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THE INTERPLAY BETWEEN CHROMOSOME STRUCTURE AND MEIOTIC INTEGRITY

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The interplay between chromosome structure and meiotic integrity

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my mother,

In honor of the sacrifices you have made for me.

Do.

Or do not.

There is no try.

~Yoda

And if all else fails…

Just keep swimming.

ABSTRACT

Sexually reproducing organisms employ a specialized cell division called meiosis to form unique, haploid gametes from a diploid precursor cell. Upon fertilization, two opposite-sex gametes fuse to create a zygote that will develop into the offspring. Fundamental to meiosis is the formation and repair of programmed DNA double-strand breaks (DSBs) during prophase. These DSBs are repaired using the homologous chromosome (homolog) as a template for homologous recombination, which creates linkages between the homologs that are essential for faithful segregation. Following prophase, two successive rounds of chromosome segregation ensue. The first, meiosis I (MI), segregates homologs and the second, meiosis II (MII), segregates sister chromatids. Recombination is dependent on chromosome structure, and events that alter the DNA landscape will impact meiotic fidelity and, in turn, genomic integrity in gametes and future offspring. Thus understanding the relationship between chromosome structure and meiosis can help gain insight into human fertility and underlying causes of genetic disorders. **It was the goal of this thesis to investigate factors known to regulate chromosome structure and determine their influence on meiosis in the budding yeast *Saccharomyces cerevisiae* and mice. In addition, we tested a novel method to search for new mammalian meiotic factors that may impact fertility.**

In **Paper I**, we identified a role for the conserved Structural Maintenance of Chromosomes (SMC) 5/6 complex during meiotic recombination in budding yeast. *smc5/6* complex mutants experienced a DSB-dependent segregation block, suggesting that the defect was caused by recombination. Consistent with this notion, Smc6-deficient cells accumulated high levels of recombination intermediates, particularly between sister chromatids, which is normally not seen in the wild type. Return-to-function studies indicated that the Smc5/6 complex was most crucial during resolution of recombination intermediates. These results suggest that the Smc5/6 complex works primarily in the resolution of recombination structures formed outside of homolog-directed pathways during meiosis.

We characterized a role for DNA topoisomerases Top2 and Top3 during meiosis in *S. cerevisiae* by using meiosis-specific mutants in **Paper II**. Cells deficient for either Top2 or Top3 experienced a segregation block. While *top3* cells were rescued completely by removing recombination, the *top2* mutant was only partially rescued. This suggests that Top3 mainly functions during meiotic recombination. In contrast, the data indicates that

Top2 has a role outside of recombination. In line with this idea, some of the segregation defects in cells lacking Top2 seemed to arise from break-independent sister entanglements. Since Top2 is known to be important in resolving sister chromatid intertwinings during mitosis to facilitate proper segregation, it is likely that it plays a similar role during meiosis.

The CCCTC-binding factor (CCTF) is an architectural protein essential for proper genome structure and function in higher eukaryotes. In **Paper III**, we created a testes-specific *ctcf* mouse mutant strain (*cctf*-cKO) in order to study the function of CTCF during gamete (sperm in males) formation in mice. CTCF-deficient mice completed meiosis and sperm specialization without any major abnormalities, though mice were infertile and had low sperm counts. Sperm from the *ctcf*-cKO had chromatin compaction defects, most likely due to lack of sperm-specific compaction factors. These findings indicate that CTCF is essential for proper chromatin organization during spermiogenesis and suggest that infertility in *ctcf*-cKO mice was a result of the chromatin defects in the sperm.

Using a method called phylogenetic profiling in **Paper IV**, we showed that new meiotic factors can be discovered by clustering proteins according to their function.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following articles and manuscripts. In the text, they will be referred to by their Roman numerals.

I. **Lilienthal, I.**, Kanno, T., Sjögren, C. (2013). "Inhibition of the Smc5/6 complex during meiosis perturbs joint molecule formation and resolution without significantly changing crossover or non-crossover levels." *PLoS Genetics* **9**(11):e1003898.

II. **Lilienthal, I.** and Sjögren, C. "Uncovering the role for topoisomerases during meiosis." *Manuscript*

III. Hernández-Hernández, A., **Lilienthal, I.**, Fukuda, N., Galjart, N., Höög, C. "Multiple roles of CTCF during spermatogenesis." *Manuscript*

IV. Tabach, Y., Golan, T., Hernández-Hernández, A., Messer, A.R., Fukuda, T., Kouznetsova, A., Liu, JG., **Lilienthal, I.**, Levy, C., Ruvkun, G. (2013). "Human disease locus discovery and mapping to molecular pathways through phylogenetic profiling." *Molecular Systems Biology* **9**:692.

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LIST OF ABBREVIATIONS

AE	Axial element of the SC
CE	Central element of the SC
CO	Crossover
CR	Central region of the SC (yeast)
D-loop	Displacement loop
dHJ	Double-Holliday junction
DSB	Double-strand break
IH	Inter-homolog
IS	Inter-sister
JM	Joint molecule
LE	Lateral element of the SC
MI	Meiosis I
MII	Meiosis II
mn	Meiotic null
NCO	Non-crossover
PAS	Periodic acid-Schiff's
SC	Synaptonemal complex
SCC	Sister chromatid cohesion
SCI	Sister chromatid intertwining
SMC	Structural maintenance of chromosomes
SSB	Single-strand break
ssDNA	Single-stranded DNA
TF	Transverse filament of the SC

INTRODUCTION

Formation of healthy gametes is essential for the propagation of sexually reproducing organisms, as it is gamete fusion that creates the offspring. Central to gamete production is a specialized cell division called meiosis, which is designed to create genetic diversity and preserve the chromosome number in a species. These features rely on chromosome structure for their accurate execution. If chromosome structure is abnormal, meiosis is defective and can lead to aneuploidy. In humans, aneuploidy is the leading cause of infertility and aneuploidy in females increases with age. The reasons for this high rate of aneuploidy are not well understood, but studying meiosis has allowed researches to gain some insight into infertility and fertility mechanisms. **The overall aim of this thesis was to investigate the interplay between chromosome structure and meiotic integrity in order to better understand factors underlying gamete formation and fertility.**

SPECIFIC AIMS OF THIS THESIS:

◊ In **Paper I**, to elucidate the function of the Smc5/6 complex during meiosis in the budding yeast *Saccharomyces cerevisiae*.

◊ In **Paper II**, to investigate the meiotic roles of topoisomerases Top2 and Top3 in the budding yeast *Saccharomyces cerevisiae*.

◊ In **Paper III**, to use a conditional knockout mouse to determine if CTCF is crucial during spermatogenesis.

◊ In **Paper IV**, to use phylogenetic profiling to find new meiosis-specific genes.

MEIOSIS

Survival of all organisms depends on their ability to grow and reproduce. Growth is achieved by cell division following DNA replication to produce two daughter cells from a single parent. This process, called mitosis, produces cells that are both identical to one another and to the precursor cell (**Figure 1**). Reproduction, on the other hand, can be achieved via several different mechanisms depending on the organism. Most eukaryotes use sexual reproduction to proliferate, which fuses the genetic material from two, often opposite-sex, individuals to create unique offspring. In order to preserve the chromosome number through generations, sexually reproducing organisms must first form gametes containing half the chromosome number as the rest of the organism. The reductive division used for gamete formation is called meiosis. In contrast to mitosis, meiosis produces cells that are haploid and genetically distinct from both the parent cell and one another (**Figure 1**). Central to this process is recombination, which is necessary for proper chromosome segregation in budding yeast and mice and also creates genetic diversity within a species. In this section, meiosis will be presented in the context of events that occur in the budding yeast *Saccharomyces cerevisiae* and mice. Gene and protein names are given for *S. cerevisiae* and are the same in mice unless otherwise specified.

General overview of meiosis

The regulatory pathways dictating the transition from the mitotic to the meiotic program are poorly conserved among species. In diploid yeast, starvation leads to irreversible entry into meiosis due to expression of the master regulator protein Ime1 (Kassir, Granot et al. 1988, Simchen and Kassir 1989, Mandel, Robzyk et al. 1994), which in turn activates a cascade of early meiotic genes. In the continued absence of nutrients, cells will then express middle and late meiotic genes to complete the meiotic program (Winter 2012). In mice, meiotic induction depends on the *Stra8* (*Stimulated by retinoic acid gene 8*) gene in both males and females (Anderson, Baltus et al. 2008).

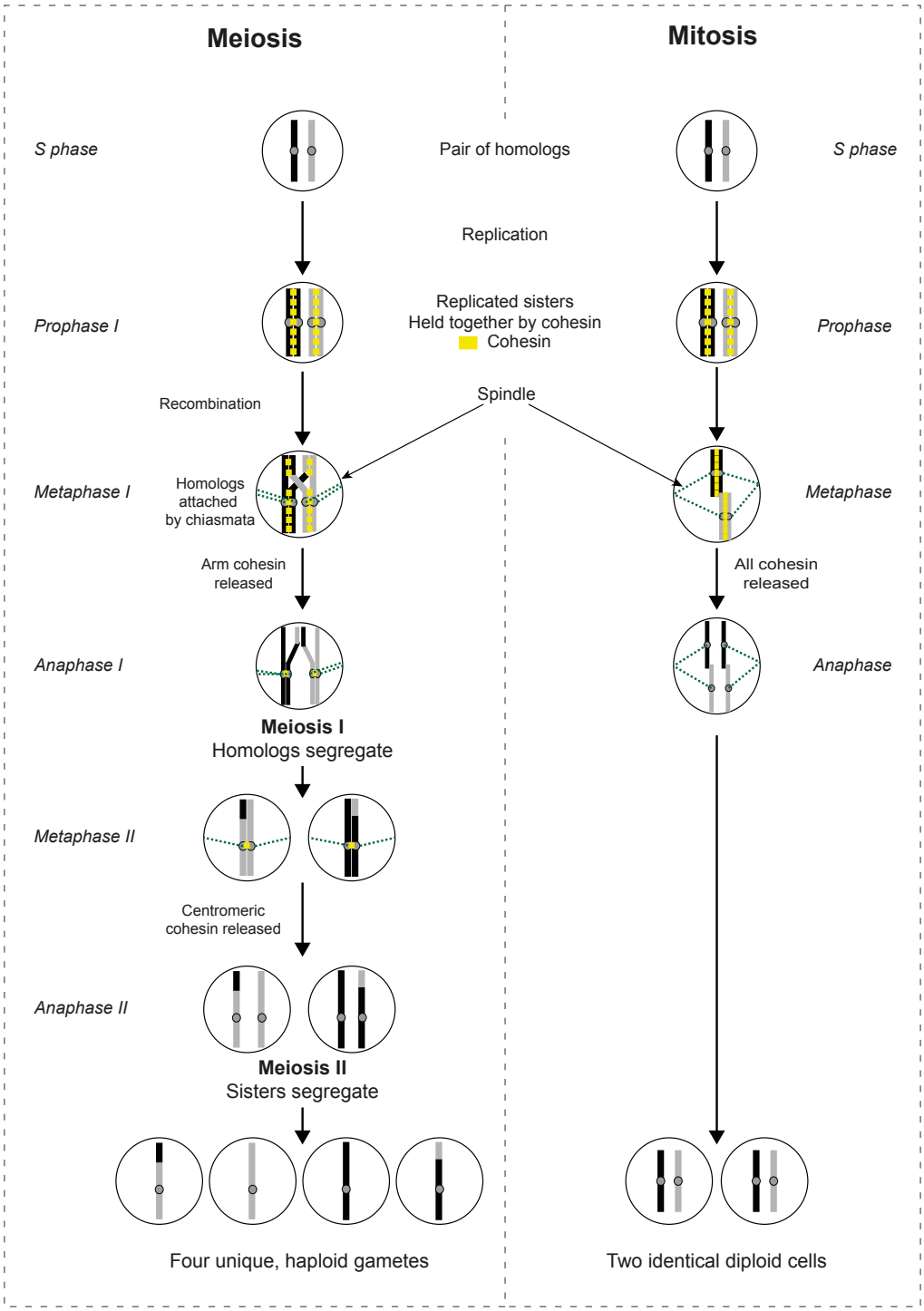

Figure 1. Meiotic and mitotic cell divisions. Cohesin presented as yellow squares, spindles shown with green dashed lines.

Following meiotic induction, meiotic S phase replicates homologous parental chromosomes (homologs) into a pair of sister chromatids and loads a complex called cohesin between sisters to form sister chromatid cohesion (SCC) (**Figure 1**). Programmed double-strand breaks (DSBs) are then induced that are repaired via homologous recombination during prophase. Whereas mitotic cells prefer the sister chromatid as a repair template, meiotic recombination favors the homolog. After prophase, two consecutive nuclear divisions ensue. The first, meiosis I (MI), segregates homologs, while the second, meiosis II (MII), segregates sister chromatids. The final meiotic products are packaged into spores in yeast while the gametes formed during mammalian meiosis are stored in the gonads. In budding yeast and mice, recombination is essential for proper homolog segregation at MI because it creates stable chromosomal connections between the homologs. These attachments, together with cohesin, ensure proper chromosome orientation at the first meiotic spindle (reviewed in Handel and Schimenti 2010).

Meiotic recombination

Meiotic recombination is the key feature of meiosis. It is initiated by the formation of programmed DNA DSBs (reviewed in (Keeney 2001). Meiotic DSBs are catalyzed by the conserved Spo11 protein in both yeast and mice (Keeney, Giroux et al. 1997, Romanienko and Camerini-Otero 2000, Baudat and Keeney 2001), which cleaves DNA via a type II topoisomerase-like reaction (for more on topoisomerases, please see the following section) to generate a transient, Spo11-bound DNA intermediate (Bergerat, de Massy et al. 1997, Keeney 2008). After break induction, Spo11 is removed and the 5' ends are resected to generate 3' DNA overhangs (**Figure 2**). To facilitate strand exchange, the 3' overhangs are coated with two conserved RecA-like recombinases. The first, Rad51, is a major repair protein in vegetative cells that favors sister-directed repair, while the second, Dmc1, is meiosis-specific and promotes the homolog as a repair template (Bishop, Park et al. 1992, Shinohara, Ogawa et al. 1992, Barlow, Benson et al. 1997, Yoshida, Kondoh et al. 1998). Despite these differences in

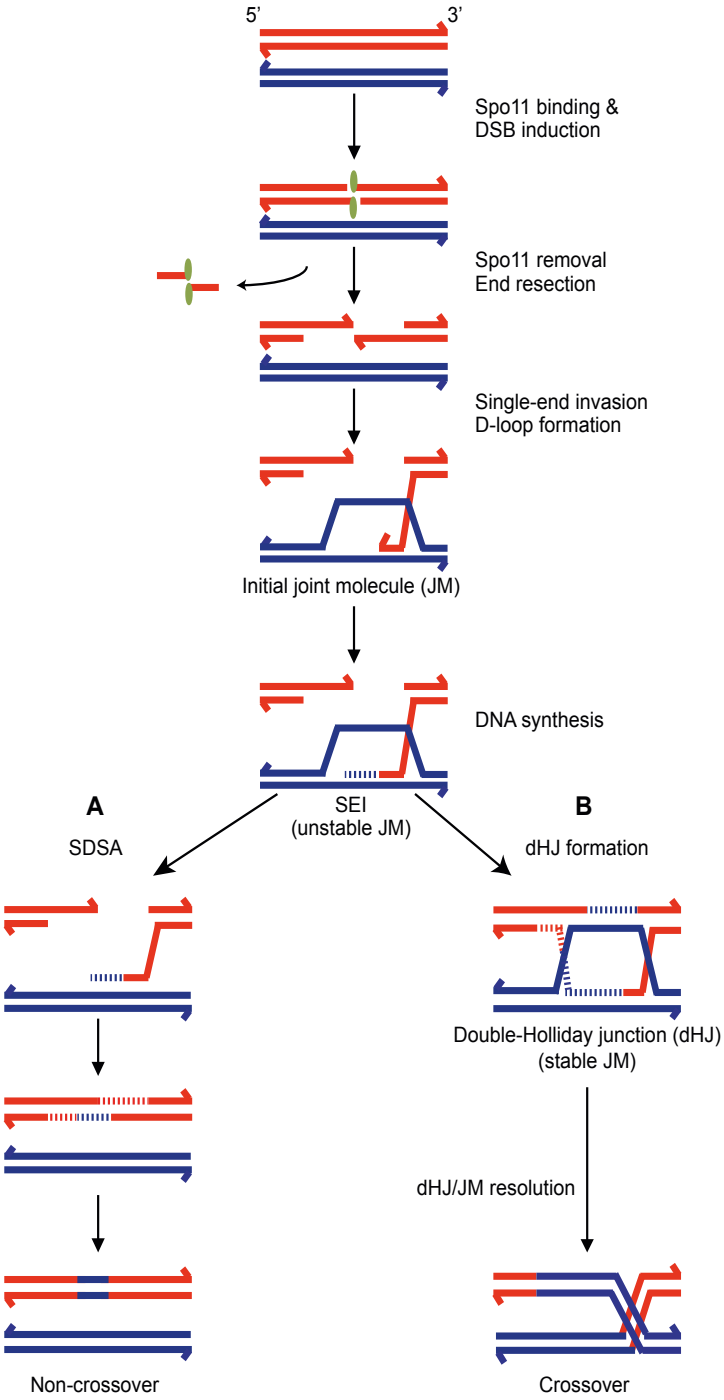

Figure 2. Schematic representation of meiotic recombination. Meiotic recombination is initiated by Spo11-catalyzed DNA double-strand breaks (DSBs). Spo11 is removed from the DNA in the form of Spo11-oligonucleotide complexes, allowing the 5' ends of the DSB to be resected to generate 3' single-stranded overhangs coated by Rad51 and Dmc1 (not shown) that can invade a homologous strand for repair. Strand invasion gives rise to a D-loop, forming an initial joint molecule (JM) intermediate. Following stabilization and DNA synthesis, the initial JM gives rise to another transient JM species called the single-end invasion (SEI). **(A)** The SEI can be quickly dissociated to re-ligate the newly synthesized DNA end to the complementary free break end in a process called synthesis-dependent strand annealing (SDSA). Additional DNA synthesis and ligation yields a mature non-crossover product. **(B)** Alternatively, the SEI can be stabilized to facilitate capture of the second 3' DSB end via engagement of the intact homologous strand. Further processing yields gives rise to a stable JM intermediate known as a double-Holliday junction (dHJ). Resolution of the dHJ yields crossover products.

template preferance, yeast and mice require both Rad51 and Dmc1 for proper homolog-directed repair during meiosis (Bishop 1994, Shinohara, Gasior et al. 1997, Tarsounas, Morita et al. 1999).

Once the broken DNA is coated with Rad51 and Dmc1, it invades an intact homologous sequence from the homolog, forming a displacement loop (D-loop) and initial DNA joint molecule (JM). After stabilization and DNA synthesis, this structure gives rise to an unstable type of JM called a single-end invasion (SEI) (**Figure 2**) (Hunter and Kleckner 2001). Further processing of the SEI in subsequent repair steps can give rise to two classes of products. The first, called non-crossovers (NCOs), repair without mutual exchange of flanking sequences, whereas the second, known as crossovers (COs), repair by mutually exchanging DNA between the homologs in order to physically attach them to one another (Mimitou and Symington 2008, Schwartz and Heyer 2011).

The majority, if not all, of meiotic NCOs are formed via the so-called synthesis-dependent strand-annealing (SDSA) pathway (**Figure 2A**) (Paques and Haber 1999, Allers and Lichten 2001, Hunter and Kleckner 2001). In SDSA, the invading strand is displaced from the D-loop to dissolve the SEI. Then, the displaced strand anneals to complementary DNA sequences on the other end of the DSB, which, after additional DNA synthesis and ligation, reseals the break to form a NCO. The Blooms helicase ortholog Sgs1 has been proposed to promote NCO formation during yeast meiosis by facilitating unwinding of the SEI (De Muyt, Jessop et al. 2012, Zakharyevich, Tang et al. 2012). Unlike NCO formation, which goes via transient JMs, CO formation involves the formation of stable JM intermediates. In the CO-forming pathway, the SEI is stabilized and the second DSB end is captured via the D-loop to form a stable JM called a double-Holliday junction (dHJ) (**Figure 2B**) (Schwacha and Kleckner 1995). In order to be processed into its products, the dHJ must be resolved. This can be achieved by either symmetric DNA cleavage by endonucleases (dHJ resolution) or by combined activity of a helicase and topoisomerase (dHJ dissolution) (Schwartz and Heyer 2011,

Youds and Boulton 2011). Even though dHJ dissolution could lead to NCO formation, NCOs and dHJs form concurrently whereas COs are found only after dHJ disappearance, suggesting that COs are the main products of dHJ processing during meiosis (**Figure 2**) (Storlazzi, Xu et al. 1995, Allers and Lichten 2001). The decision to repair a DSB via a NCO or CO is believed to occur at or prior to SEI formation and involves several factors, some of which are discussed below (Hunter and Kleckner 2001, Bishop and Zickler 2004, Borner, Kleckner et al. 2004).

Because COs establish links between homologs necessary for proper segregation at MI, CO formation is tightly regulated. As such, many factors have been identified that promote CO formation specifically. In budding yeast, the meiosis-specific ZMM proteins (Zip1-4, Mer3, Msh4, Msh5, Spo16) are essential for CO, but not NCO, formation (Borner, Kleckner et al. 2004, Lynn, Soucek et al. 2007, Shinohara, Oh et al. 2008). One way ZMM proteins may promote COs is by synaptonemal complex (SC) formation. Zip1-4 are responsible for forming the SC, which holds homologs together during recombination, thus facilitating inter-homolog repair. SC formation is also critical for proper recombination in mice (SC is discussed further in the next section). Outside of the SC, MutS homologs Msh4 and Msh5 are thought to promote crossing over early by stabilizing dHJ formation (Ross-Macdonald and Roeder 1994, Hollingsworth, Ponte et al. 1995). Mice lacking *Msh4* and *Msh5* are CO-deficient, suggesting a role for these proteins during mammalian CO formation as well (de Vries, Baart et al. 1999, Edelmann, Cohen et al. 1999). At later stages of recombination, the MutLγ complex Mlh1-Mlh3 marks crossover sites and is responsible for the majority of CO formation in budding yeast and mice (de Boer, Dietrich et al. 2007, Zakharyevich, Tang et al. 2012). How Mlh1-Mlh3 promotes CO formation was recently discovered in budding yeast by the finding that Mlh1-Mlh3 is an endonuclease that specifically binds to dHJs (Ranjha, Anand et al. 2014, Rogacheva, Manhart et al. 2014). In addition to COs designated by Mlh1-Mlh3, a minority of COs in budding yeast and mice is dependent on dHJ resolution by the endonuclease Mus81-Mms4 (de los Santos, Hunter et al. 2003, Osman, Dixon et al. 2003, Holloway, Booth et al. 2008). Interestingly, the choice of dHJ

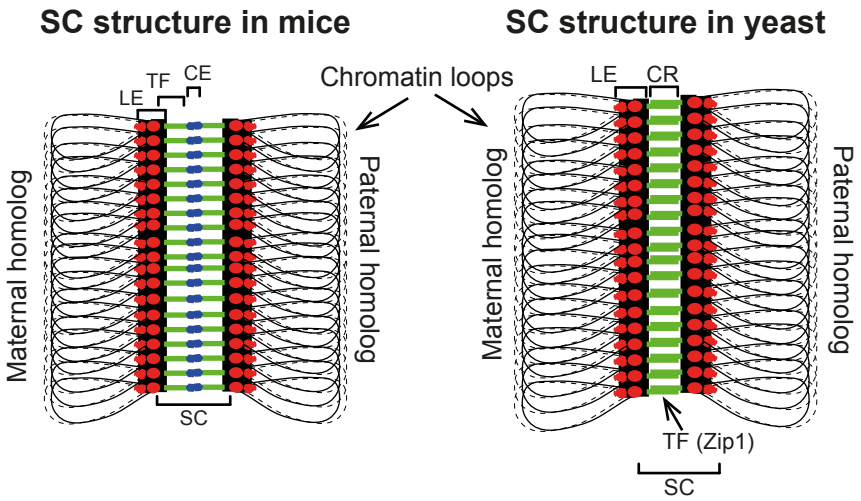

Figure 3. SC structure in mice and yeast.

resolution pathway (Mlh1-Mlh3 vs. Mus81-Mms4) in budding yeast depends on Sgs1 (De Muyt, Jessop et al. 2012, Zakharyevich, Tang et al. 2012).

All the components mentioned above must work individually and together for proper CO levels, as they alone do not dictate CO formation. The choice between CO and NCO is also decided on a chromosome- and genome-wide level. Three key elements lie behind this global CO control: assurance, interference, and homeostasis. CO assurance provides each homolog pair with at least one CO; interference distributes these COs in a non-random way on chromosomes so that they are widely spaced; homeostasis ensures a constant number of COs independent of DSB numbers (Youds and Boulton 2011). This global control of CO designation implies that whole-chromosome structure is just as important as local binding of CO-promoting factors. These two levels of CO control must work together to ensure proper recombination outcomes.

The synaptonemal complex

During meiosis, the synaptonemal complex (SC) links homologous chromosomes to one another via its protein lattice (**Figure 3**) (Fraune, Schramm et al. 2012). The SC is thought to facilitate homolog-directed recombination and CO

formation by holding homologs in close proximity during DSB repair. In line with this hypothesis, *Drosophila* males, which do not perform meiotic recombination, lack an SC (Zickler 1999). Proper SC formation in budding yeast and mice is dependent on DSB formation (Agarwal and Roeder 2000, Baudat, Manova et al. 2000, Romanienko and Camerini-Otero 2000), and meiosis in these organisms depends on SC formation, as mutating any SC components leads to CO loss and abnormal meiosis.

SC dynamics define four specific stages of prophase: leptotene, zygotene, pachytene, and diplotene (**Figure 4**). Following replication, loading of axial elements (AEs) along the length of sister chromatids defines leptotene. In mice, AEs are composed of both synaptonemal complex proteins (SYCP2 and SYCP3) and cohesin complexes (reviewed in Yang and Wang 2009). Yeast AEs are made up of cohesin complexes and the meiosis-specific proteins Red1, Mek1, and Hop1 (Page and Hawley 2004). At zygotene, transverse filaments (TFs) (SYCP1 in mouse; Zip1 in yeast) begin to form between the AEs. In mice, a set of proteins (SYCE1, SYCE2, TEX12) begins to form a longitudinal central element (CE) during zygotene that bridges the TFs projecting from each sister axis; no CE exists in yeast. Once two homologous AEs are closely aligned, a complex and coordinate protein polymerization process called synapsis takes place that tethers homologs together along their entire length by the SC. Yeast SCs lack a CE, instead forming a central region (CR) that consists of Zip1 TFs that are "zipped" together during pachytene. After synapsis, AEs

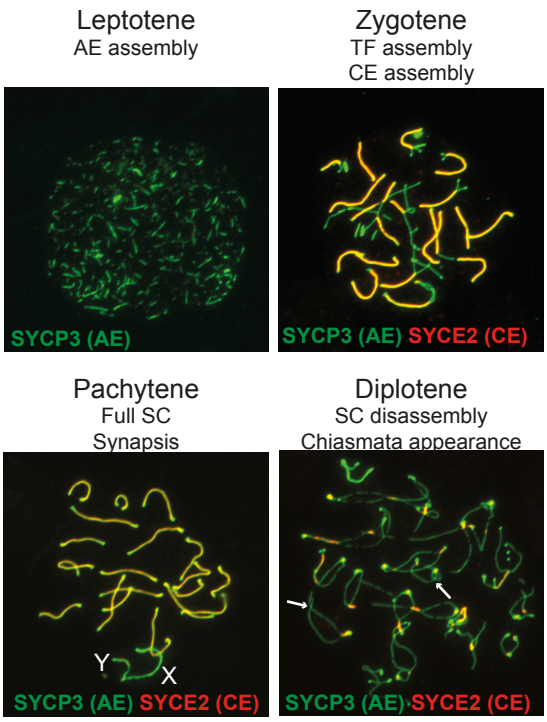

Figure 4. Stages of prophase in mouse spermatocytes.

are referred to as lateral elements (LEs). SC disassembly begins during diplotene, the final stage of prophase. By the end of diplotene, all LEs have disappeared and homologs are connected by chiasmata, the physical manifestations of crossovers at the chromosomal level.

Inter-homolog vs. inter-sister recombination in *Saccharomyces cerevisae*

Meiotic chromosomes are organized into a loop-axis configuration (**Figure 3**). It is believed that DSB formation occurs at the top of the chromatin loops while recombination is carried out at the axis. Components that shape this loop-axis structure are important for the integrity of meiotic recombination.

Unlike mitotic recombination, which favors the sister chromatid, meiotic recombination prefers the homologous chromosome as a repair template (Schwacha and Kleckner 1997). In addition to the SC's role in homolog synapsis, inter-homolog bias is due to combined efforts of mechanisms that promote homolog strand invasion, such as Dmc1, and meiotic axis components such as Red1, Hop1, and Mek1 that hinder use of the sister as a repair template by inhibiting recruitment of sister-promoting recombination factors (Bishop, Park et al. 1992, Hollingsworth and Ponte 1997, Schwacha and Kleckner 1997, Shinohara, Gasior et al. 1997, Niu, Wan et al. 2005, Carballo, Johnson et al. 2008). The meiotic axis includes the cohesin subunit Rec8 (Revenkova and Jessberger 2006). Cohesin is responsible for sister chromatid cohesion, and it is required for inter-sister recombination during DSB repair in vegetative cells (more on cohesin in following section). During meiosis, Rec8 is required for proper axis configuration, and its absence leads to altered DSB distribution thought to be a result of abnormal tethering of chromatin loops (Kugou, Fukuda et al. 2009, Kim, Weiner et al. 2010). In line with the idea that cohesin is needed to properly tether chromatin loops, mice that lack *Rec8* have shortened axes (Bannister, Reinholdt et al. 2004); this phenotype is also observed in the absence of another meiosis-specific murine cohesin component, SMC1β, which was shown to have longer chromatin loops tethered to the shortened axis (Revenkova, Eijpe et al. 2004). Interestingly, Rec8 is needed to maintain

inter-homolog bias during the SEI-to-dHJ transition, even though Rec8 traditionally aids in inter-sister recombination (Kim, Weiner et al. 2010). Homolog-promoting components of the meiotic axis counteract the inter-sister bias created by Rec8, allowing inter-homolog events to dominate (Kim, Weiner et al. 2010). Concurrent activities of these mechanisms establish a bias for inter-homolog recombination but do not eliminate inter-sister recombination altogether, with the possibility that as many as one-third of meiotic repair is directed to the sister, at least in yeast (Goldfarb and Lichten 2010, Kim, Weiner et al. 2010). Whether between sisters or homologs, all DNA linkages (i.e. JMs) must be properly resolved for accurate chromosome segregation.

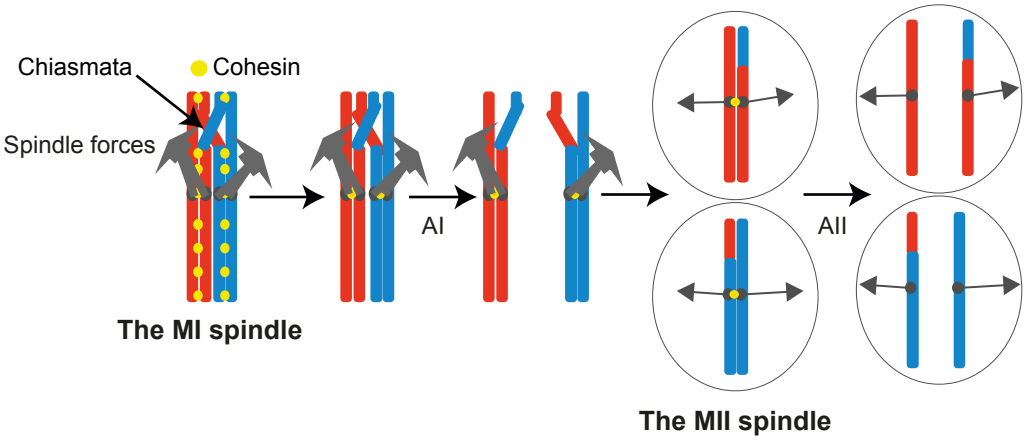

Figure 5. The MI and MII spindle forces. AI=Anaphase I; AII=Anaphase II

Meiotic segregation

Proper homolog segregation at MI depends on chiasmata formation and sister chromatin cohesion, as it is these two that oppose the pulling tensions of the first meiotic spindle to facilitate segregation (**Figure 5**) (reviewed in Handel and Schimenti 2010) (for further reading on cohesin's role in segregation in budding yeast, please see Marston 2014). Chiasmata are responsible for linking the homolog pairs at the chromosomal level so that they remain associated until segregation (reviewed in Whitby 2005). These stably attached homolog pairs are called bivalents. At the same time, sister chromatid cohesion prevents premature sister separation. Sister chromatid cohesion is

also necessary for bivalent maintenance, as early loss of cohesion on the telomere-proximal side of chiasmata can lead to untimely chiasmata dissolution. Once chiasmata and sister chromatid cohesion are properly in place, homologs can bi-orient, meaning that non-sister kinetochores orient to opposite spindle poles. Homolog bi-orientation at metaphase I, and subsequent segregation at anaphase I, is achieved by the specific loss of arm cohesion, which facilitates chiasmata resolution, and maintenance of centromeric cohesion, which prevents sister chromatid separation until MII. This is different from mitosis when all cohesion is lost at the metaphase-anaphase transition (**Figure 1**). At MII, cohesion is lost at the centromeres, allowing sister segregation (this process is reviewed in Wassmann 2013). Step-wise loss of cohesion is a hallmark of meiotic segregation and is mainly achieved by specialization of meiosis-specific cohesin complexes (reviewed in McNicoll, Stevense et al. 2013). Overall, proper segregation at MI and MII is crucial for proper gamete formation and reproductive success of an organism.

MAMMALIAN GERM CELL FORMATION

In the gonads of mammalian organisms, diploid germ cells produce haploid gametes that, after fusion with another gamete from the opposite sex, will produce the offspring. The collective process of gametogenesis (gamete formation) is known as spermatogenesis in males and oogenesis in females, and takes place in the testes and the ovaries, respectively.

Spermatogenesis

Sperm, also known as mature spermatozoa, are the final products of spermatogenesis and are the gametes used for sexual reproduction. Failure to complete spermatogenesis or the generation of improper sperm can lead to infertility or genetic disorders, making it important to understand the steps of this intricate process. Spermatogenesis takes place in the seminiferous tubules of the testes. The seminiferous

tubule is comprised of a central lumen surrounded by an epithelium arranged in layers of different germinal cell types (**Figure 6**).

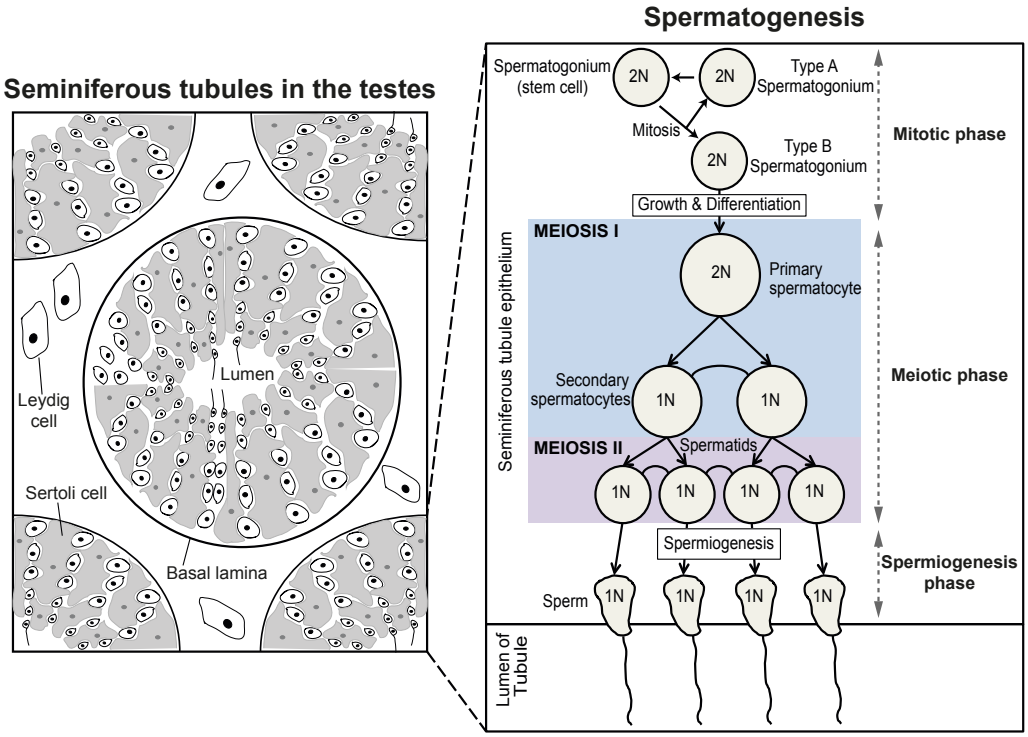

Figure 6. Spermatogenesis in the context of the seminiferious tubules.

The epithelium of the seminiferous tubules can be staged according to the cellular associations found within. In mouse, twelve (I-XII) epithelial stages have been well defined by observing the changes in the forming acrosomal region of the spermatids using the periodic acid-Schiff's reaction (PAS) (Hess and Renato de Franca 2008). For simplicity, the stages of the seminiferous epithelium in mouse are generally categorized as early (stages I-V), middle (stages VI-VIII), and late (stages IX-XII) (**Figure 7**). In addition to germinal cells, the tubules also contain somatic cells called Sertoli cells, which serve to nourish, protect, and support the spermatogenic cells (reviewed in Hess and Renato de Franca 2008, Kaur, Thompson et al. 2014). The space

between the tubules is made up of testosterone-producing Leydig cells, blood vessels, and lymphatic fluid.

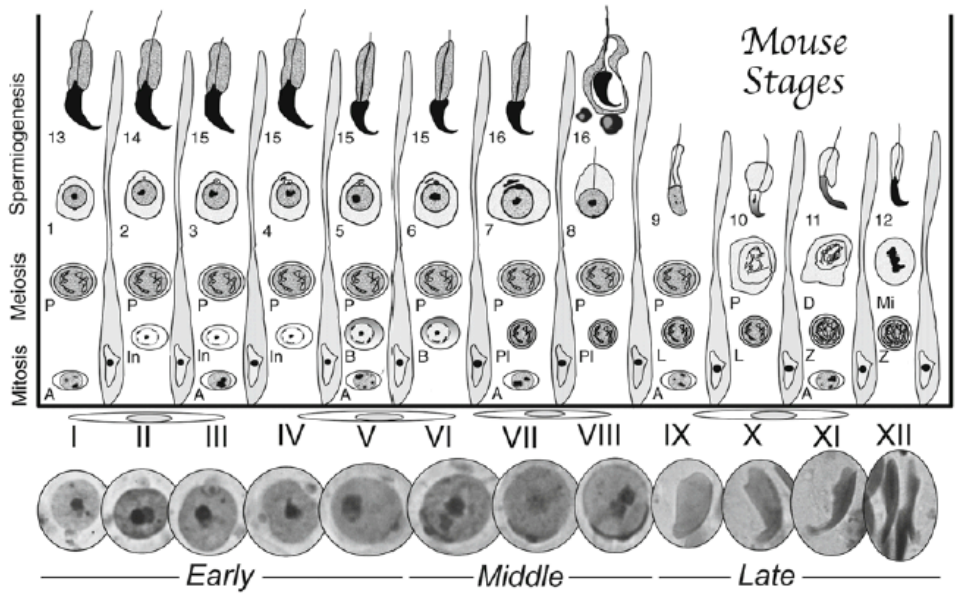

Figure 7. Stages of the seminiferous epithelium in the mouse. Sertoli cells separate each stage. Along the bottom are photos of typical cells for early, middle, and late spermatid nuclei as visualized by using the PAS reaction and hematoxylin. Spermatogonia (A, In=intermediate, B); spermatocytes (Pl=preleptotene, L=leptotene, P=pachytene, D=diakinesis, Mi=meiotic division); round spermatids (1-8); elongating/elongated spermatids (9-16). (Hess and Franca 2008)

Spermatogenesis consists of multiple rounds of mitosis, one round of meiosis, and a differentiation step called spermiogenesis (**Figure 6**) (reviewed in Hess and Renato de Franca 2008). In the mitotic phase, primordial germ cells undergo two rounds of mitosis to form type A spermatogonial stem cells, which proliferate to produce two kinds of cells: spermatogonial stem cells and differentiating spermatogonia. The differentiating spermatogonia (intermediate and type B spermatogonia) will grow and differentiate to form the primary spermatocytes that enter meiosis. During the meiotic phase, primary spermatocytes undergo replication and, following recombination during prophase, segregate their homologous chromosomes at MI to form secondary spermatocytes. These secondary spermatocytes then segregate their sister chromatids at

MII to generate haploid spermatids. The spermatids then enter the final phase of spermatogenesis, spermiogenesis.

A series of morphological changes occur during spermiogenesis that transform round spermatids into elongated, compact sperm over the course of sixteen steps (**Figure 7**). There are three types of developing spermatids in the seminiferous epithelium: early spermatids with round nuclei, intermediate spermatids with elongating nuclei, and mature spermatids with condensed nuclei (reviewed in Dadoune 2003). Central to spermatid differentiation is the attachment of specialized structures and compaction of the spermatid head, both of which allow sperm to carry out their function during fertilization. In early spermiogenesis, specialized structures called an acrosome and axoneme begin to develop at opposing poles of the round spermatids (reviewed in O'Donnell and O'Bryan 2014). The acrosome, which eventually caps the entire sperm head, arises from the Golgi apparatus and facilitates fertilization by the secretion of enzymes that digest the outer surface of the female gamete to allow nuclear fusion. The axoneme is a microtubule-based structure that is equally as crucial to fertilization, as it is the core structure of the flagellum that mobilizes sperm. Though initially formed in early stages, the acrosome and axoneme continue to develop throughout spermiogenesis parallel to nuclear reshaping.

Compaction of the spermatid nucleus begins approximately halfway through spermiogenesis with the formation of a microtubule-based organelle called the manchette. The manchette encompasses the nucleus directly below the acrosome in stage 7-8 spermatids, sliding down the nucleus in subsequent elongation steps before being dismantled in step 14 spermatids (reviewed in O'Donnell and O'Bryan 2014). This movement of the manchette facilitates nuclear compaction by altering the shape of the nucleus. DNA remodeling allows for nuclear compaction as well. During step 10 of spermiogenesis, histones begin to be replaced with protamines, which allow for tight DNA packaging due to their affinity for the minor groove of DNA (reviewed in Braun 2001). Mice have two protamines, protamine 1 (PRM1) and protamine (PRM2), both of

which are required for proper sperm formation (Cho, Willis et al. 2001, Cho, Jung-Ha et al. 2003). It is unclear why a nuclear compaction is essential for sperm formation, but it is proposed that the small head protects sperm from damage and facilitates in motility during fertilization (reviewed in Carrell, Emery et al. 2007).

Oogenesis

Unlike males, who can produce millions of new gametes each day from a replenishing pool of spermatogonial germ cells, females are born with a fixed number of cells with the potential to become mature gametes. These cells, called primary oocytes, are housed in individual primordial follicles and are arrested at a diplotene-like dicyte stage at the end of meiotic prophase at birth. Once the animal reaches sexual maturity, a hormone surge triggers the development of a subset of primordial follicles and subsequent maturation of the primary oocytes within. The oocytes complete meiosis to form mature ovum, also referred to as eggs, which are the gametes with the potential to be fertilized. The process of oocyte maturation and egg release, called menstruation, occurs once a month until menopause when menstruation ceases. (reviewed in Pepling 2006)

PROTEINS THAT SHAPE CHROMOSOMES

Though visualized under the microscope earlier, chromosomes were not linked to heredity until the turn of the 20th century, and it took scientists almost fifty more years to identify DNA as the basic unit of heredity. After Watson and Crick described the DNA double helix in 1953, research in the field boomed and provided evidence that made it impossible to deny the importance of DNA for life. We now know that proper organization of DNA into chromosomes, via the action of DNA-altering proteins, is just as important as DNA sequence itself. There are a multitude of proteins and protein families that influence overall chromosome structure and function. This section will

focus on the proteins and protein complexes most relevant to this thesis: topoisomerases, SMC complexes, and CTCF, with an emphasis on their functions in budding yeast and mice. Where relevant, findings from **Papers I-III** will be presented and discussed.

Topoisomerases

The DNA double-stranded helix must be manipulated in almost every major DNA-based cellular activity. Unwinding of the duplex during processes such as replication, transcription, and recombination can lead to DNA supercoiling (**Figure 8**), which can threaten genomic integrity if not properly managed. Other topological structures that can be deleterious to a cell are sister chromatid intertwinings (SCIs), which are the wrapping of chromatids around each other. To avoid genome instability caused by such topological structures, cells employ an essential class of enzymes known as topoisomerases.

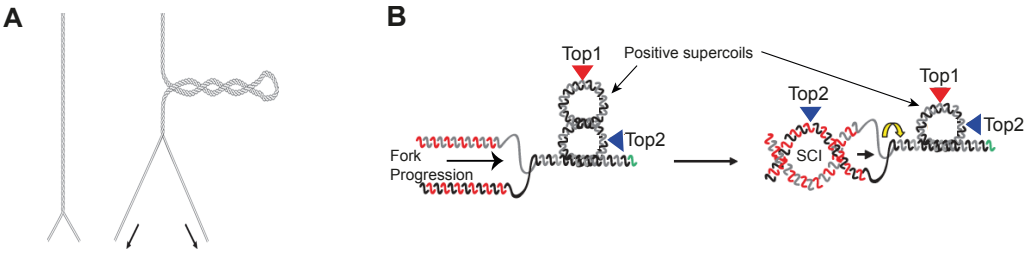

Figure 8. Topological structures must be removed by topoisomerases. (A) Illustration of twist-induced supercoiling. Pulling apart two ends of a twisted rope generates positive supercoils. (Carter and Sjögren, 2012) (B) Positive DNA supercoils accumulate in front of the replication fork, which require Top1 and Top2 for removal. Top2 also works behind the replication fork to resolve SCIs that build up behind the fork. (Kegel, Betts-Lindroos et al. 2011)

Topoisomerases facilitate DNA transformations by utilizing a reversible transesterification reaction. This reaction creates a transient break in the DNA where strand passage can occur, which, following re-ligation of the break, results in the relief of topological stress (reviewed in Chen, Chan et al. 2013). Topoisomerases are divided into two classes depending on the kind of break they induce: type I topoisomerases create a DNA single-stranded break (SSB), while type II topoisomerases create a DNA

double-stranded break (DSB) (reviewed in Wang 1996). These classes are further categorized into subfamilies A, B, or C based on amino acid sequence and reaction mechanism (reviewed in Schoeffler and Berger 2008). The topoisomerases at work in eukaryotic cells are type IA (yeast Top3), type IB (yeast Top1), and type IIA (yeast Top2). The most relevant functions of these topoisomerases as they relate to this thesis will be described for budding yeast below. For further reading on topoisomerases, please consult (Chen, Chan et al. 2013), (Wang 2002), and references therein.

Top3 (**Paper II**)

Top3 is the DNA-cleaving component of the Top3-Sgs1-Rmi1 complex. Though this complex has roles during replication, its best characterized function is during recombination in vegetative cells due to its ability to resolve double-Holliday junctions (dHJs) (reviewed in Cejka, Plank et al. 2012). A study in meiosis suggests that Top3 functions during meiotic recombination as well, since *top3* mutants experienced a recombination-dependent segregation block at MI (Gangloff, de Massy et al. 1999).

In **Paper II**, we further explored the meiotic function of Top3 by using a meiotic null (mn) allele of *TOP3*, *top3-mn*. The meiotic null allele was constructed by replacing the endogenous promoter of *TOP3* with the mitosis-specific *CLB2* promoter (Lee and Amon 2003), which is down regulated after meiotic S phase (Grandin and Reed 1993, Dahmann and Futcher 1995). In this way, replication defects are avoided using this system. Despite incomplete reduction in Top3 protein levels in the *top3-mn*, 40% of cells were unable to complete meiotic divisions and instead formed cells with one nuclear mass outside of four empty spores. This illustrates the necessity of Top3 for meiosis, as even a small loss in Top3 levels causes a phenotype. Consistent with spore formation in these cells, which indicates completion of the meiotic program in *S. cerevisiae* (Neiman 2011), Top3-deficient cells had normal spindle and synaptonemal complex (SC) dynamics.

The phenotype in *top3-mn* cells is reminiscent of that observed in *smc6* mutants (Copsey, Tang et al. 2013, Lilienthal, Kanno et al. 2013, Xaver, Huang et al. 2013) (**Paper I**) and *mus81 sgs1* and *mms4 sgs1* double mutants (Jessop and Lichten 2008, Oh, Lao et al. 2008), which accumulate recombination intermediates. Accordingly, we found that the segregation block in top3-mn was rescued when DSBs were eliminated. Given the role of Sgs1 and Top3 in mitotic recombination, it is very likely that they work in conjunction during meiosis as well. Indeed, rescue of the *top3* meiotic segregation in an *sgs1* background suggests interplay between Sgs1 and Top3 in meiosis (Gangloff, de Massy et al. 1999). However, in meiosis, accumulation of unresolved recombination intermediates and subsequent segregation block is only achieved when *SGS1* is removed along with an endonuclease component (*MUS81/MMS4*) (Jessop and Lichten 2008, Oh, Lao et al. 2008). This indicates that Top3's meiotic function depends on more than just Sgs1. Recent data suggests that Smc6 binding in mitosis partially depends on Top3 (Berta et al., unpublished). Given the recently defined role for Smc6 during meiotic recombination (**Paper I**), it may be that the phenotype in *top3-mn* cells is due to loss of Smc6 on chromosomes. However, no detectable changes in Smc6 loading onto meiotic chromosomes were detected in Top3-deficient cells by immunofluorescence. This may indicate that Top3 does not regulate meiotic Smc6 binding. Alternatively, Top3 may influence small changes in Smc6 loading. The Top3-dependent changes in mitotic Smc6 binding were detected using the more sensitive chromatin immunoprecipitation technique, making it possible that our immunofluorescence analysis was not sensitive enough to detect changes in Smc6

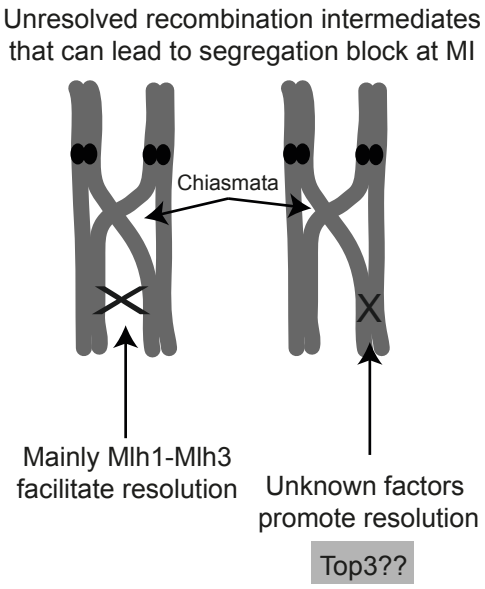

Figure 9. Possible role for Top3 in the removal of inter-sister dHJs.

loading. In conclusion, **Paper II** proposes a role for Top3 during meiotic recombination (**Figure 9**), and it will be interesting to explore this role in the future. Critical to future endeavors is the creation of a meiosis-specific *TOP3* allele that effectively reduces Top3 levels, as the *top3-mn* used in this investigation only reduced protein levels slightly. If an efficient mutant is created, it will be interesting to explore what, if any, role Top3 plays during meiotic recombination. Two lines of evidence suggest that any Top3-mediated dHJ processing would be outside of established CO-forming meiotic pathways. First, the Top3-Sgs1-Rmi1 complex dissolves dHJs into NCO products, which are not products of meiotic dHJ resolution. Second, the factors responsible for virtually all inter-homolog dHJ resolution during meiosis have been identified (Zakharyevich, Tang et al. 2012). Instead, Top3 may be needed to process *inter-sister* dHJs. Inter-sister DSB repair during meiosis is not well understood, but resolution of inter-sister recombination intermediates is required for proper segregation.

Top1

Top1 is the most important factor for removal of supercoil-induced stress in the cell (Wang 2002). Together with Top2 (see below), it works to release supercoils ahead of the replication fork (Bermejo, Doksani et al. 2007). Due to its overlapping function with Top2, Top1 is non-essential in yeast (Chen, Chan et al. 2013). The function of Top1 during meiosis was not investigated in this thesis, and to our knowledge no meiotic function has been reported.

Top2 (**Paper II**)

Chromosome replication creates two topological structures which must be handled by topoisomerases: supercoils ahead of the replication fork and SCIs behind it (**Figure 8B**). While both Top1 and Top2 can remove supercoils, elimination of SCIs relies almost solely on Top2 (**Figure 8C**), and cells lacking Top2 complete replication but are unable to segregate their chromosomes (Holm, Goto et al. 1985, Bermejo, Doksani et al. 2007, Baxter and Diffley 2008). A catalytically dead *top2* mutant,

however, leads to a checkpoint-induced replication arrest (Baxter and Diffley 2008). During mitosis, newly replicated sister chromatids must be held until their timely segregation at the metaphase-anaphase transition (Figure 1B). If sisters are not connected, not only will they fall apart prior to anaphase, they will also be unable to satisfy the spindle checkpoint due to lack of tension, causing spindle assembly checkpoint-mediated arrest. SCIs were originally proposed to be the factor linking sister chromatids, but this model was disfavored upon the discovery of proteins that functioned in the regulation of sister cohesion (Guacci, Koshland et al. 1997, Michaelis, Ciosk et al. 1997), which are know known components of the cohesin complex. More recently, cohesin was proposed to protect SCIs from Top2, suggesting once again that SCIs may play a role in keeping sisters together (Farcas, Uluocak et al. 2011). Together with the condensin complex (discussed further below), Top2 also aids in proper chromosome condensation and axis organization in vegetative cells (Koshland and Strunnikov 1996). During meiosis, Top2 binds to the meiotic chromosome axis (Klein, Laroche et al. 1992), and mutations in *top2* during meiosis lead to lack of chromosome condensation and a segregation block that is partially dependent on recombination (Rose, Thomas et al. 1990, Rose and Holm 1993). Recently, Kleckner and colleagues also reported that the structural role of Top2 makes it a key factor in meiotic crossover interference (Zhang, Wang et al. 2014).

In **Paper II**, we further characterized the meiotic role of Top2 by using the meiosis-specific mutant *top2-mn*. (This mutant was constructed as described for *top3-mn*.) The *top2-mn* displayed a mixture of two phenotypes: cells that accumulated as uninucleates (40%) and cells that formed 1-3 empty spores with one nuclear mass outside (60%). Since certain *top2* mutants experience replication defects during mitosis (Baxter and Diffley 2008), the uninucleate population in *top2-mn* could be blocked due to problems during pre-meiotic S phase. While we believe that replication defects were avoided using the meiotic null, we cannot completely exclude this possibility.

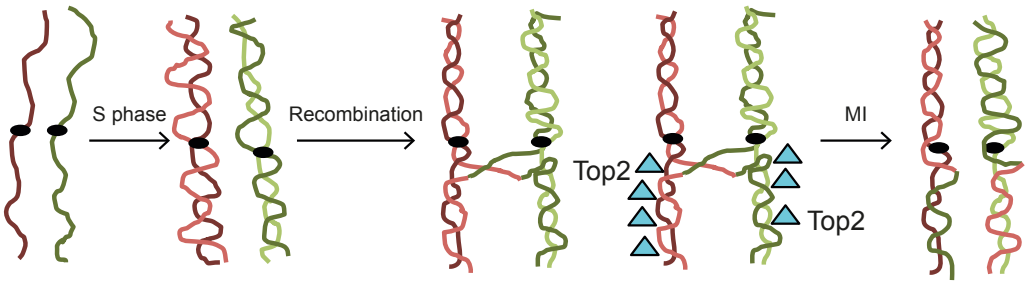

Figure 10. SCI removal by Top2 would facilitate segregation of recombined homologs. Based on similar sketch from (Rose et al., 1990).

Given the role of Top2 in SCI resolution during mitosis, it seems likely that it has a similar function during meiosis. SCI resolution is imperative for meiotic segregation, since any lingering inter-sister links on the telomere-proximal side of chiasmata will inhibit homolog segregation. In the absence of chiasmata (i.e. no recombination), a portion of sister segregation may also be blocked if SCIs are not resolved, but some cells should successfully segregate their homologs. In line with this reasoning, we found that removing recombination rescued almost the entire segregation block in the *top2-mn*, as 50% of total cells were able to complete some nuclear division (30% completed MII; 20% completed MI), compared with the 60% segregation block when *top2-mn* underwent meiosis with DSBs. The remaining cells accumulated as uninucleates (30%) or cells with 1-3 spores with an unsegregated DNA mass (20%). Top2 was not needed for recombination itself, as JM dynamics and CO/NCO formation were unperturbed in its absence. Together, these data suggest that Top2 is needed to remove SCIs that inhibit chromosome segregation as a result of chiasmata-dependent homolog links. Consistent with this idea, segregation was impeded if recombination was eliminated and cells only underwent a MII-like division (i.e. segregated sister chromatids). This suggests that recombination-independent SCIs are the source of the recombination block in Top2-deficient cells. Hence, the findings in **Paper II** point to a role for Top2 in SCI removal during meiosis. Top2 seems to be particularly important for resolving SCIs that sit downstream of chiasmata, which must be resolved for homolog segregation (**Figure 10**).

SMC protein complexes

Structural maintenance of chromosomes (SMC) proteins are members of a conserved class of proteins that are needed for genome integrity. Their unique structure and global functions make them crucial components of chromosome architecture in a cell. SMCs are large proteins (1,000-1,300 amino acids) that contain two nucleotide-binding motifs, Walker A and Walker B, which are found at the N- and C-terminal domains, respectively (**Figure 11A**) (reviewed in Hirano 2006). Bridging the termini are two long coiled-coil motifs with a hinge in the center, which facilitate folding over of the SMC monomer onto itself in order to create an ATP-binding head at one end and a hinge domain at the other (reviewed in Hirano 2006). SMC proteins interact with one another via their hinge domains and other non-SMC proteins via their head domains. In this way, the SMC proteins make up the cores for the so-called SMC complexes: Smc2-Smc4 in condensin, Smc1-Smc3 in cohesin, and Smc5-Smc6 in the Smc5/6 complex (**Figure 11B**). These complexes will be discussed further below, with an emphasis on the roles of cohesin and the Smc5/6 complex in yeast and mice, particularly during meiosis. Unless otherwise specified, the gene and protein names are given for yeast.

Condensin

Briefly, the condensin complex is essential for chromosome condensation during mitosis and meiosis (Wood, Severson et al. 2010). In addition to the core SMC proteins Smc2 and Smc4, the condensin complex includes three non-SMC elements (**Figure 11B**) (reviewed in Losada and Hirano 2005). Mutations in condensin during meiosis lead to defects in axis organization (Yu and Koshland 2003, Yu and Koshland 2005).

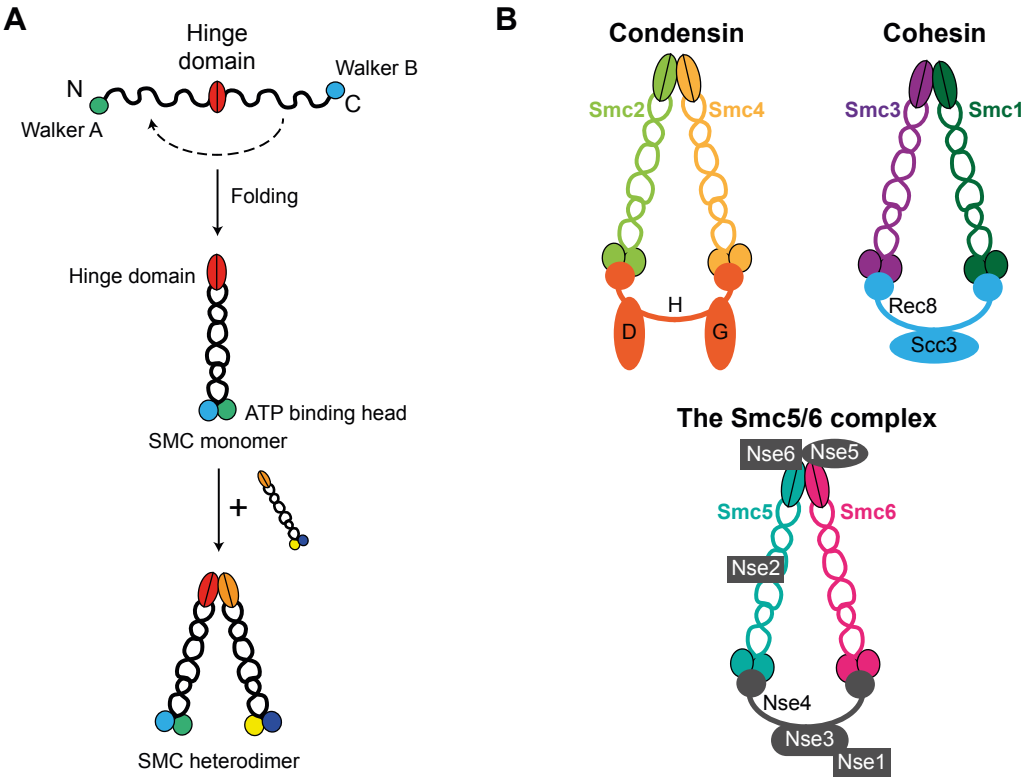

Figure 11. The SMC complexes. (A) Basic structure of an SMC protein. (B) Structure of the condensin, cohesin, and the Smc5/6 complex. See text for details.

Cohesin

As stated previously, the cohesin complex is a meiotic axis component required for proper recombination. It is also essential for sister chromatid cohesion (SCC) during mitosis and meiosis (reviewed in Peters and Nishiyama 2012). Cohesin is loaded to chromatids at telophase in metazoans and G1/S phase in yeast to prevent separation of sister chromatids until the metaphase-anaphase transition and most likely carries out its cohesive function by forming a ring encircling sisters (Michaelis, Ciosk et al. 1997, Klein, Mahr et al. 1999, Gruber, Haering et al. 2003, Haering, Schoffnegger et al. 2004, Haering, Farcas et al. 2008). Importantly, cohesin is required for DSB repair, and is recruited to DSB sites even after replication (Sjogren and Nasmyth 2001, Strom, Lindroos et al. 2004, Strom, Karlsson et al. 2007). In higher eukaryotes, cohesin also promotes genome organization together with the CCCTC-binding factor, CTCF. The

interplay between CTCF and cohesin is crucial for mammalian genome function (CTCF will be discussed more in the next section).

The cohesin complex consists of a heterodimer between Smc1 (SMC1α in mice) and Smc3 (SMC3 in mice) bridged by an α-kleisin subunit, Scc1/Mcd1 (RAD21 in mice), to which an additional non-SMC subunit binds: sister chromatid cohesion 3 (Scc3, called SA or STAG in mice) (**Figure 11B**) (yeast review in Haering, Lowe et al. 2002; mammalian review in McNicoll, Stevense et al. 2013). Vertebrates have two SA proteins, SA1 and SA2, that give rise to two functionally different mitotic cohesin complexes (reviewed in Canudas and Smith 2009). During meiosis, the kleisin subunit of cohesin is replaced with Rec8 in yeast and REC8 or RAD21L in vertebrates (Klein, Mahr et al. 1999, Bannister, Reinholdt et al. 2004, Lee and Hirano 2011, Polakova, Cipak et al. 2011). Mice have two additional meiosis-specific components for the SMC3 and SA subunits: SMC3β and STAG3, respectively (Prieto, Suja et al. 2001, Revenkova, Eijpe et al. 2001).

As mentioned earlier, cohesin is removed stepwise in meiosis (**Figures 1, 5**). At MI, arm cohesin is cleaved away in order to allow homolog segregation; centromeric cohesin is maintained until MII when sister chromatids are segregated. In addition to its role in SCC, cohesin is necessary for proper architecture of the meiotic axis (for more on the meiotic axis, please see the Meiosis section in this thesis) as well as SC formation and DSB repair in yeast and mice (Klein, Mahr et al. 1999, Pelttari, Hoja et al. 2001, Llano, Herran et al. 2012). Studies have found both unique and overlapping functions for the different meiotic subunits in mice, suggesting interplay between at least six different cohesin complexes during meiosis (Ishiguro, Kim et al. 2011, Jessberger 2011, Lee and Hirano 2011, Fukuda, Fukuda et al. 2014, Hopkins, Hwang et al. 2014, Ishiguro, Kim et al. 2014).

The Smc5/6 complex (**Paper I**)

The most enigmatic of the SMC complexes is the Smc5/6 complex. The complex consists of eight subunits, all of which are essential in yeast: Smc5, Smc6, Nse1, Mms21 (Nse2) and Nse3-6 (**Figure 11B**). These components were originally discovered in fission yeast as part of the mitotic homologous recombination repair pathway (Lehmann, Walicka et al. 1995, Fousteri and Lehmann 2000, Morikawa, Morishita et al. 2004, Andrews, Palecek et al. 2005), and was later found to function in the resolution of recombination intermediates that accumulate following damage during mitotic S phase (Branzei, Sollier et al. 2006, Sollier, Driscoll et al. 2009, Chavez, George et al. 2010). The Smc5/6 complex in budding yeast is crucial for DNA repair, and evidence suggests that one of its repair function may be due to the complex's ability to recruit cohesin genome-wide following DNA damage (Onoda, Takeda et al. 2004, Zhao and Blobel 2005, Cost and Cozzarelli 2006, De Piccoli, Cortes-Ledesma et al. 2006, Lindroos, Strom et al. 2006). Mutating genes involved in the removal of aberrant recombination structures at blocked replication forks worsens the phenotype in *smc5/6* mutants (Morikawa, Morishita et al. 2004, Hwang, Smith et al. 2008, Chen, Choi et al. 2009). Homologous recombination is not essential in yeast, however, indicating that the Smc5/6 complex, while important for recombination, plays another vital role. One possibility is that Smc5/6's crucial function is during replication, where it has been shown to reduce topological stress in vegetative cells (Kegel, Betts-Lindroos et al. 2011). The finding that removing Top2, which increases the amount of SCIs, increases the amount of Smc6 binding supports a role for Smc6 in DNA topology (Kegel, Betts-Lindroos et al. 2011). In contrast, recent studies indicate that the Smc5/6 complex's vital meiotic function is during recombination.

In *C. elegans,* Smc5 and Smc6 are required to process recombination structures in germ line cells (Bickel, Chen et al. 2010). Fission *nse1-3* are needed for proper meiotic chromosome segregation (Pebernard, McDonald et al. 2004, Wehrkamp-Richter, Hyppa et al. 2012), and mutations in *nse6* lead to the accumulation of meiotic

JMs in the form of single HJs that resemble those found in cells lacking the endonuclease Mus81 (Wehrkamp-Richter, Hyppa et al. 2012). These HJs were DSB-dependent, but the *nse6* mutant used in this study was not meiosis-specific and accumulated recombination intermediates already in mitosis and pre-meiotic S phase. Similarly, the authors of a study in budding yeast attributed the meiotic segregation defect in their *smc6* mutant to defects prior to meiotic recombination (Farmer, San-Segundo et al. 2011).

In **Paper I** (Lilienthal, Kanno et al. 2013), we identified a recombination-specific role for the Smc5/6 complex during meiosis in budding yeast. Unlike the studies described above, the mutants used in our investigation were meiosis-specific, either by conditional gene depletion by promoter replacement (using so-called meiotic nulls (mn): *smc5-mn*, *nse4-mn*, *nse2-mn*) or by temperature-dependent protein inactivation after meiotic S phase completion (using the *smc6-56* allele). This allowed us to study meiotic functions of the Smc5/6 complex exclusively. Cells deficient for Smc5/6 complex components in meiosis were unable to segregate their DNA, instead forming only cells with one DNA mass outside of four empty spores. However, when the temperature-sensitive mutant *smc6-56* underwent meiosis at restrictive temperature from the time nutrient depletion began (i.e. upon meiotic induction prior to S phase), 20% of cells remained uninucleate, suggesting a role for the Smc5/6 in meiotic DNA replication. We chose not to pursue this phenotype here, though it will be of interest to characterize this defect further in future studies. Since spores in *S. cerevisiae* form around duplicated spindle pole bodies (reviewed in Neiman 2011), the "one DNA mass outside of four spores" phenotype indicates that *smc5/6* mutants complete the meiotic program despite unsegregated DNA. In line with this, spindle duplication and elongation were normal in these mutants. In addition, SC and cohesin dynamics were unperturbed, supporting the notion that the Smc5/6 complex is needed for chromosome segregation but not meiotic progression.

Knowing the relationship between the Smc5/6 complex and the cohesin complex during mitosis, we were curious as to whether they had related functions during meiosis as well. Cohesin loading and removal at the chromosomal level was not inhibited in the *smc6-56* mutant, suggesting that the Smc5/6 complex does not regulate cohesin. In addition, the dynamics of sister chromatid cohesion were normal in these cells, further supporting the notion that the Smc5/6 complex does not affect cohesin and its functions. To determine whether the nuclear division failure was due to break-independent sister entanglements and to further explore the possibility that defective cohesin may block chromosome segregation in Smc6-deficient cells, we monitored nuclear division in cells that were forced to undergo a single mitosis-like division (i.e. segregating sisters) in the absence of recombination. In this background, the *smc6-56* mutant was able to complete nuclear divisions, indicating that the Smc5/6 complex is not needed to process recombination-independent chromatid entanglements and further supports the notion that the block in Smc6-deficient cells is a result of cohesion-independent mechanisms. In contrast to these findings, another study found that the segregation block in *smc5-mn* cells was rescued when the cohesin component Rec8 was artificially cleaved from chromosomes (Copsey, Tang et al. 2013). It may be that different subunits of the Smc5/6 complex function in different pathways during meiosis. Alternatively, cohesin removal, through elimination of SCC, may reduce the likelihood of repairing DSBs via the sister. Given that the Smc5/6 complex is required to remove such structures in mitosis, it may be that it is needed for resolution of inter-sister recombination intermediates during meiosis as well. We favor this explanation since we find that *smc6* mutants accumulate inter-sister repair intermediates that depend on Smc6 function for their removal (see below). Moreover, we found that Smc6 localization on meiotic chromosomes depended on Rec8, suggesting that Smc6 is not needed when SCC is absent. Reduction in Smc6 binding is also observed in mitotic cells when mutating the cohesin component *SCC1*, which leads to loss of cohesion (Jeppsson et al., unpublished). On the other hand, this may indicate that Smc6 loading is dependent on meiotic axis organization.

The segregation block in *smc6-56, smc5-mn, nse2-mn,* and *nse4-mn* was rescued when DSBs were eliminated, indicating that the block is recombination-dependent. Despite this, DSB repair was complete and efficient, suggesting that the accumulation of recombination intermediates may cause the problem in Smc6-deficient cells. Using return-to-function studies, we determined that the Smc5/6 complex performs its most critical functions during resolution of recombination intermediates. In line with this, we detected accumulation of 2.5-3 fold higher levels of total joint molecules (JMs) when Smc6 was dysfunctional throughout meiosis. NCO formation was not affected, but the *smc6* mutant had a higher ratio of IS-JMs to total JMs, suggesting a role for Smc6 in the prevention and/or resolution of IS-JMs. JMs persisted at later time points and although some IH-JMs remained, many lingering species seemed to be IS-JMs. Surprisingly, CO formation was normal in *smc6-56* cells despite remaining IH-JMs. It may be that the IH-JMs that persist are not enough to cause a significant change in CO levels. On the other hand, additional IH-JMs might be forming from extra DSBs. Steady-state DSB levels were slightly higher in the *smc6* mutant at one break site, which may account for extra recombination events.

To further characterize the role of Smc6 in recombination, we investigated JM dynamics in cells that had functional Smc6 during JM formation but not resolution. Under these conditions, segregation was completely blocked. Even though JMs formed at normal levels and ratios, cells were not able to resolve JMs in Smc6's absence. The remaining JMs were between both homologs and sisters, but the IS-JM: total JM ratio increased when Smc6 was inactivated, suggesting that IH-JMs, but not IS-JMs, can be resolved. Again CO and NCO levels were unaffected. When the scenario was reversed and Smc6 was functional during resolution but not formation of JMs, all

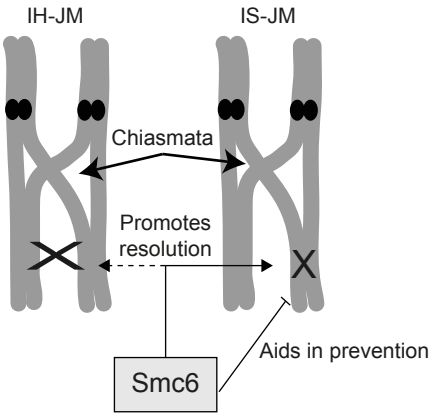

Figure 12. Possible role for Smc6 in the resolution of recombination intermediates.

JMs could be resolved despite higher and altered levels of JM species during formation. In line with the notion that JMs are the culprits of the segregation block, these cells completed nuclear divisions. These data suggest that the Smc5/6 complex is responsible for IS-JM resolution and is important in resolving a subset of non-CO forming JMs (**Figure 12**) and leads us to propose that the Smc5/6 complex is pivotal in resolving recombination intermediates that form outside of canonical meiotic pathways.

CTCF (Paper III)

Genome organization in eukaryotes requires extensive packaging of the chromatin into the fixed, confined space of the nucleus. It has been proposed that specific factors exist that ensure proper chromosome architecture and function by mediating intra- and inter-chromosomal contacts. One such candidate is the essential CCCTC-binding factor (CTCF) found in vertebrates. CTCF is a huge protein that contains a conserved DNA-binding motif consisting of eleven zinc finger domains (Klenova, Nicolas et al. 1993), which make it possible for CTCF to bind multiple DNA regions at the same time. CTCF was initially characterized as a transcription factor capable of both activating and repressing gene expression in heterologous reporter assays (Baniahmad, Steiner et al. 1990, Lobanenkov, Nicolas et al. 1990, Filippova, Fagerlie et al. 1996). Using transgenic assays, CTCF was later found to be the major DNA-binding component that establishes insulators, meaning that it buffers regions of defined gene expression and/or chromatin state (Bell, West et al. 1999). The insulator properties exhibited by CTCF were thought to be a result of its ability to modify chromosome structure. This idea is supported by the finding that many CTCF sites overlap with cohesin (Kim, Abdullaev et al. 2007), which, in addition to its roles in sister chromatid cohesion, regulates gene expression (reviewed in Merkenschlager and Odom 2013). Not long after the discovery of CTCF-cohesin binding sites, it was shown that both CTCF and cohesin are required for long-range genome interactions (Bowers, Mirabella et al. 2009, Hadjur, Williams et al. 2009), and recent evidence has further implicated the coordinated roles of CTCF and cohesin in setting up proper chromosome

structure (reviewed in Merkenschlager and Odom 2013). In addition, the recent ability to study the three-dimensional structure of genomes has generated more evidence supporting the notion that CTCF is a key architectural protein (reviewed in Ong and Corces 2014).

Although the majority of investigations into CTCF's function have focused on vegetative cells, studies suggest that it works during gametogenesis as well. In females, CTCF is required for proper meiosis and oocyte growth, and fertilized CTCF-deficient oocytes lead to offspring with defects in early embryonic development (Fedoriw, Stein et al. 2004, Wan, Pan et al. 2008). CTCF is expressed in all cell types of the seminiferous epithelium in the testes, suggesting that it may also play a role during spermatogenesis (Sleutels, Soochit et al. 2012) Though these studies hint to a role for CTCF in these processes, the function of CTCF during gametogenesis has been difficult to study due to the lack of a viable knockout mouse model.

In **Paper III**, we created a testes-specific *ctcf* knockout mouse strain (*ctcf*-cKO) to elucidate CTCF function during spermatogenesis. Excision of *ctcf* in the *ctcf*-cKO was germ-line specific and occurred from pre-leptotene cells and onward, and mice exhibited seminiferous tubule atrophy along with low sperm counts and infertility. To explore the reasons behind the low sperm counts, we analyzed CTCF function in meiosis.

Early in meiosis, γH2AX marks DSB sites. It is removed from autosomal chromosome axes concurrent with DSB repair, but a subset remains associated with the XY body, which is silenced during meiosis due to the inability for the non-homologous X- and Y-chromosomes to synapse (Hunter, Borner et al. 2001, Fernandez-Capetillo, Mahadevaiah et al. 2003, Inselman, Eaker et al. 2003). CTCF bound to meiotic chromosomes with a strong preference for the XY body, suggesting that it may play a role in meiosis, particularly at the XY body. However, *ctcf*-cKO mice had no major defects in cohesin loading, DSB formation, SC dynamics, chromosome pairing, XY body formation or recombination, suggesting a non-essential role for CTCF during

meiosis. Because the *ctcf*-cKO did not fully excise *Ctcf*, we cannot rule out the possibility that CTCF plays a role during meiosis. However, despite a normal meiosis, *Ctcf*-deficient mice had low sperm counts and were infertile, implying a role for CTCF during post-meiotic stages of spermatogenesis.

In line with this notion, elongating spermatids were apoptotic in the *ctcf*-cKO and mature *ctcf*-cKO sperm showed structural aberrations. Stage 8-10 elongating spermatids lacked a manchette, while stage 12-14 spermatids had an abnormal manchette. Later-stage *ctcf*-cKO sperm exhibited abnormal head structure and inconsistent DNA compaction, both of which may be related to the manchette abnormalities in earlier stages. Mature sperm from *ctcf*-cKO mice had less-elongated heads with visible regions of decondensed chromatin, suggesting a role for CTCF in chromatin compaction. Tail morphology was also irregular in the *ctcf*-cKO, most likely due to abnormal head shape. Consistent with a role for CTCF in chromatin compaction during spermiogenesis, CTCF-deficient mice were unable to incorporate the sperm-specific compaction factor protamine 1 (PRM1), which disrupts the protamine balance known to be crucial for sperm compaction (Cho, Willis et al. 2001, Cho, Jung-Ha et al. 2003). Thus, infertility in *ctcf*-cKO mice is likely caused by chromatin compaction defects in sperm, which result from improper protamine incorporation during spermiogenesis.

FINDING NOVEL MEIOTIC FACTORS

In 1859, Charles Darwin published his revolutionary work *On the Origin of Species by Means of Natural Selection*, which laid the foundation of evolutionary biology. Though controversial when first presented, researchers have transformed Darwin's theory of evolution into a fundamental biological dogma. Now the pressing question for many biologists is not if or why evolution happened, but rather what can we

gain from it. Namely, how can we utilize evolutionary relationships to gain insights into human health and disease mechanisms?

One way to extract information from evolution is by studying co-evolution, which is a pivotal aspect of evolutionary theory. Co-evolution is defined as the interdependence between the evolutionary changes of two interacting entities (Ehrlich and Raven 1964). Co-evolution was originally studied at the species level, but recent advances in technology and genome sequence availability have made it possible to examine co-evolution at the molecular level as well (reviewed in de Juan, Pazos et al. 2013). Of most interest in modern studies is protein co-evolution, which detects the relationship between interacting and functionally related proteins.

The co-evolution search at the protein level often makes use of phylogenetic trees, which are visual representations of the evolutionary relationships between a set of biological factors (in this case, proteins) (reviewed in Ochoa and Pazos 2014). It has been shown that structurally and functionally interacting proteins have similar phylogenetic tree patterns. That observation has led to the development of methods that use sequence input to predict protein interactions (structural and functional) based on phylogenetic tree similarity (Ochoa and Pazos 2014). Phylogenetic profiling, however, predicts protein interactions based on the joint presence or absence of proteins across species. This method is based on the notion that co-evolving populations will lose or maintain biological entities, such as proteins, as evolution, via natural selection, in that environment deems necessary (Pellegrini, Marcotte et al. 1999). Since proteins in the same phylogenetic cluster are related based on functional evolutionary conservation or divergence, rather than sequence homology, proteins that lack sequence conservation but function in the same pathway can be discovered. This feature of phylogenetic profiling make it of particular use in studying new potential meiosis-specific proteins, which are conserved on a functional but not sequence level, making them difficult to study using other established screens (reviewed in Bolcun-Filas and Schimenti 2012).

Since proteins that make up specialized complexes are expected to have a similar phylogenetic profile, it may be possible to use phylogenetic profiling to predict novel protein components of such complexes. In **Paper IV** (Tabach, Golan et al. 2013), we used phylogenetic profiling to predict protein members of the meiosis-specific synaptonemal complex (SC). As predicted, known proteins of the SC clustered strongly with other meiosis-specific proteins, demonstrating the ability of phylogenetic profiling to cluster proteins that work in the same complex. The SC proteins also shared the phylogenetic profile with other uncharacterized proteins, many of which contained a predicted coiled-coil structure that is characteristic of many SC proteins (Costa and Cooke 2007), suggesting new components of the SC. To test if these uncharacterized proteins were meiosis-specific, as would be expected for SC proteins, we analyzed the mRNA expression patterns in various mouse tissues. Several were highly expressed in the ovary and/or testes. One gene, Ccdc105 (coiled-coiled domain containing gene 105), encoded a protein most similar phylogenetic profile to SC proteins and was expressed in testes only. Using an antibody raised against CCDC105, we found that CCDC105 resides in the nuclear fraction in testes by western blotting. CCDC105 localized to the heterochromatic centromeres in in spermatocytes in late diplotene, and was also found in heterochromatic regions in post-meiotic round spermatids. These data suggest that CCDC105 may play a role in late stages of meiosis and spermiogenesis. Together, the results in **Paper IV** indicate that phylogenetic profiling can be used to find new genes that impact meiosis. Since meiosis is key for gametogenesis and reproduction, finding new meiotic factors can help researchers find ways to treat and perhaps prevent infertility.

CONCLUSIONS

The goal of this thesis was to explore the relationship between proteins that dictate chromosome structure and their function in meiosis.

In **Paper I**, we showed that the Smc5/6 complex is imperative for proper meiotic recombination in budding yeast. Using meiosis-specific alleles, we found that in the absence of Smc5/6 complex components, cells were unable to divide their nuclei due to the accumulation of recombination intermediates. Many of these intermediates were formed between sister chromatids, a situation very rarely observed in wild-type cells most likely due to the transiency of meiotic inter-sister repair. This suggests that Smc6, and the complex as a whole, facilitates proper recombination by promoting the prevention and/or resolution of inter-sister repair intermediates. In support of the latter notion, all recombination intermediates were resolved and nuclear divisions were restored when Smc6 function was turned on at the resolution step of recombination. Also in support of a sister-specific function for Smc6 during recombination were the normal levels of crossover formation in the *smc6* mutant, despite accumulating recombination structures. We therefore propose that the main function of the Smc5/6 complex during meiosis is to aid in the resolution of inter-sister recombination intermediates.

In **Paper II**, we utilized meiosis-specific mutants for topoisomerases Top2 and Top3 and found that both are necessary for proper meiotic segregation in budding yeast. In the absence of Top2, cells experienced a partially recombination-dependent segregation block. This block was not due to the accumulation of recombination intermediates as the timing of intermediate formation and removal, as well as crossover and non-crossover formation, was normal. Top2-deficient cells were not able to segregate their sisters when undergoing a meiosis II-like division in the absence of recombination, suggesting that it is recombination-independent structures which block *top2* cells. Based on these data, we conclude that Top2 is needed for the segregation of

recombined chromosomes during meiosis due to its ability to resolve sister chromatid intertwinings. The segregation block in the *top3* mutant was fully recombination-dependent. Given its role in removal of recombination intermediates in mitosis, we suggest that it works during meiosis in the processing of recombination structures as well.

We defined a role for the CCCTC-binding factor CTCF during spermatogenesis in mice in **Paper III** by creating a testes-specific knockout mouse for *Ctcf* (*ctcf*-cKO). The *ctcf*-cKO was able to complete meiosis normally, but had a very low sperm count and formed aberrant sperm. On closer examination, sperm from *ctcf*-cKO mice did not compact chromatin correctly, leading to head abnormalities and aberrant tail attachment, which most likely contributed to the infertility in these mice. In line with the lack of chromatin compaction, CTCF-deficient sperm were not able to load sperm-specific chromatin compaction factors, suggesting a role for CTCF in their recruitment and/or loading. Together, these results implicate CTCF as an important factor for proper chromatin compaction during spermiogenesis.

Finally, in **Paper IV**, we used phylogenetic profiling to discover a meiosis-specific, previously uncharacterized protein called CCDC105. CCDC105 showed an evolutionary clustering with known components of the synaptonemal complex, suggesting that it plays a role in organizing chromosome structure during meiosis. In line with this, we found that CCDC105 localized to heterochromatic regions on meiotic chromosomes in late prophase. These findings indicate that phylogenetic profiling can be used to find novel factors in pathways known to involve specific protein complexes.

In conclusion, this thesis focused on factors that uphold the integrity of gamete formation. While CTCF seems to be important for differentiation of male gametes, and the role of CCDC105 is uncharacterized as of yet, the Smc5/6 complex, Top2, and Top3 are imperative for proper meiotic segregation. Mistakes in chromosome segregation can lead to aneuploidy, which is a hallmark of tumor cells. When aneuploidy occurs in the germ line, it can lead to genetic disorders such as trisomy of chromosome 21, more

commonly known as Down's syndrome. Moreover, aneuploidy is the leading cause of infertility and spontaneous abortions in humans. Due to the increasing use of *in vitro* fertilization, researchers have been able to study aneuploidy in the germ line and its embryonic consequences more closely. This has led to a striking observation: as maternal age increases, so does aneuploidy and, in turn, maternally-contributed infertility and trisomy. The exact reason for this correlation is not yet clear, but it has been suggested that the integrity of meiotic chromosomal features, such as cohesin and chiasmata, are key predictors of aneuploidy. Since female gametes are arrested at meiosis I at birth, it may be that these factors degenerate with time. Understanding more about how chromosome structure impacts meiotic integrity is the key to treating, and perhaps preventing, infertility and genetic disorders in the future.

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My bubbly **Valentina**, my experience in Stockholm would be so much different without you. You saved me when you moved in!! Thank you for our adventures in an out of Sweden, and for becoming a life-long friend.

Ida, thank you for helping me get through the animal course and for the bond we share over Marc Jacobs.

My friends from the horse world…

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My non-science, non-horse Stockholm friends…

Charlotte, my first friend in Sweden. Thank you for always being there for me. You are my rock. Thank you for always listening and understanding and for always telling me the truth –even when I don't want to hear it! I am so excited for the new phase of your

life with little **Alvaro** and of course **Abrahan**. No matter where life takes us, we will always share a special bond. KRAM

Söta **Tove**, I am so grateful that our friendship has grown since you left CMB. Thank you for being so sweet and for always being a positive, happy influence, and for listening when I need it. Looking forward to see you grow as a mother to your new little one. Puss!

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My brilliant family in Sweden…

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Sist men absolut inte minst, till **Mormor** och **Morfar**… tack för allt. Ni betyder så mycket för mig. Jag älskar er.

All of my fantastic friends across the pond who are never than a phone or Skype call away…

My darling **Rachel**, thank you for always understanding me, no matter how crazy I may seem to the outside world. I would never have made it through these years in Sweden without our near-daily e-mails, Gchats, texts, and BuzzFeed quizzes/facts. Thanks for your support, sarcasm and wit, and for believing in me when I don't. Our friendship will most definitely make it into the beyond. #ghostfriends all the way! <3

Erica, my better half, to think it has been 10 years since we moved away to college together! A LOT has changed since then, but we have not. No matter how long we go without talking, we can always pick up where we left off. Thank you for being such a good friend, especially when I didn't deserve it. I am not me without you! Love you a million white gummy bears!

Jessie, no city is safe when we are in it together! From secret bathroom exits to random alleyways, we do know how to keep things exciting. Thank you for always making me laugh and letting me be my goofy self.

Lisa, my travel buddy, the Frodo to my Froda. From spontaneous trips to the Cape; to romantic ferry rides; to sundresses in snowstorms, we always find a way to laugh a lot together! Thanks for all the marathon Skype dates and real-life adventures. One word sums up our friendship: success.

My sparkly **Hannah**, I wish we could see each other more often. Thank you for pushing me out of my comfort zone and for encouraging me to think outside of the box. No

matter what happens, you will always be like a sister to me. In your own words… "we have our whole lives to talk, so a break here and there is no big deal." <3

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My family in the States…

Dad, I am happy and thankful that we have begun a new chapter.

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Bästa **Mamma**. You are my biggest inspiration and role model. You are living proof it is never too late to pursue your dreams and that with work and perseverance anything is possible. Your love and encouragement have shaped me, and I would not be where or who I am today without you. Thank you for your sense of humor and silliness – even though it can be embarrassing at times, I love it! There are not enough words to describe how much you mean to me. I am so very proud of you for going back to school to fulfill your dreams – you're next! Jag älskar dig med hela mitt hjärta, och jag är så otroligt stolt över att få vara din dotter. <3

And thank **YOU** for reading my thesis – or at least the Acknowledgements! Cheers! ☺

You have brains in your head.

You have feet in your shoes.

You can steer yourself

Any direction you choose.

You're on your own. And you know what you know.

And YOU are the guy who'll decide where to go.

-- Dr. Suess