD. Ticks at the top show polymorphisms tested. n_tot, number of total noncrossovers mapped. P value is Fisher’s exact test, two-tailed.
(D) Frequency in centimorgans per megabase (cM/Mb) and distribution of crossovers at pachynema (P) on top and diplonema (D) on the bottom for A3 and 59.5. Frequency is mean ± SD. Ticks at the top show polymorphisms tested. $n_{tot}$, number of total noncrossovers mapped.
Table 8. Noncrossover distributions at A3 and 59.5.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Genotype (N)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hotspot</th>
<th>Central NCOs&lt;sup&gt;b&lt;/sup&gt; (raw)</th>
<th>Distal NCOs&lt;sup&gt;b&lt;/sup&gt; (raw)</th>
<th>Central Frequency x10&lt;sup&gt;4&lt;/sup&gt; ± SD&lt;sup&gt;c&lt;/sup&gt; (CI, 95%)</th>
<th>Distal Frequency x10&lt;sup&gt;4&lt;/sup&gt; ± SD (CI, 95%)</th>
<th>Central Frequency Proportion (%) ± SD</th>
<th>Distal Frequency Proportion (%) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z&lt;sup&gt;e&lt;/sup&gt;</td>
<td>WT (3)</td>
<td>A3</td>
<td>30</td>
<td>29</td>
<td>34.8 ± 12.3 (30.4 to 39.2)</td>
<td>33.6 ± 12.6 (29.0 to 38.2)</td>
<td>50.9 ± 7.0</td>
<td>49.1 ± 7.1</td>
</tr>
<tr>
<td>P&lt;sup&gt;f&lt;/sup&gt;</td>
<td>WT (3)</td>
<td>A3</td>
<td>171</td>
<td>87</td>
<td>116.6 ± 14.5 (114.4 to 118.8)</td>
<td>60.4 ± 12.1 (57.9 to 62.9)</td>
<td>65.9 ± 8.4</td>
<td>34.1 ± 5.4</td>
</tr>
<tr>
<td>D&lt;sup&gt;g&lt;/sup&gt;</td>
<td>WT (3)</td>
<td>A3</td>
<td>87</td>
<td>32</td>
<td>111.1 ± 29 (105.0 to 117.2)</td>
<td>40.7 ± 16.2 (37.3 to 44.1)</td>
<td>73.2 ± 8.7</td>
<td>26.8 ± 4.9</td>
</tr>
<tr>
<td>Z</td>
<td>WT (3)</td>
<td>59.5</td>
<td>16</td>
<td>44</td>
<td>10.5 ± 5.2 (8.0 to 13.0)</td>
<td>30.2 ± 8.9 (27.6 to 32.8)</td>
<td>25.8 ± 5.0</td>
<td>74.2 ± 8.7</td>
</tr>
<tr>
<td>P</td>
<td>WT (3)</td>
<td>59.5</td>
<td>47</td>
<td>48</td>
<td>39.2 ± 10.5 (36.2 to 42.2)</td>
<td>31.6 ± 8.3 (29.3 to 33.9)</td>
<td>55.3 ± 7.8</td>
<td>44.7 ± 6.2</td>
</tr>
<tr>
<td>D</td>
<td>WT (3)</td>
<td>59.5</td>
<td>64</td>
<td>33</td>
<td>50.3 ± 17.9 (45.9 to 54.7)</td>
<td>25.1 ± 12.4 (20.9 to 29.3)</td>
<td>66.7 ± 8.2</td>
<td>33.2 ± 5.7</td>
</tr>
<tr>
<td>SP&lt;sup&gt;h&lt;/sup&gt;</td>
<td>WT (6)</td>
<td>59.5</td>
<td>79</td>
<td>52</td>
<td>51.2 ± 15.4 (47.8 to 54.6)</td>
<td>24.1 ± 10.7 (21.2 to 27.0)</td>
<td>68.5 ± 8.2</td>
<td>32.0 ± 5.7</td>
</tr>
<tr>
<td>P</td>
<td>Mlh3&lt;sup&gt;+/−&lt;/sup&gt; (3)</td>
<td>59.5</td>
<td>78</td>
<td>85</td>
<td>42.5 ± 12.6 (39.7 to 45.3)</td>
<td>54.9 ± 12.6 (52.2 to 57.6)</td>
<td>45.1 ± 7.1</td>
<td>54.9 ± 7.1</td>
</tr>
<tr>
<td>D</td>
<td>Mlh3&lt;sup&gt;−/−&lt;/sup&gt; (3)</td>
<td>59.5</td>
<td>107</td>
<td>95</td>
<td>80.2 ± 19.9 (76.4 to 84.0)</td>
<td>104.7 ± 19.3 (100.8 to 108.6)</td>
<td>43.3 ± 7.2</td>
<td>56.6 ± 7.1</td>
</tr>
</tbody>
</table>

A3 (no shading) and 59.5 (shading). <sup>a</sup>N, number of animals tested. <sup>b</sup>NCOs, noncrossovers. <sup>c</sup>SD, standard deviation. <sup>d</sup>CI, confidence interval. <sup>e</sup>Z, zygonema. <sup>f</sup>P, pachynema. <sup>g</sup>D, diplonema. <sup>h</sup>SP, sperm.
3.3 Most crossovers are formed in pachynema

Cytological analysis of MLH1, MLH3, and CDK2 in mouse spermatocytes shows formation of discrete foci on the synaptonemal complex during mid-pachynema (1). The frequency and distribution of these foci are similar to that of crossovers based upon chiasmata and mapping data. Similarly, mutations that abrogate crossing over also show loss of these foci. Consequently, crossovers are thought to form during pachynema, which is consistent with timing in budding yeast (48) and analysis of recombinant formation during the first semi-synchronous round of spermatogenesis at Psmb9 hotspot (64). Congruently, while we observed no crossovers at A3 and only one crossover at 59.5 in zygonema-enriched samples (Table 7), we could account for all crossovers at both hotspots in pachynema (Table 7, Fig. 12D) Similar to noncrossovers at A3, we saw a higher frequency of crossovers at 59.5 during pachynema than diplonema, while sperm showed an intermediate frequency. We suggest that some recombination events become unamplifiable by our assays during diplonema and/or some events are similarly restored to parental genotypes. Taken together, we conclude that MutLgamma-dependent crossing over primarily occurs during pachynema coincidentally with formation of central noncrossovers.

3.4. MLH3-independent crossovers form in diplonema

In mouse spermatocytes, approximately 5 to 10% of crossing over is predicted to derive from MLH1/3-independent pathways (72). Isolation of MLH1- and MLH3-independent crossovers at Psmb9 suggests these pathways produce a small fraction of total crossovers and form at the same time as MutLgamma-dependent crossovers at least in juvenile spermatocytes (64, 236). Similarly, in budding yeast, MutLgamma-dependent and –independent crossovers form contemporaneously upon expression of Polo kinase, Cdc5 (49) (237). Consistent with the previous studies, we also observed markedly fewer crossovers at A3 and 59.5 in the absence of MLH3 (70). However, we found that the frequency of these MLH3-independent crossovers was 4-fold higher at 59.5 in juvenile versus adult rounds of spermatogenesis, raising the possibility that this pathway is differentially regulated in juveniles.
To address when these MutLgamma-independent crossovers form in adults, we synchronized spermatocytes of mice lacking MLH3 and isolated cells at pachytene and diplotene stages (>97%). Recombination outcomes were analyzed at the 59.5 hotspot, which in wild type spermatocytes is highly enriched for crossovers. We found that the majority of MLH3-independent crossovers were found in the diplotene sample as compared to pachynema ($p = 0.0192$, Fisher’s exact test, two-tailed; Fig. 13A, Table 7). As such, these events occur after the majority of meiotic recombination including crossovers and noncrossovers is completed in pachynema. We suggest that MutLgamma-independent crossing over is invoked as a backup pathway to deal with unrepaired intermediates prior to chromosome segregation.

3.5. Long noncrossovers in $Mlh3^{-/-}$ spermatocytes form in diplonema

At the 59.5 hotspot, half of all recombination events are MLH3-dependent crossovers. In the absence of MLH3, designated precursors cannot mature into crossovers (crossover maturation) and alternative pathways likely act upon these sites (70). Consistent with this model, we previously described a new class of noncrossovers found at 59.5 in the absence of MLH3. These noncrossovers share features with MLH3-dependent crossover gene conversion that suggest they likely derive from a common intermediate. We characterized noncrossovers in synchronized spermatocytes lacking MLH3 to determine when this alternative noncrossover pathway may act in meiotic prophase.

In pachytene spermatocytes lacking MLH3, we saw no difference in the frequency of singletons or long noncrossovers as compared to wild-type pachynema or sperm (Table 7). In marked contrast, we observed 11-fold more long noncrossovers in $Mlh3^{-/-}$ spermatocytes at diplonema than at pachynema. Suggesting these alternative pathways, like MLH3-independent crossing over, occur after the bulk of meiotic recombination is completed in pachynema and while chromosomes are desynapsing.

In unsynchronized testes, we are able to isolate spermatocytes in the late 4C stage of meiotic prophase based upon their Hoechst fluorescence properties (70). However, our enrichment of diplonema was limited to ~60%, with the next largest fraction containing
Figure 13. Alternative repair pathways act in diplonema.

(A) Mapped MLH1/3-independent crossovers at 59.5 isolated from pachynema (top) and diplonema (bottom) Mlh3−/− spermatocytes. Frequency is mean ± SD. Ticks at the top show polymorphisms tested. n_{tot}, number of total noncrossovers mapped; kb, kilobase pairs.

(B) Mapped noncrossovers at 59.5 isolated from pachynema (left) and diplonema (right) Mlh3−/− spermatocytes. Frequency is mean ± SD. Ticks at the top show polymorphisms tested. The central 200bp are indicated by yellow bar. Here and elsewhere, the number of n_{plot} shown and corresponding length of the plotted maps is proportional to the noncrossover frequencies at diplonema. n_{tot}, number of total noncrossovers mapped; n_{plot}, proportional number of noncrossovers mapped; kb, kilobase pairs. P values, Fisher’s exact test, two-tailed.
pachynema. Given the differences in recombination activities between prophase stages, it is not surprising that we could not previously account for all recombination that occurs at 59.5 in wildtype in late 4C Mlh3−/− spermatocytes. However, with synchronized Mlh3−/− spermatocytes, we found the total recombination frequency (crossovers, singletons, and long noncrossovers) was similar between wild type and Mlh3−/− diplotene spermatocytes (wild type 211.9 ± 67.6; Mlh3−/− 185.1 ± 38.2 p > 0.05, Fisher’s exact test, two-tailed) (Fig. 13 and Table 7). Intriguingly, we observed a 1.8-fold higher frequency of singletons in Mlh3−/− spermatocytes as compared to wild type spermatocytes in diplonema. This finding suggests intermediates that cannot be processed into crossovers in the absence of MLH3 eventually become both long and singleton noncrossovers by alternative pathways, which primarily act in diplonema. Thus, synchronizing spermatocytes improves our ability to compare different mouse alleles with one another.

3.6. Hi-C analysis of mouse spermatogenesis

While chromosome conformation capture methods (Hi-C)(29, 31) have recently enabled an unprecedented exploration of eukaryotic genome structure and regulation, analysis of mammalian meiotic prophase by Hi-C has been limited by an inability to isolate pure populations of meiotic prophase cells. To overcome this challenge, we purified large numbers of highly-synchronized mouse spermatocytes (66, 218) (R.K. and F.C., unpublished) (Fig. 14A-C) and performed Hi-C in both early prophase (zygonema) and late prophase (late pachynema/diplonema) (Fig. 15A, Table 5). To capture inter-homolog contacts during recombination and synapsis, we isolated spermatocytes from C57BL6/J (B) x M. castaneus (CAST/EiJ; CAST) F1 hybrid mice, which possess 0.83% overall single-nucleotide polymorphism (SNP) density between haplotypes. We performed Hi-C using 100-base paired-end sequencing reads, theoretically allowing us to unambiguously assign B6 vs. CAST haplotype for over half of individual reads, and over a quarter of paired-end reads. We generated 351 million Hi-C contacts for zygonema from two independent samples, and 487 million contacts for pachynema from three independent samples (Fig. 14E-G, Table 6). The resulting Hi-C contact maps from the two prophase stages were visually distinct, yet maps from
Figure 14. Synchronization and isolation of meiotic prophase spermatocytes.

(A) Experimental workflow. C57BL/6 x CAST/EiJ F1 hybrid male mice were injected daily from 2-8 days post-partum with WIN18,446, then injected with retinoic acid at 9 days post-partum to synchronize spermatogenesis. Treated animals were allowed to recover for 21-47 days, then spermatocytes were isolated at time-points enriched at specific stages of prophase (189). The zygonema/pachynema stages for each wave of spermatogenesis are indicated by color: red (second wave), orange (third), green (fourth), and blue (fifth).

(B) Spermatocytes were isolated, stained with Hoechst 33342 and sorted by FACS to further enrich for either zygonema or pachynema-stage cells (66) (1C: spermatids; SP: spermatogonia). (C) Samples of FACS-sorted cells were removed for chromosome spreads, and stained for SYCP3 and H1T for stage scoring (Table 5). Scale bar = 10 µm.
(D) Graph showing total measured chromosome axis length in pachynema spermatocytes of B6 x CAST F1 hybrid mice (orange; 48 cells from two animals) and B6 x DBA F1 hybrid mice (blue, 334 cells from two animals).

(E) Genome-wide Hi-C contact map for ES cells in interphase. Atypical contacts between chromosomes 8 and 14 indicate that a small percentage of analyzed cells possess a translocation between these two chromosomes.

(F) Genome-wide Hi-C contact map for zygonema cells, showing X-shaped inter-chromosomal contact patterns between all chromosomes.

(G) Genome-wide Hi-C contact map for pachynema cells. Inter-chromosomal contacts are reduced in pachynema relative to zygonema, and the X chromosome in particular is isolated in pachynema as it is packaged into the XY body.
Figure 15. Hi-C analysis of the meiotic prophase genome.

(A) Experimental workflow. C57BL/6 x CAST/EiJ male F1 hybrid mice were treated with WIN18,446 followed by retinoic acid to synchronize spermatogenesis, then spermatocytes were isolated and purified by FACS (Fig. 14). Samples of 600,000-800,000 cells were analyzed by Hi-C using 100-base paired end sequencing followed by a haplotype-aware analysis pipeline (Table 5 & 6).

(B) Hi-C contact maps for cells in interphase (E14 cell culture cells), early zygonema, and late pachynema. Compared to interphase cells, meiotic prophase chromosomes lose all long-range (> 10 Mb) contacts. For all panels is white (zero Hi-C contacts per kb) to red (indicated CPKM (contacts per kb per billion mapped contacts; see Methods) or higher Hi-C contacts per kilobase. See Fig. 14E-G for genome-wide Hi-C contact maps.

(C) Pearson correlation matrices for chromosome 3 in interphase, zygonema, and pachynema. These matrices graphically illustrate the correlation between different chromosomal regions' Hi-C contact patterns. Red indicates strongly-correlated contacts, and blue indicates strongly anti-correlated contacts. The observed red-blue checkerboard patterns strongly indicate the presence of compartments in all datasets.

(D) Eigenvector analysis of chromosome 3 in interphase, zygonema, and pachynema. Correlations were calculated using a two-tailed non-parametric Spearman correlation coefficient. See Fig. 18 for Eigenvector analysis of additional chromosomes.
biological replicates showed high reproducibility (Fig. 16), demonstrating the robustness of our synchronization and purification method. We could assign 3.3% of zygonema read pairs (11.7 million) and 3.6% of pachynema read pairs (17.7 million) as unambiguous inter-homolog contacts (Methods, Table 6). As a control, we used a recent Hi-C dataset from unsynchronized cultured mouse embryonic stem cells (hereafter termed “interphase”) (45) Overall, our data provide an unprecedented picture of dynamic genome reorganization in mammalian meiotic prophase.

3.7. Meiotic prophase chromosomes maintain compartment structure but lose topologically associating domains

The eukaryotic genome is organized in all developmental and cell-cycle stages to achieve the particular needs of each cell. In interphase, chromosomes occupy individual “territories” in the nucleus, and also show multiple levels of internal organization. Dynamic DNA binding, loop extrusion modulated by chromosome-bound CTCF, and dissociation from DNA by cohesin complexes gives rise to megabase-sized topologically associating domains (TADs) with high local interaction propensity (238) (37-39, 239-242). Interphase chromosomes are also arranged into “compartments,” with the gene-dense and transcriptionally-active “A” compartment physically separated from the gene-poor, heterochromatic “B” compartment (31, 243). In contrast to TADs, compartments are not formed through dynamic loop extrusion and do not depend on cohesion (37, 229, 244), rather they likely form through the tendency of heterochromatin to self-associate through a phase separation-like mechanism(38, 239).

In meiotic prophase, we observe a near-complete loss of very long-range contacts (over ~5-10 Mb) consistent with the known organization of meiotic chromosomes as linear arrays of loops anchored to the meiotic chromosome axis (Fig. 15B). We also observe “X”-shaped inter-chromosomal contact patterns consistent with the alignment of chromosomes into the prophase bouquet, which are particularly strong in zygonema but also detectable in pachynema (Fig. 17A-B). Despite the reorganization of chromosomes into loop arrays, we find that meiotic prophase chromosomes maintain strong A/B compartment identity, observable in Hi-C contact
Figure 16. Reproducibility of Hi-C contact maps in biological replicates.

(A) Hi-C contact map for combined zygonema data (top), and individual maps for samples #1 and #2 (bottom). The dotted box indicates the chromosome region shown in Fig. 20B.

(B) Hi-C contact map for combined pachynema data (top), and individual maps for samples #1, #2, and #3 (bottom).
Figure 17. Inter-chromosomal contacts reveal the meiotic bouquet.

(A) Schematic of the bouquet present in early meiotic prophase, and its expected signature in inter-chromosomal Hi-C contact maps.

(B) X-shaped inter-chromosomal contacts between meiotic prophase chromosomes, a result of physical alignment in the bouquet. Inter-chromosomal contacts are particularly strong at the centromeric ends of chromosomes, confirming reports of early-prophase centromere clustering in the mouse (245) (246) (247). Consistent with the loss of the bouquet upon homolog synapsis in pachynema, the X-shaped inter-chromosomal contact patterns are strongly reduced in this stage compared to zygonema. Color scale for all panels is white (zero Hi-C contacts per bin) to red (indicated number or higher Hi-C contacts per bin).

(C) Inter-chromosomal contacts between chromosome 3 and chromosome X reveal isolation of the X chromosome into the XY body in pachynema.
maps as a checkerboard pattern near the diagonal axis (Fig. 15B). A/B compartments are also clearly visible in chromosome-wide Pearson correlation matrices (Fig. 15C), and are remarkably consistent with interphase compartments (Fig. 15D, 18A-C). Thus, despite the reorganization of chromosomes into loop arrays in meiotic prophase, the fundamental organization of chromatin into A/B compartments is maintained.

We next examined TADs, which are visible in Hi-C contact maps as squares with high contact propensity, often with strong corner signals that result from looping interactions between TAD boundaries (39). We find that in meiosis, TADs are mostly lost despite the continued presence of cohesin on chromosomes (Fig. 19A) (4, 240). A few loci show evidence of looping interactions between TAD boundaries (Fig. 19B), but most loci show a complete loss of both the square and corner TAD signals. These data suggest that if cohesin-constrained loops are present in meiotic chromosomes, as ample cytological and electron microscopy data suggest (4), the locations of these loops most likely vary from cell to cell. This may arise from a reduction in CTCF’s influence on loop positioning, or from modulation of cohesin activity upon association with the filamentous chromosome axis “core” proteins. We propose that association with the chromosome axis reduces the dynamics of chromosome association and dissociation by cohesin, leading to the formation of a stable loop array (293). Our data do not reveal whether cohesin-mediated loop extrusion activity is reduced upon axis association, though the increase in average loop size as cells progress from zygonema to pachynema (see below) suggests that loop extrusion continues through prophase (Fig. 19C). Our data indicating a lack of reproducible loop positions in meiosis contrasts with recent Hi-C analyses of S. cerevisiae meiosis, which showed strong looping interactions between cohesin binding sites across the genome in pachynema (241) (294). While binding sites for S. cerevisiae meiotic cohesin complexes are highly reproducible (23, 242), likely leading to these strong looping signals, there is so far no evidence of reproducible cohesin binding along chromosomes in mouse spermatocytes.
Figure 18. Consistent compartment identity in interphase, zygonema, and pachynema.

(A–C) Eigenvector analysis of chromosomes 3 (A), 6a (B), and 12 (C) in interphase, zygonema, and pachynema Hi-C contact maps. A and B compartments are shown in blue and green, respectively. For each chromosome, DSB hotspots (110) and annotated genes are also shown. Correlations were calculated using a two-tailed, non-parametric Spearman correlation coefficient.

(D) Eigenvector analysis of chromosome X in interphase, zygonema, and pachynema. While the calculated correlation coefficient between interphase and zygonema is low (0.18), the overall compartment structure is similar. Compartment structure is completely lost in pachynema.
Figure 19. Loss of TADs in meiotic chromosomes.

(A) High-resolution view of a region of chromosome 2, showing loss of topologically-associating domains (blue boxes) in meiotic prophase.
(B) High-resolution view of a region of chromosome 6, showing establishment of a stable loop array in pachynema with loop bases (blue circles) corresponding to interphase TAD boundaries.
(C) Model for assembly of meiotic chromosomes. Association of dynamic cohesin complexes (grey) with chromosome axis core proteins mediates assembly of the axis and reduces cohesin dynamics (chromatin association and dissociation, and loop extension) as cells enter leptonema/zygonema, then loops further extend in coordination with axis compaction as cells enter pachynema. At some loci, the bases of stable loops in pachynema coincide with interphase TAD boundaries (yellow).
3.8. Formation of transcription “hubs” on meiotic chromosomes

While we observe a near-complete loss of TAD signal in meiotic chromosomes, a large fraction of the genome shows looping or clustering interactions at the 1-10 Mb scale, which are present in zygonema but very pronounced in pachynema (Fig. 20A-B, 21). When we overlaid Hi-C contact maps with RNA Polymerase II-bound loci in both prophase stages (238), we found that the clustered loci correspond to loci undergoing active transcription in both meiotic stages (Fig. 20A). Some clusters also correspond to highly-transcribed clusters of piRNAs, short RNAs with specialized roles in transposon silencing and sperm development (Fig. 20B) (248, 249). These data suggest that transcribed loci self-associate or condense within the meiotic chromosome structure to form clusters or “hubs” (Fig. 20C). While prior studies have shown that transcription machinery can localize to “transcription factories” (250) and form phase-separated condensates within the nucleus (251-254), the strong interactions evident in our Hi-C contact maps suggest that meiotic prophase chromosomes are particularly susceptible to these influences. Supporting the idea of transcription hub formation in meiotic prophase, several prior studies have shown that RNA polymerase II (255-257) and nascent RNA transcripts (257, 258) form highly punctate localization patterns in mouse and human spermatocytes.

3.9. Global organization of meiotic chromosomes

To characterize the global organization of meiotic chromosomes, we next analyzed genome-wide Hi-C contact probability \( P \) as a function of genomic distance \( s \). We find that for genomic distances less than \(~5\) Mb, contact probability \( P(s) \) follows a power-law scaling proportional to \( s^{-0.5} \), dramatically different from the typical scaling of interphase chromosomes (between \( s^{-1} \) and \( s^{-1.5} \)) (31, 259) (Fig. 22A, 23). The \( P(s)\sim s^{-0.5} \) scaling we observe in meiosis is similar to prior findings on mitotic chromosomes, which are organized as helical arrays of loops by cohesin-related condensin complexes (44, 260, 261). Meiotic chromosomes are also morphologically similar to early mitotic prophase chromosomes, being individualized and compacted, but much longer than mitotic prometaphase or metaphase chromosomes (262). In agreement with this idea, the \( P(s) \) curves of meiotic prophase cells are most similar to those of
Figure 20. Transcription-mediated interaction hubs in meiotic chromosomes.

(A) High-resolution view of a region of chromosome 9 in interphase, zygonema, and pachynema. Shown in green are RNA Polymerase II peaks detected at 10 dpp (zygonema) or 16 dpp (pachynema) (238).

(B) High-resolution view of a region of chromosome 7 in interphase, zygonema, and pachynema. Shown in blue are piRNA clusters transcribed at 12.5 days post-partum (dpp), during pachynema of the first wave of spermatogenesis (piRNA clusters measured at 14.5 dpp, later in pachynema, were nearly identical) (232), and shown in green are RNA Polymerase II peaks detected at 10 dpp (zygonema of the first wave of spermatogenesis) or 16 dpp (pachynema).

(C) Left: Model for assembly of transcription-mediated interaction hubs. In the absence of dynamic cohesin complexes disrupting chromatin-chromatin interactions, highly-transcribed loci (green) will condense through cooperative self-interactions into interaction hubs. Right: Schematic of Hi-C contact maps resulting from assembly of interaction hubs. Highly-transcribed
regions show depletion of short-range contacts with non-transcribed regions, and increased interactions with highly-transcribed regions up to several Mb away. See Fig. 23 for additional examples of transcription-mediated interaction hubs.
Figure 21. Transcription-mediated interaction hubs in meiotic chromosomes.

(A-C) Regions of chromosome 1 (A), 2 (B), and 4 (C) in interphase, zygonema, and pachynema. Shown in blue are piRNA clusters transcribed at 12.5 dpp (232), and shown in green are RNA Polymerase II binding peaks at 10 dpp (zygonema) or 16 dpp (pachynema)(238).
Figure 22. Global organization of chromosomes and detection of inter-homolog contacts in meiotic prophase.

(A) Genome-wide contact probability versus genomic distance \((P(s))\) in zygonema (green) and pachynema (purple) versus interphase (black), with dotted lines corresponding to \(P(s)\sim s^{-0.5}\) and \(P(s)\sim s^{-1.5}\). The observed scaling is consistent across all chromosomes (Fig. 23A, B, D) and for both B6 and CAST chromosomes (Fig. 23F-G). Lower panel: Plot of the slope of the \(P(s)\) curves shown above reveals average loop size in zygonema (0.8-1.0 Mb) and pachynema (1.5-2.0 Mb).

(B) Schematic model of chromosome organization and homolog synapsis in meiotic prophase, with the chromosome axis (gray line) constraining sister chromosomes as aligned loop arrays (two shades of blue in inset). Notably, loops likely extend in all directions from each axis (see cross-section view at right), resulting in the interdigitation of loops from homologous chromosomes (blue and yellow). Synaptonemal complex (SC) transverse filaments are shown in gray.

(C) Inter-homolog Hi-C contact maps for chromosome 5 in zygonema. Close-up views of boxed regions 1 (middle of chromosome) and 2 (end of chromosome) are shown in lower panels.

(D) Inter-homolog Hi-C contact maps for chromosome 5 in pachynema, as in (c). See Fig. 24 for additional examples.

(E) \(P(s)\) curves for inter-homolog contacts (zygonema green, pachynema purple), with dotted line corresponding to \(P(s)\sim s^{-0.16}\). Modeling the convolution of two \(P(s)\sim s^{-0.5}\) functions, representing the physical alignment and interdigitation of two loop arrays in pachynema, gives
a function proportional to $s^{-0.206}$ (Fig. 25). Intra- versus Inter-homolog $P(s)$ functions are illustrated in magenta in panel (b).
Figure 23. Contact probability versus distance by chromosome and haplotype.
(A-E) Contact probability ($P(s)$) plots for E14 ES cells (A), zygonema (B), zygonema inter-homolog (C), pachynema (D), and pachynema inter-homolog (E). For each graph, genome-wide data is shown in black, and individual chromosomes are shown in rainbow colors, offset in Y by 0.1 units for each chromosome to improve clarity. The unique scaling of the X chromosome in pachynema is also shown in Fig. 27D. The X chromosome is not shown in inter-homolog graphs, as these cells contain only one X chromosome.

(F) Contact probability versus distance plot for zygonema, showing all data (black) and data for each haplotype (B6 orange, Y offset 0.2 units; CAST purple, Y offset 0.4 units).

(G) Contact probability versus distance plot for pachynema (calculated from pachynema sample #1, see Table S1), colored as in (a).

(H) Contact probability vs. genomic distance ($P(s)$) curves for the X chromosome (green/purple) vs. autosome (black) in zygonema.

(I) Contact probability vs. genomic distance ($P(s)$) curves for the X chromosome (green/purple) vs. autosomes (black) in pachynema.
chromosomes in early mitotic prophase, which have lost detectable TADs and are organized as linear arrays of loops, but have not yet formed the highly compacted helical arrays characteristic of metaphase chromosomes (260). While contact probability in mitotic prophase chromosomes drops sharply beyond ~2 Mb (260), meiotic chromosomes retain a \( P(s) \sim s^{-0.5} \) scaling relationship up to ~5 Mb (Fig. 22A). Chromosomes in pachynema show high contact probability at slightly longer distances than in zygonema, suggesting that cohesin-constrained loops may continue to extend through zygonema until final stabilization of the loop array in pachynema. This model agrees with prior reports of axis compaction as cells progress from zygonema to pachynema, and the more general inverse relationship between loop size and axis length in mutants of both meiosis-specific cohesin subunits (e.g. Smc1β) and chromosome axis core proteins (SYCP3) (25, 27, 263). To estimate average loop length genome-wide, we examined plots of the slope, or derivative, of the \( P(s) \) function, maxima in which have been shown to correlate with average loop lengths inferred from polymer simulations (264). This analysis suggests that average loop lengths are 0.8-1 Mb in zygonema, and extend to 1.5-2 Mb in pachynema (Fig. 22A, lower panel). To estimate average loop density along chromosomes, we measured the total length of synapsed chromosome axis in B6 x CAST pachynema spermatocytes at 215 +/- 33 µm (Fig. 14D). If the entire 2.8 Gb (haploid) genome is contained within loops averaging 1.5 Mb in length, this suggests an average loop density of ~10 loops per micron of chromosome axis in pachynema.

3.10. Hi-C captures homolog pairing in meiotic prophase

Meiotic prophase is the only developmental stage in mammals where homologous chromosomes are physically associated along their lengths. The 0.83% single-nucleotide polymorphism (SNP) density between B6 and CAST haplotypes in our F1 hybrid mice allowed us to assign 3.3% of zygonema read pairs (11.7 million) and 3.6% of pachynema read pairs (17.7 million) as unambiguous inter-homolog contacts, enabling analysis of inter-homolog contacts genome-wide (Methods, Table 6). Hi-C contact maps constructed using only inter-homolog contacts showed strong diagonal signal in all intra-chromosomal maps, clearly
indicating that homologs are aligned along their lengths (Fig. 22C-D, 24). This general relationship was true in both zygonema and pachynema, despite the fact that chromosomes are only partially synapsed in zygonema. Preferential association within A/B compartments, visible as a checkerboard pattern in the inter-homolog Hi-C maps, was also evident along the entire lengths of most chromosomes (Fig. 22C-D, 24A-B). This finding supports a model in which the chromatin loops of paired homologs are extensively interdigitated (Fig. 22B), allowing preferential self-association of the A and B compartments between these chromosomes. In agreement with this idea, we also observe evidence of transcription-mediated interactions between homologs (Fig. 24C-D).

We next plotted contact probability versus genomic distance specifically for inter-homolog contacts (Fig. 22E). The inter-homolog $P(s)$ function shows a significantly shallower slope than the intra-homolog $P(s)$ function, with a power-law scaling roughly proportional to $s^{-0.18}$ (Fig. 22E). When considering the structure of a synapsed homolog pair, we envision that two factors may contribute to this shallower slope. First, synapsed homologs are aligned and juxtaposed arrays of chromatin loops, whose bases are held apart by the SC but which can likely extensively interdigitate (Fig. 22E). The effect of this loop interdigitation can be modeled mathematically as a convolution of two $P(s)=s^{-0.5}$ functions, which results in a power-law scaling function proportional to $P(s)=s^{-0.2}$ (Fig. 25). Second, chromosomes are unlikely to be held in perfect juxtaposition by the synaptonemal complex. Local variation in packing density due to differences in loop size and positioning, plus variations in axis structure, likely give rise to small displacements of aligned homologs relative to one another. The effect on interhomolog $P(s)$ would be to increase long-range contacts relative to short-range contacts, as we observe (Fig. 22E). Overall, our data support a model in which synapsed homologs are closely aligned along their length, while individual loci within the aligned loop arrays retain significant freedom to access sequences on the homologous chromosome within a +/- 5-8 Mb region.
Figure 24. Inter-homolog Hi-C contact maps.
(A) Hi-C contact maps showing inter-homolog contacts for chromosome 3 in zygonema and pachynema.
(B) Hi-C contact maps showing inter-homolog contacts for chromosome 6 in zygonema and pachynema.
(C) Overall Hi-C contact map for a region of chromosome 7 showing strong clustering of piRNA loci in pachynema. Dotted box indicates the region shown in Fig. 20B, and blue circles indicate strong clustering interactions.
(D) Hi-C contact maps showing interhomolog contact maps (at two different contrast levels) of the same region as panel C. The maps show evidence of transcribed-loci clustering between homologs, despite their low resolution and signal-to-noise ratio.
Figure 25. Modeling inter-homolog contacts as a convolution of $P(s)$ functions.

(A) Graphical illustration of the mathematical convolution of two power-law functions.
(B) Applicability of the convolution to inter-homolog interactions in meiotic chromosomes: Intra-homolog contact probability versus genomic distance follows a power-law scaling function (top). Interactions between two juxtaposed and aligned loop arrays with identical power-law scaling can be modeled by a convolution of the respective scaling functions, resulting in a wider and shallower scaling function for inter-homolog contacts (bottom).
(C) Plot of the function $P(s)=s^{-0.5}$ (orange), and a convolution of this function with itself, $P(s) * P(s)$ (gray, normalized to 1 at $x=1$). Both functions are symmetrical with respect to the Y axis (as in panels A and B), but only positive values are shown. The convolution was calculated using integer values for $s$ in the interval from -30 to 30. Since the value of $0^{-0.5}$ is infinity, we used the value of $0.1^{-0.5} = 3.16$ for the purposes of this calculation. Fitting the convolution data series (gray) to a power-law trendline yields a function with scaling proportional to $s^{-0.206}$, close to the observed scaling of inter-homolog contacts in meiotic prophase chromosomes.
(D) Log-log plot of the graph shown in (C); this plot is equivalent to the log-log $P(s)$ plots in Fig. 22.
3.11. Meiotic recombination frequency is strongly correlated with compartment structure

Spo11-catalyzed DSBs, which initiate meiotic recombination, occur preferentially in “hotspots” whose locations are dictated by a combination of chromatin structure and protein factors, and in yeast correlate with high-GC content regions (23, 103, 265, 266). In most mammals, hotspot locations are controlled by PRDM9, a histone methyltransferase that generates trimethylated histone H3 lysine 4 and 36 (H3K4me3, H3K36me3) marks in chromatin near its binding sites (267). PRDM9 has been shown to direct recombination away from functional elements like promoters at the fine scale (95), but control of DSB formation at larger scales is not well understood in mammals. Prior reports that the recombinase RAD51 preferentially localizes to R-band (A compartment) chromatin in meiotic prophase (268), and also that meiotic chiasma appear more frequently in R bands in mouse spermatocytes (269), have hinted that compartment identity may play a role in mammalian meiotic recombination. More recently, PRDM9 was shown to bind and promote DSB formation more effectively in euchromatin than in heterochromatin or lamin-associated regions (270), suggesting that chromatin accessibility may directly affect meiotic recombination rates through differential PRDM9 binding. Finally, genome-wide maps of meiotic DSBs have shown a bias toward nucleosome-depleted regions flanked by H3K4me3- and H3K36me3 nucleosomes in euchromatin (117).

To further explore the connection between chromosome compartments and meiotic recombination, we overlaid the chromosome compartment structure with a previously-reported map of meiotic DSB hotspots in B6 x CAST F1 hybrids (110). We found that both hotspot density (Fig. 26A-B, 10A) and relative intensity (Fig. 26C, 10E) are significantly higher in the A compartment compared to the B compartment. The A compartment is also enriched in both PRDM9-bound sites (Fig. 10B) and H3K4me3 peaks (Fig. 10C) in B6 x CAST spermatocytes (108). Finally, a set of ~800 crossovers between B6 and CAST chromosomes in the multi-species Collaborative Cross (227) also shows a strong bias toward the A compartment (Figure 10D). Overall, these data indicate that the meiotic recombination landscape, while controlled at
Figure 26. Meiotic DSB hotspots show strong compartment bias.

(A) Eigenvector analysis of chromosome 3 in interphase, zygonema, and pachynema, as in Fig. 15D, with the addition of DSB hotspot locations and intensities(110).
(B) Fraction of each chromosome in the A compartment (blue circles, genome-wide data at top) and the fraction of DSB hotspots in that chromosome that are located in the A compartment (open circles). Every chromosome shows a strong bias toward the A compartment (Fig. 10A).
(C) Cumulative distribution of hotspot intensity by compartment (A blue, B green) in chromosome 3 (left) and genome-wide (right). P values calculated using a Kolmogorov-Smirnov test. See Fig. 10E for graphs of each individual chromosome.
the fine scale by the location of PRDM9 binding sites, is strongly correlated at the megabase scale with compartment identity and chromatin state.

3.12. Isolation and silencing of the X-chromosome in pachynema

In mammalian meiosis, chromosomes that fail to pair and synapse are subject to a pathway termed meiotic silencing of unsynapped chromatin (MSUC), in which these regions obtain repressive chromatin marks and are transcriptionally silenced (199, 271-273). In male mice, the X and Y chromosomes pair, synapse, and form crossovers in a ~1 Mb “pseudo-autosomal region”, but the bulk of these chromosomes remain unpaired. As spermatocytes enter pachynema, the unsynapsed regions of the X and Y are silenced by MSUC, also termed “meiotic sex chromosome inactivation” (MSCI) (274), and become isolated from other chromosomes as they are packaged into the “sex body” or XY body. Our Hi-C contact maps clearly illustrate the reorganization of the X chromosome in pachynema. In zygonema, the X chromosome behaves equivalently to autosomes, showing strong “X”-shaped inter-chromosomal interaction patterns (Fig. 17C) and maintaining compartment structure while losing visible TADs (Fig. 27A-B). While these features are maintained through pachynema on autosomes, however, the X chromosome shows dramatic changes. First, the X chromosome becomes strongly isolated from all autosomes in pachynema, completely losing the “X”-shaped inter-chromosomal contact pattern observed in zygonema (Fig. 17C). Second, the X chromosome’s compartment structure is completely lost in pachynema (Fig. 27B, 18D). Third, consistent with the idea that the looping or clustering interactions we observe on autosomes are linked to transcription, we observe a near-complete loss of this clustering on the X chromosome as it becomes transcriptionally silenced in pachynema (Fig. 27B).

While these data reveal significant reorganization of the X chromosome in pachynema, its underlying structure as a linear array of loops appears mostly unaffected. We plotted \( P(s) \) for the X chromosome in both zygonema and pachynema, and found that while the X chromosome shows a subtly different contact probability curve in pachynema compared to autosomes, the overall shape and slope of the curve is largely unchanged from zygonema (Fig. 23H,I). Thus,
Figure 27. X chromosome organization in pachynema.

(A) Hi-C contact maps for the X chromosome in interphase, zygonema, and pachynema. Dotted boxes indicate the area shown in close-up in insets.

(B) Closeup view of a region of chromosome X that shows transcription-mediated clustering of loci in zygonema, which is largely lost in pachynema. Shown in green are RNA Polymerase II binding peaks at 10 dpp (zygonema) or 16 dpp (pachynema)(238).

(C) Pearson correlation matrices for the X chromosome in interphase, zygonema, and pachynema. See Figure 18D for Eigenvector analysis of the X chromosome.
the pachynema X chromosome can be considered to represent a “basal state” of meiotic chromosome organization, in which the axis-associated loop structure is unperturbed by either transcription-mediated clustering of loci or A/B compartment structure. In agreement with the idea that meiotic chromosome axis-associated chromatin loop locations are mostly stochastic, we observe no evidence of reproducibly-located loops along the pachynema X chromosome in our Hi-C contact maps (Fig 27A-B).
Chapter 4: Discussion and Future Directions

4.1 Success of synchronization method

Defining the temporal progression of meiotic recombination is critical for understanding how crossover and noncrossover pathways are interrelated and regulated. The ease of synchronizing meiosis in SK1 yeast cells enables genetic analysis of recombinants at discrete meiotic time points. However, a temporally-based molecular analysis of meiotic recombination outcomes at each stage of prophase I in a mammalian system has been hampered by the continuous nature of spermatogenesis. Although we can assume evolutionary conservation of some recombination pathways based upon current knowledge of mechanistic details in budding yeast, there are enough notable exceptions that we cannot simply assume that mammals behave identically to budding yeast.

Previous attempts to isolate pure populations of spermatocytes at specific stages of prophase I have relied on STA-PUT or the first juvenile spermatogenic wave. STA-PUT takes advantage of the fact that different cell types can be separated by sedimentation velocity; however this method can only enrich for cells in pachynema at high purity (275). Additionally, the first juvenile wave of spermatogenesis does not resemble adult spermatogenesis, particularly with regard to recombination pathway used (70). Recently, a novel method was developed that enables the isolation of enriched populations from spermatogonia to spermatids but this requires the use of transgenic mice in addition to synchronization and FACS sorting (276).

To tackle this issue, I developed a novel in vivo assay by combining two pre-existing methods: synchronization of mouse spermatogenesis by using retinoic acid inhibitors followed by injection of retinoic acid (218), then let the mice recover for 10-50 days before isolating synchronized prophase I cells by flow cytometry following Hoechst 33342 staining that when combined allows separation of cells based on DNA ploidy and size. Compared to the 15-20 minute window between zygonema and pachynema in SK1 yeast cells (48), my assay offers a much wider window of time for the isolation cells in three prophase I stages of interest:
zygonema (2 days), pachynema (7 days) and diplonema (1 day) In addition, my synchronization protocol does not require transgenic mice, increasing accessibility for many researchers.

This novel assay allowed the investigation of specific stages of prophase I in a metazoan system for the first time. My results reveal novel mechanistic insights into how meiotic pathways are regulated and interrelated, while also providing insight into how molecular recombination events depend on and correlate with chromosome behavior.

4.2 Role of early distal noncrossovers during meiosis

A temporal analysis performed in budding yeast revealed that noncrossovers and crossovers can be molecularly identified by PCR at pachynema and that noncrossovers appear earlier than crossovers. However, only central polymorphisms were probed in this study, thus both noncrossovers and crossovers were of similar length (noncrossover 1.8 kb and crossover 2.0 kb) (59)

Prior attempts to study the timing of noncrossover and crossover formation in a mammalian system took advantage of the semi-synchronous nature of juvenile mouse spermatogenesis to isolate spermatocytes from 11- to 21-dpp (days post-partum) and amplify noncrossovers and crossover recombinants at different time points at the Psmb9 hotspot located on mouse autosome 17 (64). During the first wave of spermatogenesis, 100% of 11-dpp spermatocytes are in leptonema/zygonema and 67% of 16-dpp spermatocytes are in pachynema, and by 20-dpp, 81% of spermatocytes are in pachynema and 7% in diplonema, indicating the first wave of semi-synchronous spermatogenesis lasts from approximately 11-dpp to 21-dpp (SYCP3 and H1T were used in staging) (170). Parallel measurements of both noncrossovers and crossovers at each time point showed that approximately 73% of total noncrossovers formed at 17-18-dpp as compared to only 23% crossovers. This suggested that, similar to budding yeast, both crossover and noncrossovers pathways occur in mid-to-late pachynema. These results led the authors to suggest that noncrossovers likely form earlier than noncrossovers. Although this interpretation is consistent with what was observed in
budding yeast, it lacks convincing molecular evidence. Moreover, recent works have reported that the juvenile spermatogenic wave in mice is very different from adult waves, both at the cytological and molecular levels (70, 277, 278).

My novel timing assay revealed a new noncrossover recombination pathway. Approximately half of all noncrossovers formed at 59.5 appear during zygonema and are noticeably underrepresented in the center of the hotspot. Such distal noncrossovers are also observed at A3 but in lower proportions, making up ~1/3 of total noncrossovers. This difference can be attributed to a higher proportion of noncrossovers formed at A3.

Considering the stage when these distal noncrossovers form and the chromosomal behavior observed at this stage, there are two at least two possible roles for these distal noncrossovers during prophase I: either 1) distal noncrossovers are involved and aid in interhomolog pairing; or 2) distal noncrossovers appear during zygonema at partially synapsed regions. It has been hypothesized that strand invasion is required for homologs to find each other. By contrast, based on previous results from budding yeast and mice (48, 64), formation of the synaptonemal complex along the chromosome axis has been suggested to be a prerequisite for the completion of any recombination.

If the former is true, then one can argue that meiosis uses these early distal noncrossovers as a means to scan the chromosomes to find the right partner for proper pairing, and consequently, synapsis. This could be advantageous during meiosis because noncrossovers that derive from NAHR are generally not deleterious, and these distal gene conversions can provide additional sources of genetic diversity. In other words, distal noncrossovers may act as a prerequisite for pairing during zygonema. However, my results only show that these early distal noncrossovers occur contemporaneously with pairing and partial synapsis, and I cannot exclude the possibility that distal noncrossovers arise from partially synapsed regions. Another possibility is that even though full synapsis may not be required as it is in budding yeast, some level of synapsis, even partial synapsis, may be required for any recombination to occur in a mammalian system. In support of this argument,
partial interhomolog pairing is observed in both budding yeast and mice even in the absence of DSBs (125-127). However, the partial interhomolog pairing in mice lacking functional SPO11 is lost by zygonema, thus supporting an important role for recombination in maintaining and completing interhomolog pairing in a mammalian system.

I favor the model that distal noncrossovers play an important role in promoting proper pairing/synapsis because that model explains why so many DSBs are formed at the beginning of meiotic prophase I when only 10% are repaired as crossovers. Presumably, most DSBs are repaired as noncrossovers, and noncrossovers do not guarantee proper chromosome segregation. Therefore, it is unclear why so many DSBs are made if they do not to help ensure proper segregation. One possibility is that they enable greater genetic diversity. However, as noncrossovers are formed during zygonema at the time chromosomes are finding each other and engaging in interhomolog interaction, it seems that meiotic chromosomes must be able to scan for the right partner, and strand invasion and extension provide a mechanism for scanning. This model also explains why the pre-DSB pairing observed in the aforementioned mutants (in 1.4.3. Pairing, synapsis and recombination) lead to neither stable pairing nor synapsis (125-127).

Regardless, the identification of distal noncrossovers during meiosis at early prophase I is an important finding and how these noncrossovers influence other recombination pathways must now be considered by the field. This finding has already prompted other scientists to study meiotic recombination outcomes with distal polymorphisms in budding yeast during zygonema (e.g. Dr. Michael Lichten, unpublished data). Interestingly, his group observed a similar phenotype consistent with distal noncrossovers forming during zygonema, indicating that this pathway is likely evolutionarily conserved throughout eukarya.

4.2.1 Possible mechanisms of distal noncrossover formation

The discovery of distal noncrossovers raises the conundrum of how distal noncrossovers form when approximately ~80% of DSBs occur in the central 200 bp of a meiotic hotspot? The frequency of these early distal noncrossovers is too high (greater than 20%) to
Figure 28. Model of meiotic recombination in relation to cytological timing based on my data.

Left panel: $L = \text{leptonema}; Z = \text{zygonema}; P = \text{pachynema}; D = \text{diplonema}$
have come from the remaining ~20% of DSBs. Three potential models that could explain how distal noncrossovers may be achieved are detailed below (Fig. 28).

One potential mechanism of distal noncrossovers formation relies on initial invasion into the sister chromatid and extension of the 3’ end using the identical sister sequences. Then, the extended 3’ end ejects and switches templates by invading into the homolog and further extends using the homolog. The final repair outcome is a noncrossover located distal to the center of hotspot. This mechanism has been suggested previously by Dr. Neil Hunter (47), and has been observed in vivo in budding yeast and mouse spermatocytes (57, 83). A limited number of central noncrossovers observed at both hotspots during zygonema could result from a minimal extension using the sister chromatid (~1-2 bp) before quickly switching the template to the homolog. Therefore, it is likely that meiotic recombination has homolog bias for the completion of repair, rather than recombination per se.

Alternatively, differential mismatch repair could produce distal noncrossovers. Upon DSB formation in the center, the broken 3’ end would immediately invade into the homolog and extend from the center to create a heteroduplex tract, which can either be restored or converted to the donor (homolog) genotype by mismatch repair. It is possible that the abundance of distal noncrossovers could be explained by unique mismatch repair dynamics in which restoration is favored near the center but full gene conversion is favored at the distal region.

4.3. Central noncrossovers as a reservoir for the crossover/noncrossover decision

The other half of noncrossovers do not appear until pachynema. This suggests that there may be a mechanism that suppresses the completion of recombination in the hotspot center until full synapsis is achieved. Importantly, central noncrossovers form at the same time as crossovers, which are also largely formed in the center of hotspots. Because most DSBs do not become crossovers, it is possible that central noncrossovers arise from the crossover/noncrossover decision at the time of crossover maturation during pachynema and
that both crossovers and central noncrossovers come from the same intermediate stabilized by MSH4/5.

This model is also supported by cytological assays. During zygonema, there are ~300 RAD51 and/or DMC1 foci marking DSBs, but this number is reduced to ~100-150 intermediate foci marked by pro-crossover factor MSH4/5 complex by late zygonema/early pachynema. Thus, approximately ~33% - 50% of foci have disappeared. This number correlates well with the frequency of distal noncrossovers observed during zygonema at least at two hotspots. At the 59.5 and A3, the total noncrossover frequency at zygonema makes up 20% (59.5) and 33% (A3) of the total recombination frequency observed at diplonema. Therefore, as intermediate foci identified by MSH4 staining appear, any DSB sites not marked with MSH4 (~33 - 50% of total DSBs) have likely repaired their breaks as distal noncrossovers. Some of the foci stabilized by the MSH4/5 complex have presumably been designated as future crossover sites. It is likely that any DSB intermediate marked by MSH4/5 foci that was not selected for crossover designation will be repaired as central noncrossovers during crossover maturation in pachynema (Fig. 29).

4.4 Crossover formation at the pachytene stage aids proper repair and avoids NAHR

In alignment with cytological data, crossovers could be detected by PCR amplification during pachynema. One noticeable difference is that the crossover frequency observed in pachynema is unusually high compared to that observed during either diplonema or in fully mature sperm. There are several possible explanations for this observation. First, these amplified products could represent extended D-loops that appear as crossovers in our assay but later get nicked and form heteroduplex intermediates, which then are restored to the parental genotype by mismatch repair, rendering the outcome invisible to the assay at later times. Second, these may represent heteroduplex intermediates at the central polymorphism. The majority of crossovers convert in the central 200 bp of meiotic hotspots as observed at both the A3 and 59.5 hotspots. Since most crossovers occurring during pachynema also occurred in the center, it is possible that these may represent heteroduplex in the central region,
Figure 29. Distal noncrossovers vs. central noncrossovers.

DSBs (yellow) that do not become stabilized by MSH4/5 (red) are repaired as distal noncrossovers that promote pairing and synapsis during zygogena. Only a small number of intermediate foci stabilized by MSH4/5 (red) will become designated as future crossovers. Foci not selected by the designation process will repair its DSBs as central noncrossovers.
which later gets restored to the parental genotype by mismatch repair. To test this hypothesis, I gathered crossover molecules from pachynema samples to genotype them using both B6 and DBA alleles at polymorphisms. Then, we calculated the number of mixed tracts that were positive for both B6 and DBA at particular polymorphisms. These mixed tracts indicate either a single crossover molecule with a retained heteroduplex tract or two or more crossover molecules in a single well. By using a Poisson approximation, I can estimate the number of wells that likely contain more than one crossover molecules. When the number of wells with mixed tracts exceeds the expected number based on the Poisson approximation, it suggests that these mixed tracts represent single crossover molecules with retained heteroduplex. However, the number of wells with crossovers containing mixed tracts in pachynema did not exceed expectations, suggesting that the crossovers we observe in pachynema do not contain heteroduplex tracts in the central polymorphisms. Third, it is possible these are crossovers that fail prior to the crossover assurance check point and undergo apoptosis at pachynema. This, however, is also unlikely because there are few apoptotic cells present during pachynema in wild-type animals based on TUNEL staining performed on testes tubules (279). Finally, it is possible that the PCR assay used fails to amplify certain crossovers from cells in diplonema or from fully mature sperm for reasons that are unclear. In any case, by comparing the crossover frequencies of pachynema to diplonema and sperm, it appears that all crossovers can be accounted for by pachynema.

As mentioned previously, crossover designation occurs during zygonema when homologous chromosomes start to pair and show partial synapsis. Since our assay cannot recover crossovers until full synapsis is achieved, it suggests that crossover-designated sites are blocked from completing repair until synaptonemal complex formation has completed for the entire chromosome. This model is attractive as it would be advantageous if spermatocytes evolved mechanism(s) to suppress crossover formation until all homologs are fully aligned along their lengths to avoid NAHR (203).
In addition, it further solidifies the crucial role the synaptonemal complex proteins play in promoting crossover maturation. Studies using loss-of-function mutations in ZMM proteins that promote synapsis formation revealed that synaptonemal complex formation is required for crossover formation but exactly how has been elusive. In the absence of SYCP1 in mice, spermatocytes pair properly but fail to synapse and recruit MLH1/3 complexes (10, 163, 280), whereas the loss of MutS homolog 4 (MSH4/5), another ZMM protein, leads to an even earlier collapse of meiotic progression by a failure to pair and align homologs (164). Moreover, yeast Zip3 mutants manifest a severe defect in synaptonemal complex formation, although abrogation of RNF212 (the mammalian Zip3 ortholog) in spermatocytes does not affect synapsis (151, 152) (unpublished data). According to Woglar & Villeneuve (71), during late pachynema in C. elegans, the two layers of the central region of the synaptonemal complex forms spatially distinct, bubble-like structures that encase designated crossover sites. The intermediate repair proteins BLM, MutS and COSA-1 likely stabilize and help prevent the dismantling of crossover-specific intermediates into noncrossovers by dissolution, which does not ensure chromosome segregation. They further confirmed their hypothesis by analyzing synaptonemal complex mutants that only show transient “bubbles” lacking BLM, indicating that these transient structures likely have defaulted to becoming a noncrossover. Therefore, crossover designation and execution are finely coordinated with synaptonemal complex formation to ensure crossover repair with the correct homolog partner and that the required proteins are assembled near the crossover-designated sites.

4.5. The difference in the timing of switch from distal to central noncrossover dominance at A3 and 59.5 may be due to PRDM9

Our temporal analysis reveals differences in the timing of noncrossovers formation at the 59.5 and A3 hotspots. At the 59.5 hotspot, the number of distal noncrossovers is much greater than the number of central noncrossovers formed during zygonema; however the numbers parallel each other and then reverse during pachynema and beyond, and this pattern is retained in sperm. However, at the A3 hotspot, the number of distal and central
noncrossovers is roughly equivalent reflecting the central/distal division of pachynema spermatocytes at 59.5. Then, central noncrossovers become dominant during pachynema and is equivalent to that observed in diplonema and sperm. This suggests that the program switch from distal to central noncrossovers occurs faster at A3 than 59.5.

We propose that this difference in the shift between distal and central noncrossovers between the two hotspots may be attributed to differential binding of PRDM9 to the A3 and 59.5 hotspots. At the A3 hotspot, relatively equal amount of DSBs are generated on both the B6 and DBA alleles (65, 66, 70). In contrast, approximately ~90% of DSBs are found on the B6 chromosomes at the 59.5 hotspot, indicating PRDM9 binding at the 59.5 hotspot is highly asymmetric (70, 95, 117). Asymmetric binding of PRDM9 is thought to delay synapsis as these sites frequently show persistent DMC1 expression (111, 118). Based on these observations, we propose that the 59.5 hotspot shows delayed synapsis (and likely repair) relative to the A3 hotspot, and that this delay is reflected by a slower central/distal program switch at 59.5.

4.6. Long noncrossovers and SSE-dependent crossovers act as a backup pathway

The presence of long noncrossovers in high frequency and a few crossovers in spermatocytes lacking MLH3 at the diplotene stage is consistent with data in yeast that these are likely products of backup pathways that become active at a later stage to deal with unrepaired DSBs in order to condense and compact the chromosomes for segregation (49, 80, 214, 215). Unrepaired dHJs especially poses severe threat to germline genome integrity because it will cause chromosomes to tear apart upon segregation. This is in contrast to what was previously reported by (64) where both MLH1/3 dependent and SSE-dependent (Class I & II) crossovers appeared contemporaneously in the first semi-synchronous juvenile wave. Again, juvenile spermatogenesis is not equivalent to adult spermatocytes, and that likely explains the discrepancies. These backup pathways are likely suppressed at pachytene stage to prevent crossover designated sites from illegitimately forming noncrossovers that will not guarantee proper chromosome segregation. Indeed, timely expression and activity of proteins is critical during meiosis and tightly controlled. For example, premature activation of Yen1 during
prophase I, an SSE protein that is normally active during anaphase II in budding yeast leads to premature resolution of joint molecules as crossovers that are non-interfering MLH1/3 independent crossovers (215). Disruption in crossover distribution and patterning can lead to unstable spindle formation and impaired segregation of chromosomes.

Another important evidence derived from my results adds insight into the crossover intermediate structure commonly known as dHJs (48). The prevailing model is established mostly from studies in budding yeast and suggests the crossover-specific intermediate is a fully ligated dHJ. My data suggests otherwise, since if that was true, I should have been able to PCR-amplify long noncrossover-like molecules during pachynema that make up a part of dHJ (Fig. 4), particularly at the 59.5 hotspot where such intermediates should be common. Rather, my data suggests that either the designated crossover intermediate is an unligated dHJ with a nicks on both strands, as suggested by Marsolier-Kergoat and colleagues (57), or is a fully ligated dHJ that is very transient and short-living. The former assumption is more favored since a fully ligated dHJ structure suggests the intermediate upon second-end capture gets fully ligated, only to make a nick again for resolution. By leaving the intermediate unligated (i.e., a nick), the resolution of dHJ can be achieved more efficiently.

4.7. MLH3 may have an earlier role prior to crossover maturation

The proportion of central noncrossover recombination was significantly underrepresented in Mlh3 mutants compared to wild type, which was a phenotype that was overlooked in our prior analysis (70). Moreover, Mlh3 null mice exit out of pachynema prematurely and stay longer in diplonema (unpublished). These data suggest an additional role that MLH3 may play during meiotic recombination. Interestingly, in the absence of RNF212, which is proposed as a crossover designation factor, we observe an identical phenotype (unpublished), which suggests that MLH3 may play an earlier role, potentially as early as zygonema. MLH1 has been shown to be involved in resolving interlocks during zygonema in Sordaria macrospora to facilitate proper pairing (17). Because MLH1 is an obligate heterodimeric partner of MLH3 for the MutLγ complex, we hypothesize that MLH3 may play the
same role at an earlier stage in spermatocytes even though we do not observe discrete foci by cytology.

If we assume that MLH3 is active during zygonema, could it be possible that MLH3 is involved in crossover designation, which we also know happens during zygonema? Could it be that the crossover designation step regulates the central and distal distribution of noncrossovers? There is evidence that MLH3 and MSH4 interact together in both mouse meiotic cells and human testis (281), although the timing of their interaction during prophase I is not clear. In the absence of MSH4, mouse spermatocytes do not pair or synapse properly and MSH4 is known to form a heterodimeric complex with MSH5 to form MutSγ. On the other hand, genetic ablation of Mlh3 in mouse spermatocytes do not exhibit any pairing or synaptic defects (70). Therefore, if MLH3 and MSH4 do form a complex at early prophase I stage and are responsible for the distribution of noncrossovers, then in the absence of MLH3, this may cause a disruption in noncrossover distribution.

Another possible explanation for MLH3 affecting the distribution of noncrossovers would be a potential promiscuous activity of MLH1 as a result of MLH3 loss. For example, MLH1 can form complexes with multiple other proteins, such as MLH2 to form MutLβ (MLH1/2), which interacts with the Mer3 helicase (HFM1 in mammals) that recognizes D-loops and limits gene conversion lengths genome-wide in budding yeast. Additionally, MLH1 can dimerize with PMS1 to form MutLα (MLH1/PMS1) that bears an endonuclease activity during MMR (PMS2 in humans) (165). Differential activity of MLH1 and MLH3 has also been shown previously in budding yeast, where the deletion of one or the other leads to different outcomes. For example, mlh3 mutants have a higher spore viability than mlh1 mutant (80% to 50%, respectively), and the residual crossovers observed in the mlh3 mutant retain stronger interference than mlh1 mutant (215). Similarly, mouse spermatocytes exhibit differential temporal expression of MLH1 and MLH3: MLH3 expression can be detected by cytology during early pachynema but MLH1 expression can be detected at mid pachynema (282). It is possible that MLH3 may be present
at zygonema but cannot be detected by cytology because they have not formed discernable aggregates.

4.8. Loss of MLH3 leads to more singleton noncrossovers

Another striking result that we obtained from our temporal assay and did not observe from our previous bulk 4C prophase I assay in Mlh3 null mice was the increased frequency of singleton noncrossovers at 59.5 hotspot that only converted one polymorphism. This is consistent with findings in budding yeast from various mlh3 mutants that were created by site-directed mutagenesis. All mlh3 mutants analyzed in this experiment showed genome-wide increase in noncrossovers (283). This result implies one of the four possibilities: 1) these singleton noncrossovers are the result of dissolution of dHJs that only incorporate a single polymorphism, 2) the SDSA pathway continuously functions during the progression of prophase I in the absence of MLH3 in mouse spermatocytes, 3) these are the byproducts of hDNA-retaining outcomes of dHJ resolution or dissolution that then become restored by mismatch repair, only leaving a singleton noncrossover outcome (Fig. 30), or 4) these singleton noncrossovers are derived from a novel recombination pathway that is yet to be identified.

4.9. Chromosome organization during meiotic prophase I

In addition to the discovery of temporally and spatially distinct individual meiotic recombination pathways at a DNA level in relation to cytological evidence, the genome wide Hi-C showed that the higher order chromosome organization and structure during zygonema and pachynema is very dynamic and highly regulated.

Employing both contact probability maps and direct measurements of axis length, the average loop size and dynamics were calculated for the first time in mouse spermatocytes, which seems to be evolutionarily conserved to 8.5 loops per micron of axis (294). Short loops formed during mitotic interphase of Patski cells lead to longer axes, therefore, a steep decline of contact probability at shorter distance than observed in meiotic zygonema or pachynema.
In the absence of MLH3, dHJ dissolves, leaving heteroduplex DNA configuration as above. Mismatch repair restores all the heteroduplex tracts back to parental DNA, leading to short noncrossovers.

Figure 30. Short (singleton) noncrossover formation in the absence of MLH3
Because there is a peak transcription that occurs during mid-pachynema, one can suspect that TADs may be maintained during meiosis. However, as in mitotic cells, TADs are lost during zygonema and pachynema but unlike mitosis, compartment structures are maintained. This is consistent with what Avalattam and colleagues (284) found in their Hi-C maps of pachynema enriched spermatocytes by STA-PUT method, although these cells displayed weaker compartmentalization than somatic cells, round spermatids or sperm.

Moreover, the meiotic hotspots were largely enriched in compartment A. It has long been known that PRDM9 identifies meiotic hotspots based on its consensus sequence motif and that its PR/SET domain deposits H3K4me3 at that site to open up the chromatin for SPO11 to make breaks. However, the conundrum regarding PRDM9 regulation still remained: 1) the consensus motif of zinc finger array of PRDM9 evolves very quickly, 2) some sequence motifs that are perfect targets for zinc finger array do not form DSBs by SPO11, 3) some sequence motifs that are poor fit for zinc finger array do form DSBs by SPO11, and 4) there are more H3K4me3 sites than there are DSBs during meiosis. In fact, approximately 30% of DSB sites do not have matching sequences to the motif of zinc finger (95, 107, 110, 129, 130). Therefore, the sequence motif alone could not explain how the meiotic hotspot landscape is determined. Based on our data, in addition to sequence information, the architecture of chromosomes seems to influence the location of meiotic DSBs.

Although our data does not directly show that PRDM9 preferentially deposits H3K4me3 in open and gene active compartment A regions of the genome, previous reports indicate that PRDM9 more readily binds to euchromatin than heterochromatin (270). In agreement with this finding, SPO11 is mostly making its DSBs on H3K4me3 sites within compartment A, indicating that chromatin accessibility also participates in shaping the meiotic DSB landscape. It may be that the meiotic program favors DSB formation mainly in compartment A to minimize the search grid for the homologous partner chromosome. By largely excluding compartment B, homologs may find each other more efficiently and progress to pairing and synapsis. Consistent with this hypothesis, asymmetric hotspots that have impaired synapsis and slower repair kinetics may
reflect regions in which the two parental homologs reside in different compartments. This is consistent with Gregorova and colleagues’ finding (118) that a minimum of 27 Mb of homologous sequence is required to successfully synapse homeologous (or not sufficiently homologous) chromosomes. Considering the haploid genome size is ~ 2.8 Gb in mice, only a small portion of homology is thus required for interhomolog interaction. Furthermore, compartment B is enriched with repetitive sequences (285), which are prone to illegitimate recombination by NAHR and deleterious chromosomal rearrangements. Therefore, engaging in interhomolog interaction and recombination by largely targeting compartment A may provide an auspicious environment for meiotic cells to ensure chromosome segregation and reduce erroneous meiotic activities.

Interestingly, interhomolog interactions in zygonema and pachynema are almost identical. This is ironic because chromosomal behavior in zygonema is very different from pachynema: zygonema lacks complete synaptonemal complex formation, and the distance between homologs are closer during pachynema as a result of synapsis. Due to low resolution, the current Hi-C method cannot capture synapsis but rather only the paring behavior, which explains the striking similarities of Hi-C contact maps between two samples. Our proposed model of interdigitated structure that form during interhomolog interaction is derived from the fact that homologous chromosomes can retain a high contact probability at longer distance (5-8 Mb) than genome wide contact probability (~2 Mb). Because our recombination data suggest that recombination may be critical for homolog search, it is our hypothesis that homologous chromosomes need to form a structure that allows the 3’ invading end to scan the chromosome over a larger range than a single loop.

In addition, our data suggest that loop formation is highly variable between cell to cell. This argument is supported by Gassler and colleagues (259) in single cell Hi-C of oocyte data where stable loop formation was present in individual cells. Therefore, in population data, high variability in the location of stable loops produces very weak signal and therefore renders them undetectable. The variability of stable loops probably provides additional flexibility to better
achieve homolog pairing and synapsis. If all cells were programmed to form stable loops only at restricted regions, any complications that arise at these particular regions will hinder further meiotic progression. This is in contrast to what was observed in budding yeast, where stable loops were detectable (241). One major difference between budding yeast and mammalian genome is the size. Because yeast genome size is much smaller compared to mammals, they may bear no need for such flexibility since the cost of pairing and synapsis is likely miniscule compared to mammals.

How can mid-pachynema cells regulate transcription in the absence of TADs? Our data suggest that meiotic cells regulate transcription by forming clusters or a “hub” of transcription loci as loops extrude along the axis during pachynema (250). The retention of compartmental organization is probably also contributing to transcription regulation to some extent so that the gene active compartment A could be utilized for transcription of genes necessary for downstream events. Moreover, TADs are by no means the only factor regulating transcription. Evidence in support of this comes from the cohesin SMC1beta and CTCF knock out mice where transcription disruption was not grossly disrupted, likely because of compartments being well maintained (37, 229, 244). Therefore, it is not surprising that the disappearance of TADs coincides with active transcription.

The meiotic Hi-C results were in comparison to somatic mouse kidney embryonic Patski cells in interphase. This provides nice juxtaposition to mitosis and how higher order chromosomal organization during meiotic prophase I differs from mitosis. However, a more proper control for our samples is spermatogonia that are their mitotically dividing precursors and share the same niche as spermatocytes. Our spermatogonia Hi-C results resemble the mitotic interphase phenotype with obvious compartment separation and visible TADs with no evidence for pairing between the homologs. In spite of similarities, the signal for TADs is weaker in spermatogonia than Patski interphase cells (Fig. 31). This is also consistent with what Wang and colleagues (286) found in primate spermatogonia where TAD signal is relatively weaker in comparison to fibroblasts.
Figure 31. Hi-C map of spermatogonia

Spermatogonia, as expected, do not show significant interhomolog interaction.
Since much of Hi-C analysis has been performed in abnormal cancer cells or embryonic stem cells that do not represent mammalian cells in vivo, the result is not surprising. We suggest that this distinction could be due to the fact that 1) our spermatogonia samples are primary cells, which is expected to be different than cancer cells or embryonic stem cells, and 2) the spermatogonia cells may have already begun to lose their TAD structures to prepare for meiotic entry.

4.10. Clinical implications of my findings

Correlations between advanced age and disease risk has long been known: older females bear a higher risk of having a child with Down Syndrome (134), whereas older fathers bear a higher risk of having a child with schizophrenia and autism (287). Contrary to popular beliefs, male fertility and disease risk of offspring is just as affected by age. The laboratory of Dr. Kari Steffanson performed a genome wide sequencing from Icelanders to investigate the rate of de novo germline mutations in both males and females and reported that males accumulate mutations at four times higher rate than females (287). This is consistent with continuous mitotic division of male germ cells that increases the likelihood of introducing mutations over time by replication errors. On the other hand, being too young carries disease risks as well. In 1983, Rocker and Huether (288) reported an unexpected negative correlation between younger fathers in Ohio and trisomy on chromosome 21. This was later recapitulated in 2015 in Swiss men (186). Congruent with this finding, our lab have previously reported that juvenile male spermatocytes harbor more univalent chromosomes, and that the mechanism behind it is likely due to an increase in alternative repair that gives rise to noncrossovers at the expense of crossovers, which will fail to ensure proper chromosome segregation (70). Because my timing analysis showed that these alternative repair pathways in adult spermatocytes do not become activated until diplonema, the juveniles may have precocious activation of alternative repair pathways that skews crossover-designated intermediates to form noncrossovers.
4.11. Future directions

My results indicate that meiotic prophase I is orchestrated by highly dynamic molecular pathways and higher order structural organization. We are the first group to identify temporally and molecularly distinct meiotic recombination pathways in a metazoan system that suggest distinct roles during meiosis. This was possible due to successful establishment of a synchronization assay, which allowed us to further study the higher order chromosome organization at specific stages of meiotic prophase I. These discoveries will now provide additional parameters for investigators to consider when studying meiosis prophase I. More importantly, the clinical implication of my results warrants further exploration. There are some questions that my results raise regarding pairing, synapsis, and crossover assurance mechanisms that will be important to address for future discoveries:

Q1) Are distal noncrossovers required for pairing or are they a byproduct of partially synapsed regions?

The role pairing plays during meiotic prophase I is critical, and I discovered a molecular recombination pathway associated with pairing. It will be interesting to investigate the role of distal noncrossover recombination in ensuring proper pairing. To address this question, we can analyze a Msh5 ATPase Dead mutant and Msh5 null mice that we obtained from the laboratory of Dr. Paula Cohen from Cornell University. Msh5 null mice pair properly but do not synapse, whereas Msh5 ATPase Dead animals pair properly but only make partial synapsis before going through apoptosis at pachynema. If distal noncrossover recombination is required for pairing, then I anticipate to observe distal noncrossovers in both the null and ATPase dead mutants. However, if distal noncrossovers are coming from partially synapsed regions and that synapsis is a prerequisite for noncrossover outcome, then I will only observe distal noncrossovers in ATPase dead mutants but no recombinants in the null.

Q2) What is the relationship between synaptonemal complex proteins and higher order chromosomal organization?
The role of synapsis during meiosis is undeniable. Msh5 ATPase dead mutants will be useful to look further into higher order chromosomal organization and transcriptional regulation by Hi-C. RNA-seq in pachynema and zygonema enriched samples will establish a baseline of future research. This is necessary to compare our adult synchronized samples to transcription data since both RNA pol II and pi-RNA ChIP-Seq were performed in juvenile mice. RNA pol II ChIP-Seq especially needs to be performed in synchronized pachynemas since the Hi-C map presented in our paper is from whole testicular extracts from juvenile mice. I do not anticipate to detect any active transcription during zygonema, and therefore could be used as our negative control. Performing Hi-C in leptonema and early zygonema cells will also be interesting to investigate the timing and the dynamics of loop formation.

Q3) Does RA treatment eliminate the juvenile characteristics of spermatogenesis?

Even though the synchronization protocol did not affect the overall recombination outcomes, my data implicates that synchronization may obliterate the juvenile characteristics of recombination that we observed in unsynchronized juvenile spermatocytes previously (70). This may be a result of RA treatment and reflect differential RA activity in juvenile spermatocytes from adults. Prior studies indicated that WIN 18,446 treatment followed by RA treatment did not affect the body weight of these mice (which was confirmed by my experiments) nor the testosterone level present in the serum (218). Seven-day treatment with WIN 18,446 sans RA injection increased the total number of Sertoli cells and undifferentiated spermatogonia arrested in G0 stage (188, 289), although whether these changes has any impact on recombination phenotype is unclear. Sertoli cells are “nurse” cells that are known to nourish germ cells to develop into sperm by secreting important substances, such as transferrin and hormones, but their in vivo roles are not well understood (290). The first wave of spermatogenesis triggered by WIN 18,446 protocol undergo five mitotic divisions instead of six as in adults, which resembles the normal first round of spermatogenesis that is 2.5 days shorter than the following rounds of spermatogenesis (188). To definitively confirm whether the synchronization protocol influences the recombination phenotype in juveniles, cytology could
be utilized to count MLH1 foci in 13-16 dpi spermatocytes that corresponds to first spermatogenic wave after WIN 18,446 and RA treatment. If juvenile recombination features are retained even after synchronization protocol, I expect to observe the average number of MLH1 foci in synchronized juvenile spermatocytes that are significantly less than those observed in unsynchronized adult spermatocytes (70). Furthermore, I aim to perform RNA-seq in synchronized and unsynchronized juvenile vs adult mice at specific stages to investigate whether a bolus of RA produces significantly different gene expression profiles that could potentially influence the recombination pathways.

Q4) Are the crossover intermediates in mouse spermatocytes unligated dHJs?

To address this, I could perform an experiment to seal the nicks and generate amplifiable products. To do this, I would synchronize animals, isolate cells at late zygonema and/or early pachynema and treat the cells with DNA ligase and/or DNA polymerase and ligase. I can then perform hotspot analysis to see if long noncrossovers can be amplified at late zygonema/early pachynema. As a negative control, I could test spermatocytes lacking designated intermediates such as $Rnf212^{-/-}$.

Q5) Do juvenile spermatocytes have precocious activity of backup pathways?

To answer this question, I would take advantage of the first semi-synchronous spermatogenesis in wild type mice. Normally, 13-16-dpp mice are considered to be mostly enriched in pachynema. Therefore, I can sort the juvenile pachynema samples to analyze and see if a significantly higher number of long noncrossovers are formed at that stage. In addition, I can use juvenile spermatocytes as a model to make inferences about female meiotic recombination since the chromosome organization and behavior observed in human juvenile spermatocytes were similar to that observed in adult female oocytes: human juvenile spermatocytes had longer axis length than adult human spermatocytes that was similar to human oocyte axis length (70). Considering the difficulties associated in studying female meiosis, this will be an excellent tool to gain mechanistic insights on how female meiosis is regulated.
In conclusion, my synchronization protocol led to critical discoveries of meiotic prophase I regulation. Addressing the questions above will further our understanding on mechanistic details of meiotic prophase I in a mammalian system. In the long run, expansion of our knowledge in meiotic prophase I regulation in mice may help us understand meiotic defects observed in humans that can pave ways for solution.
References


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