The Link between Recombination Initiation, Pairing and Synapsis in S. cerevisiae

by

Lina S. Yisehak

Faculty Advisor: Dr. Amy J. MacQueen

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Abstract

Crossover recombination during meiosis is accompanied by a dramatic chromosome reorganization. In *Saccharomyces cerevisiae* homologous chromosomes enter meiosis unaligned, with their centromeres engaged predominantly in non-homologous associations; the onset of meiotic recombination by the Spo11 transesterase leads to stable pairwise associations between homologous centromeres followed by the intimate alignment of homologous axes via synaptonemal complex (SC) assembly. However, the molecular relationship between recombination and global meiotic chromosome reorganization remains poorly understood. In budding yeast, one question is why SC assembly initiates earliest at centromere regions while the DNA double strand breaks (DSBs) that initiate recombination occur genome-wide.

We targeted the site-specific HO endonuclease to various positions on *S. cerevisiae*’s longest chromosome, in order to ask whether a meiotic DSB’s proximity to the centromere influences its capacity to promote homologous centromere pairing and SC assembly (Chapter 2). We show that repair of an HO-mediated DSB does not promote homologous centromere pairing nor any extent of SC assembly in *spo11* meiotic nuclei, regardless of its proximity to the centromere. DSBs induced en masse by phleomycin exposure likewise do not promote homologous centromere pairing nor robust SC assembly. Interestingly, in contrast to Spo11, HO-initiated interhomolog recombination is not constrained to use the meiosis-specific Dmc1 recombinase. Our results strengthen the previously proposed idea that specialized properties of Spo11 DSB machinery activate mechanisms that reinforce homologous chromosome alignment, and that two unique outcomes of Spo11-associated DSBs are homologous
centromere pairing and the establishment of Dmc1 as the primary strand exchange enzyme.

DNA recombination matures within SC. Zip1 protein, a component of SC, promotes interhomolog crossovers. Although the absence of Zip1 shows a reduction in MutSγ-mediated crossovers, how Zip1 promotes recombination is not known. A larger deletion of 244-511 amino acids in Zip1, zip1-M1, was previously implicated in crossover function. I examined crossovers in various zip1 alleles, that encode a protein with small ~10—20 amino acids deletion in Zip1’s M1 region. Our analysis shows that zip1-M1 may play an important role in crossover interference. We found that a smaller deletion in M1 region, zip1-B, formed excess crossovers suggesting this region may limit crossovers.
Chapter 1

General Introduction
Mitosis and Meiosis: Similar but different

Cell division is a means of reproduction through which cells produce a brand-new copy of themselves. The process by which an identical copy is created is called mitosis, whereas meiosis generates new cells carrying only half of the genomic information present in the original mother cell (Figure 1).

Ploidy reduction occurs during meiosis because homologous chromosomes segregate apart from one another during the first meiotic division. Mitosis involves only a single cell division that segregates the sister chromosomes (Figure 1). Errors in homologous chromosome segregation during meiosis lead most often to inviable embryos, and are associated with various disorders of clinical significance in humans. At least one third of miscarriages in humans result from aneuploid gametes (HUNTER 2015). Aneuploid gametes that do not cause miscarriage can cause chromosomal birth defects such as Down’s syndrome (trisomy 21), Patau’s syndrome (trisomy 13) and Edward’s syndrome (trisomy 18) (HERBERT et al. 2015).

Chromosome ploidy is reduced during the first division in meiosis

Meiosis involves two rounds of divisions, Meiosis I (MI) and Meiosis II (MII). The reduction in chromosome ploidy, from 2n to 1n, occurs in the first meiotic division, MI. The events that ensure accurate segregation of homologous chromosomes such as chromosome pairing, DNA recombination and synaptonemal complex (SC) assembly occur during prophase I. Based on the chromosome structures and dynamics,
prophase is divided into five distinct stages called leptotene, zygotene, pachytene, diplotene and diakinesis (Figure 2).

**Figure 1.** A diagram of meiosis and mitosis. (A) Meiosis involves two divisions, Meiosis I and Meiosis II (Left). Two chromosomes, a long and a short chromosome are shown in the diagram (red/green). Chromosome ploidy is reduced in Meiosis I (2n to 1n). Sister chromatids are divided in an equational cell division in Meiosis II. (B) Mitosis division resembles Meiosis II, in that the sister chromatids are divided for every chromosome, resulting in the same ploidy (2n) as parent cells (Right). Meiosis
results in four haploid cells, but mitosis results in two cells that are identical to the parents. Image adapted from (macroevolution.net).

During leptotene stage, chromosomes start to condense and begin to form axes (Figure 2, i). The chromosome gets organized into loops of varying length and each loop is attached to axis sites called axial elements (Moens and Pearlman 1988). The axial elements are chromosome sites at which proteins such as Red1, Hop1 and Mek1 localize (Hollingsworth et al. 1990; Rockmill and Roeder 1991; Smith and Roeder 1997) (Figure 3). Sister chromatids are held together in cohesion complexes that includes the cohesin subunit, Rec8 (Klein et al. 1999). Recombination is initiated by formation of double strand breaks (DSBs) on the loops of chromatin (Keeney et al. 1997; Blat et al. 2002). DSB formation increases the mobility of DNA inside the cell as recombination repair begins (Mine-Hattab and Rothstein 2013). Leptotene is also a time when telomeres associate with the nuclear envelope, in a “bouquet” formation, a process that is thought to facilitate the pairing process (Zickler and Kleckner 2015).

In early zygotene, the synaptonemal complex (SC), a multimeric structure, starts to initiate between chromosomes (Figure 2, ii). The SC structure is made up of Hop1 and Red1 that occupy the axis sites; and several proteins including Zip1 that form the central region (Figure 3, also discussed below) (Cahoon and Hawley, 2016). Following DSB, Mek1 is recruited to the chromosome axes (Figure 3). The intimate alignment of the chromosomes within the context of SC is known as synapsis.
Synapsis initiates earliest from the centromeres, and from various sites that are proposed to be recombination sites on the chromosome (Agarwal and Roeder 2000; Tsubouchi et al. 2008). DSBs disappear before the pachytene stage as repair proceeds (Padmore et al. 1991).

During pachytene stage, SC is complete and all the homologous chromosomes are paired full-length (Figure 2, iii) (Zickler and Kleckner 2015). Recombination intermediates mature within the SC. For instance, double Holliday junctions, which are four-way recombination intermediates, are resolved to crossover or non-crossovers by the end of pachytene (Zickler and Kleckner 2015). At this time, crossovers physically connect each chromosome to its homolog, in addition to the SC structure.

During diplotene, SC disassembles from all the chromosomes. At this stage, all homologous chromosomes remain connected by crossovers or “chiasmata” (Zickler and Kleckner 2015). Cells enter the last stage of prophase, diakinesis, where chromosomes get further condensed. The nuclear envelope disintegrates and spindles begin to form. In humans, oocytes are arrested at this final stage of prophase until puberty in females. Thus, the prophase stage establishes the chromosome connections that are crucial for accurate segregation of each chromosome.
Figure 2. Progression through prophase I. (A) Images show rye microsporocytes. Images adapted from (Zickler and Kleckner, 2015). Chromosomes condense and DSB initiates in leptotene stage (i). Chromosomes pair with one another, as early recombination steps take place. Synapsis initiates in zygotene and it is coordinated with recombination repair (NIMONKAR et al.). Synapsis is complete by pachytene and chromosomes are seen fully aligned as a single unit. In wild-type cells, crossovers mature in the context of SC. Crossover formation is completed by diplotene and SC disassembles. Chromosomes further condense and are held together with chiasmata in diplotene and diakinesis, preceding the first meiotic division.
Figure 3. Cartoon shows the localization of axis proteins and Zip1 during prophase progression. Chromosomes are condensed and the two sister chromatids of each homolog are held together by cohesins. Chromatids are shown in light and dark blue. The meiosis-specific cohesin, Rec8 is shown in gold. Red1 and Hop1 localize to chromosome axis sites, shown in red and purple. Following DSB initiation, Mek1 (cyan) is recruited to the chromatin. Zip1 polymerizes in between the homologs as part of the SC structure, shown in green.
Meiotic recombination overview

At the heart of meiosis in most organisms is homologous recombination, which is initiated by a large number of programmed double strand breaks (DSBs) (Keeney 2008) (Figure 4A). There are two major recombination pathways for DNA breaks formed during meiosis; the double strand break repair (DSBR) pathway and the synthesis dependent strand annealing (SDSA) pathway. For either pathway, following DSB formation, DNA is resected by the Mre11-Rad50-Xrs2 (MRX) complex at DSB locations on chromosomes, leaving 3’ DNA single-strand overhangs (Symington and Gautier 2011) (Figure 4B). After DNA resection, DNA strand exchange is facilitated by the coordinated action of RecA homolog strand exchange proteins.

In budding yeast, plants and mammals, strand exchange is coordinated by two RecA-like proteins, Rad51 and Dmc1 (Bishop et al. 1992; Shinohara et al. 1992). While Rad51 functions in mitosis and meiosis, Dmc1 is meiosis-specific and has 26% amino acid identity with RecA (Bishop et al. 1992; Shinohara et al. 1992). Similar to a RecA protein, both proteins are ATPases and possess a site that has a high affinity for single strand DNA which allows them to form nucleoprotein filament on the 3’ DNA overhangs. These nucleoprotein filaments promote homology search and invasion of a homologous DNA template, and both Dmc1 and Rad51 have been proposed to occupy both sides of a DNA DSB during recombination (Brown et al. 2015). Rad51 and Dmc1 also possess a second site that has low affinity to double strand DNA, and this facilitates homology search (Chen et al. 2008; Danilowicz et
The genes encoding these two proteins diverged after the whole-genome duplication event in *S. cerevisiae* (*Wolfe and Shields 1997; Kellis et al. 2004*).

Strand invasion of the duplex DNA with a Rad51 and Dmc1 nucleoprotein filament forms a displacement loop (D-loop) structure, which is an unstable recombination intermediate (Figure 4C). DNA is synthesized at the end of the single-strand annealed to the template, which functions as a primer for DNA synthesis, and this process extends the D-loop (*Brown and Bishop 2014*). There are two different ways that this D-loop can be processed. If the invading strand and the D-loop disengage by the action of helicases, a limited DNA synthesis takes place to fill in the gaps, and the repair pathway is called a synthesis dependent strand annealing (SDSA) (Figure 4D) (*McMahill et al. 2007*). SDSA pathway accounts for most of the non-crossovers formed in meiosis in budding yeast (*McMahill et al. 2007*). On the other hand, if the initial D-loop is not disengaged and the DNA extension (synthesis) continues, it creates a metastable single-end invasion (SEI), which is a more stable form of the D-loop. SEI intermediates fully connect with the homologous template, creating a DNA structure called, double Holliday junction (dHJ) (*Holliday 1964*). This pathway is known as double-strand break repair (DSBR) and it was proposed in 1983 (*Szostak et al. 1983*). Although, it was proposed initially that there are two outcomes upon resolution of dHJs, non-crossover (NCO) or a crossover (CO) recombinant (Figure 4G, H), most dHJs are thought to give rise to COs in budding yeast (*Allers and Lichten 2001*). dHJs are stabilized with ZMM proteins (discussed below) and utilize distinct pro-crossover resolvases (*Zakharyevich et al.*).
2012). Crossovers are key outcomes of meiotic recombination and physically connect homologous chromosomes until they segregate during anaphase of meiosis I. Both crossovers and non-crossovers can give rise to gene conversion due to synthesis of new DNA using the homologous chromosome as a template.

Figure 4. The DSBR and SDSA recombination pathways. (A) DNA double strand break (DSB) formation is shown. (B) DNA is resected, revealing 3’ DNA overhangs. (C) Strand invasion is promoted by Rad51 and Dmc1. Based on current working models, both proteins co-occupy strands, shown in green and yellow. The displacement loop (D-loop) formation is shown. (D-E) The synthesis dependent...
strand annealing (SDSA) pathway is shown which forms non-crossovers. (F-H) The double strand break repair (DSBR) pathway is shown. (F) shows the formation of the double Holliday junctions. Arrows indicate the possible cleavage sites for resolution. (G) shows a crossover recombinant and (H) shows a non-crossover recombinant product. Image adapted from (Sung and Klein, 2006).

**Double strand break repair (DSBR) factors in S. cerevisiae**

Recombination in budding yeast is initiated by the widely conserved, meiosis-specific transesterase, Spo11, in conjunction with at least nine other proteins (Keeney et al. 1997; Lam and Keeney 2014). DSBs are not formed if any of the nine accessory proteins are deleted, even if Spo11 is present. Spo11 contains a tyrosine residue (Tyr-135) which is essential for its catalytic function to form a double strand break on DNA; this residue is conserved across all characterized Spo11 homologs (Bergerat et al. 1997; Diaz et al. 2002) (Figure 5). Similar to topoisomerases, Spo11 likely forms a DNA break via a nucleophilic reaction of Tyr-135 with a phosphate group in DNA’s sugar-phosphate backbone, resulting in the covalent attachment of Spo11 to the 5’ end of the broken DNA strand (Keeney 2008) (Figure 5, Bottom). Two units of the Spo11 homodimer carry out this reaction on each strand of the DNA duplex to form a DSB (Keeney 2008). Spo11 is removed in subsequent steps (Ma et al. 2015). In addition to the catalytic factor Spo11, cells require the accessory proteins Ski8, Rec114, Mei4, Mer2, Rec102, Rec104, Mre11, Xrs2 and Rad50 (MRX) in order to
initiate recombination during meiosis (Alani et al. 1990; Malone et al. 1991; Ajimura et al. 1993; Rockmill et al. 1995a; Gardiner et al. 1997). Rec102, Rec104, Rec114, Mei4 are meiosis-specific, while Ski8, Mre11, Rad50 and Xrs2 have meiosis and mitotic functions (Murakami and Keeney 2008). Mer2 is transcribed at low levels in vegetative cells, but its transcript is efficiently spliced only during meiosis (Keeney 2008).

Although the role of these Spo11 accessory proteins in not well-understood, some genetic dependencies and interactions between them have been identified (discussed below).

Figure 5. A model for Spo11-induced break formation. Cartoon depicts Spo11 protein modeled after the structure of Topoisomerase VIA. Two subunits of Spo11 are shown in green and blue which are docked on DNA (light blue). Top shows Spo11 prior to DNA cleavage. Bottom shows both Spo11 units covalently attached to a phosphate by their respective tyrosine residues immediately after DSB formation. The cleavage of
DNA by Mre11’s endonuclease activity is shown. Image adapted from (Keeney, 2008).

The Mre11-Rad50-Xrs2 (MRX) Complex

Mre11, Rad50 and Xrs2 (MRX) are required for both formation of DSBs and creating the earliest recombinant intermediate, resected DNA, during meiosis. Mre11 has both endonuclease and exonuclease activity and forms a complex with Rad50 and Xrs2 (Johzuka and Ogawa 1995; Usui et al. 1998). Rad50 belongs to a structural maintenance of chromosome (SMC) protein family that binds DNA in an ATP-dependent manner (Alani et al. 1990; Hopfner et al. 2000). While Mre11 and Rad50 are found in prokaryotes and eukaryotes, Xrs2 is only found in eukaryotes (Stracker and Petrini 2011). Xrs2 regulates the translocation of Mre11 into the nucleus (Tsukamoto et al. 2005). Xrs2 (Nbs1 in human) has regions that physically interact with Mre11, as well as with Sae2 protein, an endonuclease involved in DNA resection (Williams et al. 2009). In the absence of any of the MRX proteins, strains have low viability both in meiosis and mitosis (Tsoubouchi and Ogawa 1998; Bressan et al. 1999). During meiosis, point mutations that only affect the exonuclease activity of the MRX complex allow DSB formation, but the accumulation of unresected DSBs in these separation-of-function mutants demonstrate the dual role of MRX proteins in both DSB formation and repair (Alani
et al. 1990; Tsubouchi and Ogawa 1998). However, the specific role of MRX complex in DSB initiation is not known.

Spo11 removal from the ends of DNA requires MRX function and this requirement appears to be conserved across organisms, while the DSB initiation function of the MRX complex is not well-conserved. For example, MRX is required for break initiation by Spo11 or its homolog in S. cerevisiae and C. elegans, but it is not required for break initiation in fission yeast or plants (Chin and Villeneuve 2001; Bleuyard et al. 2004; Young et al. 2004).

Other Spo11 accessory proteins

The other Spo11 accessory proteins also form functional subgroups that interact and depend on one another for chromatin localization (reviewed in (Lam and Keene 2014)). It is thought that Mer2 gets recruited to the chromatin by the axis proteins, Red1 and Hop1 (Panizza et al. 2011), however a low level of Mer2 is associated with chromatin before cells enter meiosis (Henderson et al. 2006). Mer2 physically interacts with Mei4 and Rec114, and the recruitment of Mei4 and Rec114 to DSB sites depends on Mer2 (Maleki et al. 2007; Panizza et al. 2011). Rec102 and Rec104, which are required for the nuclear localization of Spo11, interact with Ski8 and Mei4-Rec114, and thus have been proposed to bridge the Rec114-Mei4-Mer2 subcomplex with a Spo11-Ski8 subcomplex (Arora et al. 2004; Kee et al. 2004; Lam and Keene 2014). Mre11’s localization to DSBs requires all the accessory proteins except Rad50, thus MRX is thought to be recruited to DSB initiation sites.
following the localization of Spo11 and the other accessory factors (BORDE et al. 2004).

**Double-strand break (DSB) initiation sites**

Spo11 creates DSBs on chromosomes without regard to DNA sequence but in a non-random manner, with certain chromatin regions preferred over others (BAUDAT AND NICOLAS 1997; KEENEY et al. 1997; GERTON et al. 2000; PAN et al. 2011). In yeast, Spo11 forms numerous DSBs on each chromosome and the sites where Spo11 frequently makes DSBs are commonly referred to as “hot spots”, which are 100-200bp in length (PAN et al. 2011). Hot spots are enriched in the promoter regions of genes and are less frequent in telomere and centromere regions (GERTON et al. 2000; BLITZBLAU et al. 2007; PAN et al. 2011). The accessory proteins may directly or indirectly play a role in targeting Spo11 to different chromosomal sites in budding yeast meiosis, but specific factors that direct Spo11 have not been identified to date. Interestingly, DSB initiation sites are conserved even among divergent Saccharomyces species (LAM AND KEENEY 2015). Different species of birds also appear to have a well-conserved recombination hotspot map (SINGHAL et al. 2015).

**Factors that influence DSB number**

Apart from the nine accessory proteins that are absolutely required for Spo11-mediated DSB formation, additional factors are required for robust recombination initiation in budding yeast meiosis. As described above, meiotic chromosome axes
are enriched with meiosis-specific proteins, such as Red1 and Hop1, and meiosis-specific cohesin complexes (HOLLINGSWORTH et al. 1990; SMITH AND ROEDER 1997). Null mutations in axis proteins reduce meiotic recombination initiation to 5% of wild-type levels (ROCKMILL AND ROEDER 1990; MAO-DRAAYER et al. 1996). The role of axis proteins is also conserved in mammals; mutations in HORMAD1 (one of the mammalian axis proteins) reduces the number of DSBs on chromosomes by 76% (SHIN et al. 2010). Additionally, the meiosis-specific cohesin Rec8 is important for proper DSB resection, but absence of the cohesin, rec8, does not affect the kinetics of DSB formation (KLEIN et al. 1999).

**DSB regulation**

Downstream consequences of recombination between homologous chromosomes, in otherwise wild-type budding yeast meiosis, inhibit further DSB formation (KAUPPI et al. 2013; THACKER et al. 2014). Following DSB formation, Spo11 remains covalently attached to the cleaved ends of DNA. A fragment of the single strand DNA is removed along with Spo11 in subsequent endonuclease processing. Thus, oligos linked to Spo11 are a direct product of DSB formation. Such “Spo11-oligos” are increased in mutants that fail to properly process recombination intermediates into crossovers (THACKER et al. 2014), suggesting that breaks continue to form which results in more “Spo11-oligo” amount. Thus, homologous chromosome engagement either through DNA recombination or synapsis may function as a feedback control to shut off DSB formation.
The phosphorylation of accessory proteins downstream of DSB formation can inhibit their function, indicating that early steps in the processing of DSBs can inhibit additional recombination initiation activity. For example, phosphorylation of Rec114 (a Spo11 accessory protein) by Mec1/Tel (homologs of mammalian ATR/ATM kinase), which are kinases activated by DSB formation, inhibits DSB formation (Carballo et al. 2013). Studies in mice also show that ATM limits DSB formation in mammals, possibly through the phosphorylation of Spo11 accessory proteins or proteins that control meiotic cell cycle progression (Lange et al. 2011). DSB activity may also be regulated through the phosphorylation of Mer2 by cyclin-dependent kinases (CDK) (Henderson et al. 2006); It is thought that phosphorylation could modify the interaction of Mer2 with other accessory proteins (Henderson et al. 2006), which can be a mechanism to regulate breaks. CDK levels are at low or intermediate levels in pre-meiotic replication and rise as cells progress through meiosis (Grandin and Reed 1993; Stuart and Wittenberg 1998), suggesting that Mer2 interactions may be modulated as cells progress through meiotic prophase.

Tel1/Mec1 kinase

The formation of a DSB and subsequent steps involves regulation by the DNA checkpoint kinases Mec1 and Tel1, homologs of mammalian ATR and ATM respectively (Subramanian and Hochwagen 2014). Mec1 and Tel1 phosphorylate substrates at serine or threonine sites in response to DSB formation (DNA damage) (Figure 6). These kinases have numerous overlapping targets that are involved in
regulating DSB repair. Tel1 (ATM) is thought to be activated by blunt DSBs or breaks that load MRX (or Mre11-Rad50-Nbs1, see below) complex (Lee and Paul 2005), whereas Mec1 (ATR) is activated by Replication protein A (RPA)-coated single strand DNA during early DSB repair (Zou and Elledge 2003).

DNA damage (DSB) is sensed by the MRE11-RAD50-NBS1 (MRN) complex in mammals and likely by the analogous MRX complex in yeast, which activates Tel1 kinase (Usui et al. 2001; Maréchal and Zou 2013). This initial DSB signal leads to a phosphorylation signal cascade by both Mec1 and Tel1 that modulates meiotic recombination and chromosome structure in the following ways. One early target of Mec1/Tel kinases is the Sae2 protein, an endonuclease that functions in chromosome resection together with MRX complex (Cartagena-Lirola et al. 2006). Phosphorylation of Sae2 by Mec1/Tel1 kinase is necessary for DSB repair as demonstrated by meiotic repair defects of sae2 mutants that cannot be phosphorylated (Cartagena-Lirola et al. 2006). Another important direct target of Mec1 and Tel1 kinase is the axis protein Hop1 (Carballo et al. 2008). Hop1 phosphorylation allows it to recruit Mek1, the meiosis-specific paralog of the effector kinase Rad53 (Carballo et al. 2008) (Figure 6). Mek1, a meiosis specific protein autophosphorylates, and acts on multiple direct and indirect targets to promote interhomolog recombination and synaptonemal complex assembly in between chromosomes (Rockmill and Roeder 1991; Hollingsworth 2016).
Figure 6. Mec1/Tel Signaling pathway during meiosis. DSBs are initiated on chromosomes by Spo11 (orange) during meiosis. The breaks are sensed by early recombination proteins such as MRX (yellow). The ATM and ATR homologs, Tel1 and Mec1 (magenta) are the transducers of the DSB signal. Mec1/Tel1 are kinases.
that have numerous targets in response to breaks. One of these targets is the meiosis specific Hop1 protein (green). Phosphorylation of Hop1 by Me1c/Tel1 enables it to recruit Mek1 kinase (cyan, effector kinase). Mek1 has numerous targets including Rad54, Hed1, Histone H3 and Zip1. The Mec1/Tel1 signal cascade initiated by DSBs modulates numerous meiotic processes that includes, interhomolog DNA repair and synapsis.

**Interhomolog bias**

Rad51 is required for homologous recombination repair during vegetative cell growth and Rad51 utilizes the sister chromosome as a template for repair (GAME AND MORTIMER 1974; SHINOHARA et al. 1992). However, the meiosis-specific Dmc1 provides most strand exchange activity during meiosis, where the nonsister chromatid is the preferred template for repair (BISHOP et al. 1992). One mechanism by which meiotic interhomolog repair bias is established is through the phosphorylation of Rad54 by Mek1 kinase, which inhibits Rad51’s strand exchange activity (NIU et al. 2009). Rad54 is a highly conserved ATPase motor protein that interacts with and stimulates Rad51 (CEBALLOS AND HEYER 2011). Failure to downregulate Rad54 changes meiotic interhomolog repair into predominantly intersister repair in *S. pombe* (TRICKEY et al. 2008). Thus, Mek1 phosphorylation of Rad54 functions to disrupt the Rad51-Rad54 functional interaction and shift intersister repair into interhomolog repair.
Another molecular mechanism that promotes interhomolog bias is the Mek1-dependent phosphorylation of Hed1 (CALLENDER et al. 2016), a meiosis-specific protein that binds Rad51 and inhibits its strand exchange activity during meiosis (TSUBOUCHI AND ROEDER 2006). The inhibitory activity of Hed1 on Rad51 depends on phosphorylation (BUSYGINA et al. 2008). In the absence of Hed1, Rad51 strand exchange activity is increased and can restore interhomolog recombination to a \textit{dmc1} mutant by promoting a “Rad51-only” recombination repair (LAO et al. 2013). However, despite the restoration of interhomolog recombination, interhomolog:intersister bias is diminished in the \textit{hed1 dmc1} mutant (LAO et al. 2013). Thus, similar to Rad54 phosphorylation by Mek1, Hed1 phosphorylation also functions to downregulate Rad51 in order to promote interhomolog repair during meiosis.

**Rad51 is an accessory for Dmc1**

Bishop and colleagues demonstrate that Rad51 is an essential accessory factor for Dmc1, identifying a distinct role from its canonical RecA-like strand exchange activity (CLOUD et al. 2012). RecA proteins contain three positively charged amino acids that create a basic patch, which contacts the phosphate backbone of DNA for strand exchange activity. Cloud and colleagues (2012) mutated these residues in Rad51 (\textit{rad51-II3A}) and showed that Rad51-II3A itself is incapacitated for strand exchange, while Rad51-dependent, Dmc1 strand exchange activity remains robust in the context of the Rad51-II3A protein. Consistent with the idea that Rad51 is an
accessory for Dmc1, a rad51 null mutation diminishes interhomolog crossovers and interhomolog bias during meiosis (SHINOHARA et al. 1997). Furthermore, recent studies show that Dmc1 and Rad51 colocalize on meiotic recombination intermediate DNA substrates (BROWN et al. 2015) affirming that these two proteins cooperate during meiosis.

**Recombination and homolog pairing**

Recombination initiation through the formation of DSBs is necessary for chromosome pairing and synapsis in yeast (PADMORE et al. 1991). Mutations in strand-exchange protein Dmc1 delays meiotic progression and reduce pairing of homologous chromosomes from 95% in wild-type to 60% (ROCKMILL et al. 1995b). Consistent with the requirement of Rad51 for Dmc1 function, mutation in the strand exchange protein Rad51 reduces pairing similar to dmc1 (ROCKMILL et al. 1995b). These observations suggest that steps in recombination initiation and repair mediate homologous chromosome recognition and pairing, which is ultimately important for accurate segregation of chromosomes. In organisms such as yeast and mammals, homolog pairing is intimately linked to DSB initiation.

**Initial homolog pairing interactions**

As discussed above, the early stage of prophase stage, especially the leptotene/zygotene stage, involves chromosome movement that culminate in synapsis between chromosomes. In early prophase, chromosomes associate with the nuclear
envelope and aggregate in a small area of the nucleus, creating an organization called the bouquet (ZICKLER AND KLECKNER 2015). While these movements occur, recombination is also initiating on chromosomes. Prior to DSB initiation, the SC transverse filament protein Zip1 localizes to meiotic centromeres and promotes a pair-wise association of non-homologous chromosomes known as centromere “coupling” (TSUBOUCHI AND ROEDER 2005) (Figure 7A). A shift from non-homologous “coupling” to homologous centromere “pairing” is thought to be facilitated in part through the concerted action of Mec1/Tel1 signaling and a phosphatase, PP4, on Zip1 (FALK et al. 2010). The phosphorylation of Zip1 on serine 75 is thought to destabilize Zip1’s non-homologous association, “coupling”, which allows the chromosomes to search for homology. Then, the de-phosphorylation of serine 75 by PP4 phosphatase allows Zip1 to solidify pairing with the correct partner, by removing the destabilizing phosphorylation (Figure 7C). The early chromosome movements such as bouquet formation and the centromere association through Zip1 are mechanisms that promote interaction between homologs which could facilitate chromosomes pairing. However, chromosomes that cannot couple in mutants such as zip1, eventually segregate accurately as long as they receive DSBs. Thus, earlier pairing interaction may facilitate homolog association, but the initiation of recombination by Spo11 is absolutely necessary for accurate pairing and segregation of chromosomes.
**Figure 7.** *Zip1 at centromeres mediates centromere association pre-DSB and post-DSB.* Diagram shows the transition of meiotic chromosomes from non-homologous “coupling” to homologous “pairing”. (A) Before DSB initiation, chromosomes are coupled at the centromeres with their non-homologous partners. Coupling is dependent on Zip1 (Tsubouchi and Roeder 2005). Green shows Zip1; red, blue,
and yellow show chromatids. (B) DSB initiation activates Mec1 kinase which phosphorylates Zip1. Phosphorylation of Zip1 destabilizes the coupling interaction at the centromeres (FALK et al. 2010). (C) Following DSB formation, centromeres associate with their homologous partners.

The Synaptonemal Complex (SC) in budding yeast

Homologous chromosomes form an intimate alignment with the assembly of a tripartite structure called the synaptonemal complex (SC), a widely conserved feature of meiotic chromosomes (SYM et al. 1993). SC structures from divergent species are ~100 nm in width, and are assembled by proteins that, while not ancestrally related, are typically characterized by the presence of one or more extensive coiled-coil regions (Figure 8) (PAGE AND HAWLEY 2004; ZICKLER AND KLECKNER 2015). Such SC structural proteins connect the axial cores of homologous chromosomes (referred to as lateral elements within the context of the SC) along their entire length. These proteins include transverse filaments, which are rod-like, coiled-coil proteins that assemble with their long axis perpendicular to the long axis of the chromosome, and SC central element proteins, which appear to organize the transverse filament proteins at the midline of the SC (DONG AND ROEDER 2000). The budding yeast SC transverse filament protein is Zip1 (SYM et al. 1993), while Ecm11 and Gmc2 have been recently identified as budding yeast SC central element proteins (Figure 8) (HUMPHRYES et al. 2013). Two Zip1 coiled-coil parallel dimers or higher order unit span the distance
between homologous lateral elements within the budding yeast SC, organized at the midline of the SC by Ecm11 and Gmc2 (SYM et al. 1993; HUMPHRYES et al. 2013).

**SC function**
The SC appears to be linked to many aspect of homologous recombination repair. First, the formation of SC between chromosomes may limit DSB formation. Evidence for this comes from studies in yeast and mouse that show that DSBs continue to form on chromosomes that fail to undergo complete synapsis (KAUPPI et al. 2013; THACKER et al. 2014). Furthermore, the idea that synapsis decreases interhomolog recombination during break repair comes from a recent study that shows that Mek1, which normally promotes interhomolog repair, is excluded on synapsed chromosomes (SUBRAMANIAN et al. 2016). This study suggests that synapsis changes the repair template choices available at DSB site. Second, in *C. elegans*, SC facilitates crossover interference, the phenomena that prevents crossovers from occurring too close to one another on chromosomes. Depletion of the SC structure in *C. elegans* showed loss of interference (LIBUDA et al. 2013). However, interference is intact in SC-deficient budding yeast meiosis when Ecm11 or Gmc2 is absent (VOELKEL-MEIMAN et al. 2016) and Zip3 foci display an interfering distribution even when Zip1 is absent (FUNG et al. 2004). These contrasting interference phenotypes of SC-deficient mutants in different organisms suggests that the influence of Zip1 on crossover interference varies between organisms.
**Figure 8.** *The synaptonemal complex (SC) assembly.* Top: Cartoon shows the components of the proteinaceous SC structure. Lateral element proteins (Red1, Hop1) anchor the condensed DNA loops of the four sister chromatids. Zip1 assembles in between the lateral elements, with its N-terminus (dark blue, center) and the C-terminus (light blue, axis). Central element proteins, Ecm11-Gmc2 (Purple) and SUMO (yellow) are shown at the center. Image adapted from (Voelkel-Meiman, 2015). Bottom: EM image shows a central element, transverse filament and the two lateral elements of the SC structure from *D. melanogaster* chromosomes. Scale bar, 100 nm. Image by F. Guo (Cahoon and Hawley, 2016).
Zip1 is phosphorylated at four serine sites at the C-terminus in a Mek1-dependent manner and this phosphorylation is required for synapsis (Chen et al. 2015). Alanine mutations that prevented phosphorylation drastically decreased crossover formation. These mutants also had increased level of DSBs, suggesting that phosphorylation of Zip1 functions as a negative feedback to regulate Spo11 break formation.

A function that has been (in the absence of direct evidence) long attributed to the SC, promoting crossover recombination events, has recently been ruled out for the budding yeast SC. Chromosomes that are unable to synapse as a result of missing residues 21-163 of Zip1’s N-terminus (zip1-N1) were capable of promoting robust Msh4-dependent crossovers. Similarly, strains with mutations in central element proteins ecm11 and gmc2, that are not capable of assembling SC had robust crossovers (Voelkel-Meiman et al. 2016). Also in support of this conclusion is the fact that the ancestrally related K. lactis Zip1 protein can rescue SC-associated crossing over, but not SC assembly when expressed as the sole source of Zip1 in S. cerevisiae (Voelkel-Meiman et al. 2015).

While the budding yeast SC structure per se is dispensable for MutSγ-associated crossover events, the SC transverse filament protein, Zip1, is required for them. This is evident by the fact that early recombination intermediates are drastically diminished, and MutSγ crossing over is abolished in zip1 null mutants (Sym et al. 1993; Storlazzi et al. 1996; Tung and Roeder 1998). Zip1 protein increases the abundance of stable DNA joint molecule structures when Msh4-Msh5 is absent.
(VOELKEL-MEIMAN et al. 2015), suggesting its activity acts directly on recombination intermediates, but the precise role of Zip1 in crossover recombination remains to be defined.

Zip1 has furthermore been implicated in the segregation of non-recombinant (non-exchange) chromosomes. Zip1 localizes to the centromeres prior to recombination initiation and promotes coupling of non-homologous chromosomes (TSUBOUCHI AND ROEDER 2005) and this is thought to facilitate pairing. After recombination initiation, in yeast, the segregation of non-exchange chromosomes is dependent on Zip1 function at the centromeres (NEWHAM et al. 2010). In this study, they show that the segregation of chromosomes that cannot recombine (due to absence of breaks) required Zip1. Additionally, Zip1 promotes the segregation of chromosomes in mutants that have crossover defect such as msh4 and msh5 (CHAN et al. 2009), suggesting that Zip1 plays a role in segregation of chromosomes.

**SC initiation sites**

The process of SC assembly is called synapsis. Synapsis occurs downstream of DSB signal and is regulated with a combination of structural and non-structural proteins in yeast (reviewed in (CAHOON AND HAWLEY 2016)). Zip1 is prevented from assembling SC in part by a proline isomerase protein, Fpr3, and the E3 SUMO ligase-like protein, Zip3 (MACQUEEN AND ROEDER 2009). In addition to DSBs, SC initiation requires Zip2, Zip3, Zip4 and Spo16 which are known as synapsis initiation complex, SIC, proteins. These proteins colocalize with one another on chromosomes (AGARWAL
Following recombination initiation by DSBs, SC initiates earliest from centromeres (Tsubouchi et al. 2008). As meiosis progresses, additional SC assembly events appear to occur at recombination sites, based on the following evidence. First, in budding yeast, the earliest Zip1 colocalizes with SICs and SICs colocalize with recombination protein such as Mre11 on chromosomes (Chua and Roeder 1998; Agarwal and Roeder 2000; Tsubouchi et al. 2006). Zip3 has also been shown to interact with a number of recombination proteins, including Rad51 and Msh4 (Agarwal and Roeder 2000), through yeast-2-hybrid and co-immuno precipitation experiments. Second, mutants that reduce the number of crossover recombination sites in S. macrospora show a decrease in synapsis initiation sites (Zickler et al. 1992). In budding yeast, mutations in Spo11 that decrease the number of DSBs, decreased the number of Zip3 foci and the number of synapsis initiation to the same extent (Henderson et al. 2004). Third, SICs show interference on chromosomes just as crossovers do, which suggests that synapsis initiation sites are crossover sites (Fung et al. 2004).

SC-associated crossing over

The majority of meiotic crossover recombination events in budding yeast are formed through a pathway that involves SC-associated proteins. These proteins, referred to as the ZMMs, include Zip1, Zip2, Zip3, Zip4, Msh4, Msh5, Mer3 and Spo16, are required for over half of meiotic crossovers as well as robust SC assembly (Sym et al. 1993; Chua and Roeder 1998; Nakagawa and Ogawa 1999; Agarwal and...
The ZMMs are thought to be the pro-crossover factors that promote the resolution of DNA intermediate into crossover (Jessop et al. 2006). On synapsed chromosomes, ZMM proteins form foci that roughly correspond to the number of crossover sites. For example, Zip3 forms discrete foci in between the two chromosome axes (Figure 9B).

ZMMs as a whole appear to be important for the formation of stable interhomolog joint molecule (dHJ) recombination intermediate structures (Borner et al. 2004), but their individual molecular roles in this function remain unclear. Zip2 and Spo16 were recently shown to bind branched DNA, and form a larger complex with Zip4 (De Muyt et al. 2018). Chromatin immuno-precipitation experiments show that these proteins depend on one another to localize to chromosomes, particularly to localize to Spo11 hotspots (DSB initiation sites) (De Muyt et al. 2018). In this study, they could detect a physical interaction between Msh5 and Zip4, suggesting that Zip4 may be a bridge that connects the other ZMMs to Msh4-Msh5 machinery. These interactions link synapsis initiation to crossover maturation, as Msh4-Msh5 are known to stabilize double Holliday junctions (Snowden et al. 2004). Out of the ZMMs, Mer3 which is a DNA helicase, Msh4-Msh5 (MutS family protein) and Zip2-Spo16 are known to bind DNA directly (Hunter 2015; De Muyt et al. 2018).

Exo1 nuclease and the mismatch repair Mlh1-Mlh3 (MutLγ family) proteins are the main resolvases that form the majority of class I crossovers (Argueso et al. 2004; Zakharyevich et al. 2012). Mlh3 has been shown to directly bind dHJs and is
necessary for crossover formation (NISHANT et al. 2008; RANJHA et al. 2014). Exo1’s interaction with Mlh3 is required for its pro-crossover function, but not its nuclease activity (ZAKHARYEVICH et al. 2010). Mutations in either Exo1 or Mlh1-Mlh3 diminish crossover levels in budding yeast (ZAKHARYEVICH et al. 2012).
**Figure 9.** The ZMM proteins. (A) Cartoon (top, left) depicts Zip2, Zip3, Zip4, Msh4, Msh5, Mer3 and Spo16 (ZMM) proteins. The ZMM proteins co-localize with one another on synapsed chromosomes (top, right). (B) Surface spread chromosomes of a wild-type strain at 24 hours into sporulation (pachytene stage) carrying one copy of Zip3-myc is shown. DNA is shown by DAPI staining (Top, left), Red1 localizes to the two axis sites for each set of homologous chromosome (lower, left). Zip3-myc (green) forms punctate foci on chromosomes. The merged image shows that these foci lie in between the Red1 sites (top, right). Structured illumination microscopy images by Amy MacQueen.

**SC-unassociated crossing over**

Although over half of crossovers in budding yeast meiosis form through a crossover pathway that is coupled with SC assembly, a small fraction of meiotic crossovers in budding yeast form through a “class II” crossover pathway that does not involve SC-associated proteins (LYNN et al. 2007). This pathway utilizes structure-selective nucleases such as Mus81-Mms4, Slx1-Slx4 and Yen1 and other DNA processing enzymes to resolve recombination intermediates. Such processing factors engage with the dHJ in a manner that results in a 1:1 outcome of crossovers and noncrossovers (DE LOS SANTOS et al. 2001; EHMSEN AND HEYER 2008; ZAKHARYEVICh et al. 2012) Genetic experiments show that SC-associated (class I) and the class II pathways are distinct: Double mutations of mms4 zip4 and mms4 msh5 have an additive effect in
reducing crossovers compared to the single mutants (DE LOS SANTOS et al. 2003; ARGUESO et al. 2004). Furthermore, crossovers that are formed in the SC-independent pathway do not show interference as class I crossovers do (ARGUESO et al. 2004).

In addition to class I and class II resolvases, helicases facilitate the resolution of all joint molecules. For example, Mms4 and Sgs1, a RecQ helicase resolve aberrant structures including joint molecules created as a result of multi-chromatid recombination or single dHJs (OH et al. 2007; OH et al. 2008).

**SC assembly is coordinated with meiotic recombination**

Chromosomes start to pair with one another as recombination initiation occurs in the leptotene stage of prophase. This pairing is reinforced downstream of break initiation by the assembly of SC between homologous chromosomes. Chromosomes transition from non-homologous associations to pair-wise association, only after the initiation of recombination. However, how Spo11’s earliest recombination initiation steps are coordinated with correct pairing of chromosomes is poorly understood. Studies that show the physical interactions of ZMM proteins to recombination intermediates suggest that SC initiation is coordinated with recombination process (DE MUYT et al. 2018).

DSBs are necessary to initiate recombination and assemble SC in yeast. Although many of the genetic requirements are known for recombination, how recombination events and pairing is coordinated in yeast is largely unknown. Breaks are initiated on chromosomes by a conserved protein, Spo11, and occur in certain preferred locations
(“DSB hotspots”) on the chromosome. How the location of the DSBs or the breaks themselves uniquely bring about the signal cascade necessary to recruit many meiosis-specific factors remains a mystery. Two important events promote global re-alignment of chromosomes into homologous pairs, the coupling of homologous centromeres and the assembly of SC between homologous chromosomes. The molecular mechanism(s) that underlie how the process of meiotic recombination promotes these global pairing processes remain mysterious.

Questions addressed in this thesis

The work described in this thesis aims to gain insight into the molecular mechanisms that promote accurate segregation of homologous chromosomes. Chromosomes start non-homologous associations prior to DSB formation in a process called “coupling”. The transition to correct homologous association, “pairing”, occurs concurrently while the earliest recombination process takes place. It is known that the initiation of recombination by Spo11 is absolutely necessary for this transition in budding yeast. This places the crucial step in meiosis as the DSB initiation step and thus, raises the questions of whether any source of DSB would promote successful pairing between homologous chromosomes.

Another important molecular feature of successfully paired chromosomes is the assembly of the SC which intimately aligns homologous chromosomes. SC requires DSB initiation and originates from the centromeres. After chromosomes undergo “coupling” through centromere association, SC originates from the centromere
downstream of break initiation, designating the centromeres as the most likely sights on the chromosome to pair between homologs first. This raises several questions: Is a DSB nearby or distal to centromeres sufficient for this initial homologous association? Is there a threshold level of DSBs necessary to promote homologous pairing and synopsis?

The capacity of artificial breaks to partially promote meiotic function was first tested decades ago by Thorne and Byers (1993) who showed that irradiation of spo11 meiotic cells partially rescues the spore viability defect of spo11 cells. This study suggested the tantalizing idea that some aspect of Spo11 breaks can be substituted. More recent studies that utilized site-specific endonucleases such as HO endonuclease and VDE endonuclease found that an artificial break can promote crossover recombination (Malkova et al. 2000; Fukuda et al. 2003; Medhi et al. 2016). These studies found that the presence of Spo11 increased the likelihood of crossing over at an artificial break site, suggesting that Spo11 in trans may promote any break, regardless of the source, to repair with a homologous template to result in a crossover. Spo11 initiates breaks in a non-sequence specific manner but some chromatin regions are favored more than others and these are named “hotspots”. Spo11 “hotspots” are enriched with axis proteins, such as Hop1 and Red1. This raises the questions of whether artificial breaks placed in a Spo11 hotspot location would influence the repair outcome. Does an artificial break placed in a Spo11 “hotspot” location promote recombination and SC? Can over expression of axis components promote SC by recruiting the necessary pro-crossover factors?
To investigate the molecular mechanism(s) that couple meiotic recombination with global pairing processes, I carried out the following: I analyzed the capacity of artificial DSBs, positioned close to or distal from centromeres, to restore global meiotic chromosome pairing interactions; this study additionally revealed interesting insight into the molecular factors that process such non-Spo11 DSBs during meiosis in budding yeast (Chapter 2).

Zip1 protein promotes crossing over independent of its ability to form SC structure (Voelkel-Meiman et al. 2015; Voelkel-Meiman et al. 2016). Structure-function analysis of Zip1 in prior studies showed that regions of Zip1 interrupting the coiled-coil region may be functionally important for crossing over (Tung and Roeder 1998). Tung and Roeder showed that a large allele, zip1-M1, which removed amino acids 244-511 in Zip1 reduced spore viability, reduced crossing over and resulted in abnormal SC. These findings raised the question of whether smaller regions within the zip1-M1 promote crossing over. I characterized several zip1 deletion alleles that remove an interesting domain that interrupts the central, coiled-coil containing region of Zip1, within the 244-511 region, to assess whether residues in this region function in crossover recombination (Chapter 3).
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Chapter 2

*HO endonuclease-initiated recombination in yeast meiosis* fails to promote homologous centromere pairing and is not constrained to utilize the Dmc1 recombinase
**Introduction**

Sexually reproducing organisms rely on the meiotic cell division cycle to halve their chromosome ploidy. Successful ploidy reduction during meiosis requires that chromosomes efficiently search for, recognize, and establish a stable connection with their homologous partners, allowing them to orient and segregate properly to opposite poles of the meiosis I spindle apparatus (Page and Hawley 2003). Accordingly, a characteristic feature of meiotic prophase nuclei is a large-scale reorganization in which homologous chromosomes transition from an unpaired distribution to an intimate, lengthwise alignment, usually in the context of an elaborate proteinaceous structure, the synaptonemal complex (SC). However, the connection that homologous chromosomes (homologs) ultimately rely on for their proper segregation is more discrete: this crucial link is nearly always provided by a crossover recombination event. Thus, the central task of meiosis is to coordinate a dramatic change in the spatial distribution of chromosomes with the formation and repair of DNA double strand breaks (DSBs) in a manner that promotes crossing over (Pawlowski and Canede 2005; Hunter 2015; Zickler and Kleckner 2015). The molecular basis for how DNA repair and chromosome pairing mechanisms are coordinated during meiosis remains poorly understood.

Meiotic recombination is normally initiated via DSBs created by the conserved transesterase, Spo11, in conjunction with several accessory proteins (Lam and Keene 2014). Each end of a Spo11-mediated DSB undergoes nucleolytic processing to generate 3’ single stranded DNA; these single-stranded termini associate with
homologs of the *E. coli* RecA recombinase protein in order to assemble nucleoprotein filaments capable of catalyzing strand exchange with homologous duplex DNA (Brown and Bishop 2014; Lam and Keeney 2014; Hunter 2015). In budding yeast meiosis, a subset of nascent strand exchange events undergo dissolution after limited DNA synthesis, and DNA repair is completed for these events via a synthesis dependent strand annealing (SDSA) mechanism to form a noncrossover (Allers and Lichten 2001a; McMahan et al. 2007). Another subset of strand exchange events become stable single end invasion intermediates, and then mature into double-Holliday junction (dHJ) containing joint molecule structures, after “capture” of the second end of the DSB (Schwacha and Kleckner 1994; Schwacha and Kleckner 1995; Allers and Lichten 2001b; Hunter and Kleckner 2001). In budding yeast, the interhomolog crossovers that are critical for proper chromosome segregation form predominantly by the resolution of dHJs (Allers and Lichten 2001a).

The diploid meiotic cell offers three homologous templates that could be targeted for strand exchange by either end of a DSB: One sister and two homologous non-sister chromatids. In contrast to vegetative cells (Kadyk and Hartwell 1992; Bzymek et al. 2010) homologous recombination in meiotic cells preferentially utilizes a non-sister chromatid (the homolog) as a template for DNA repair (Schwacha and Kleckner 1994; Schwacha and Kleckner 1995; Hunter and Kleckner 2001; Hong et al. 2013). One meiosis-specific mechanism that promotes interhomolog versus intersister DNA repair involves the specialized, concerted action
of two RecA homologs: The strand exchange activity of a meiosis-specific RecA homolog, Dmc1 (Bishop et al. 1992) in conjunction with a supporting activity of the mitotic RecA protein, Rad51 (Game and Mortimer 1974; Game et al. 1980; ShinoHara et al. 1992; Schwacha and Kleckner 1997; ShinoHara et al. 1997a; Cloud et al. 2012). In the absence of either Dmc1 or Rad51, meiotic interhomolog recombination is dramatically diminished and residual DSB repair occurs primarily using the sister chromatid (Bishop et al. 1992; ShinoHara et al. 1992; ShinoHara et al. 1997a; Hong et al. 2013; Lao et al. 2013). During normal meiosis in budding yeast, Rad51’s strand exchange activity is diminished and the preferential use of a Dmc1 recombinase pathway is ensured in part by the inhibition of the Rad54 motor protein (Niu et al. 2005; Busygina et al. 2008; Niu et al. 2009) and the stabilization of an interaction between Rad51 and its inhibitor, Hed1 (Tsubouchi and Roeder 2006; Lao et al. 2013); both of these pathways rely on the activity of the meiosis-specific kinase, Mek1 (Xu et al. 1997; Hong et al. 2013; Callender et al. 2016; Hollingsworth 2016).

In budding yeast, meiotic recombination not only generates interhomolog crossovers but also promotes homologous chromosome synapsis - the assembly of SC between lengthwise-aligned chromosomes (Page and Hawley 2004; Cahoon and Hawley 2016). The SC has a conserved, tripartite structure in which rod-like transverse filament proteins assemble in perpendicular orientation to the long axis of the chromosome. Transverse filament proteins bridge chromosome axes (called lateral elements within assembled SC) and a distinct substructure, the central element,
assembles at the midline of the SC. The transverse filament of the budding yeast SC is comprised of the Zip1 protein, which has an extensive central coiled-coil motif that is predicted to fold into a rod-shaped homodimer or tetramer (SYM et al. 1993; SYM AND ROEDER 1995; DONG AND ROEDER 2000). The interacting Ecm11 and Gmc2 proteins assemble the central element substructure of budding yeast SC (HUMPHRYES et al. 2013; VOELKEL-MEIMAN et al. 2013).

Synapsis initiates at multiple discrete points along the length of chromosomes, many of which are likely sites of interhomolog recombination (CHUA AND ROEDER 1998; AGARWAL AND ROEDER 2000; HENDERSON et al. 2004). Interestingly, however, the earliest SC assembly events in budding yeast meiotic cells occur predominantly from centromeres (TSUBOUCHI et al. 2008). Spo11-dependent SC assembly from centromeres raises the mechanistic question of how this class of synapsis events is coupled to meiotic recombination, given that centromeres are not thought to correspond to sites that undergo frequent interhomolog recombination in budding yeast meiosis (LAMBIE AND ROEDER 1986; LAMBIE AND ROEDER 1988; BLITZBLAU et al. 2007; CHEN et al. 2008; PAN et al. 2011; VINCENTEN et al. 2015).

One explanation for initial SC assembly from centromeres in budding yeast may relate to the existence of an SC-independent “coupling” mechanism that can reinforce pair-wise interactions between homologous centromeres. The SC transverse filament protein, Zip1, mediates two-by-two associations between centromeres, regardless of homology and independent of Spo11 activity, at the onset of meiosis in budding yeast (TSUBOUCHI AND ROEDER 2005). Zip1 also mediates pair-wise associations between
homologous centromeres in a Spo11-dependent manner during later meiotic prophase (KEMP et al. 2004; FALK et al. 2010; NEWNHAM et al. 2010; KURDZO et al. 2017).

Zip1’s centromere pairing activity does not involve a conventional SC structure, as the SC central element protein Ecm11 is dispensable for both Spo11-independent and Spo11-dependent centromere pairing (HUMPHRYES et al. 2013; KURDZO et al. 2017). However, the local abundance of Zip1 at centromeres perhaps bestows these chromosomal regions with an increased capacity to assemble SC in response to trans acting signals from recombination sites.

How are meiotic DNA repair processes connected to specialized chromosome pairing and synapsis outcomes? Thorne and Byers (1993) showed that the capacity for X-ray induced DSBs to partially rescue the low spore viability of spo11 meiotic cells depends on the meiosis-specific chromosomal protein, Hop1; this result indicates that recombination intermediates might ensure meiotic chromosome pairing and/or segregation outcomes at least in part by interfacing with meiotic factors that function at the chromosome axis. Consistent with this notion, a handful of meiosis-specific proteins that localize within the SC, including Zip1, Zip2, Zip3, Zip4, Spo16, and the MutSycomplex Msh4-Msh5, are critical for normal levels of dHJs and crossing over during meiosis (SYM et al. 1993; AGARWAL AND ROEDER 2000; NOVAK et al. 2001; TSUBOUCHI et al. 2006; SHINOHARA et al. 2008). Furthermore, meiosis-specific chromosome axis proteins such as Red1, Hop1 and Rec8 have been associated with promoting interhomolog interactions (SHINOHARA et al. 1997a; KIM et al. 2010; HONG et al. 2013).
Further insight into how DNA repair machinery is connected to specialized chromosome pairing and synapsis outcomes in budding yeast is provided by elegant studies that compare recombination outcomes in meiotic versus mitotic cells. Preferential use of the sister chromatid ensures an extremely low frequency of interhomolog crossovers arising from homologous recombination in vegetative cells (Kadyk and Hartwell 1992; Schwacha and Kleckner 1997; Johnson and Jasin 2001). Malkova et al. (2000) found that an HO-mediated DSB is four times more likely to repair as an interhomolog crossover in spo11 meiotic cells than in mitotic cells; these results, similar to the aforementioned results of Thorne and Byers, suggest the possibility that Spo11-independent mechanisms ensure that any DSB (no matter the source) is processed in a manner that is “meiotic-like” in budding yeast meiotic cells. However, it was also noted that Spo11 activity has a trans effect on HO DSB repair: The presence of Spo11 correlated with a shorter gene conversion tract length and an even higher likelihood of crossing over relative to when Spo11 is absent (Malkova et al. 2000). Independent studies have provided additional evidence that Spo11 can increase the likelihood of interhomolog repair (Neale et al. 2002) and reduce gene conversion tract length in trans (Neale et al. 2002), as well as influence the repair factors utilized by a VDE recombinase-mediated DSB (Medhi et al. 2016). Furthermore, when assessed, prior studies found no evidence that an HO-mediated or VDE-mediated DSB promotes SC assembly in spo11 mutant meiocytes (Malkova et al. 2000; Neale et al. 2002), although partial SC assembly may have been missed because of the small size of the chromosomes sustaining the DSB. Taken
together, these findings support the idea that unique properties of meiosis-specific DSB machinery in conjunction with recombination-independent features of the meiotic nucleus ensure that recombination is accompanied by robust homolog engagement and crossing over in budding yeast. Our understanding of the meiotic chromosome pairing processes that are uniquely regulated by Spo11, however, remains incomplete.

In this study, we specifically addressed the question of whether Spo11-mediated DSBs are unique in their capacity to promote homologous centromere pairing and/or homolog alignment. We evaluated the genetic and cytological behavior of meiotic cells devoid of Spo11 but capable of HO endonuclease-mediated DSBs at several distinct positions along budding yeast’s longest chromosome (IV), including positions that Spo11 utilizes frequently. We asked whether HO endonuclease-mediated DSBs positioned nearby or distal to centromere IV, or alternatively whether en masse phleomycin-induced DSBs, are capable of promoting homologous centromere pairing and/or any extent of SC assembly in spo11 mutant meiotic nuclei. Our investigation revealed that HO-mediated DSBs fail to promote homologous centromere pairing and even partial SC assembly, regardless of their proximity to the centromere. Our results shed light on additional distinctions between the processing of HO- versus Spo11-initiated recombination intermediates in meiotic cells, including independence from the meiosis-specific Mek1 kinase and Dmc1 recombinase by HO meiotic DSBs. Our findings add to mounting evidence that one or more specialized properties of Spo11 act to couple a recombination-based homology recognition process with mechanisms.
that reinforce homolog pairing in yeast meiosis, and that one unique outcome of
Spo11-mediated DSBs is the establishment of Dmc1 as the primary recombinase.

Materials and Methods

Strain Construction

Yeast strains used in this study are isogenic to BR1919-8B (ROCKMILL et al. 1995); Table S4 lists their genotypes. Strains were constructed using standard genetic and transformation methods. Primers are listed in Table S5.

To build a plasmid to integrate HO endonuclease driven by SP013 promoter sequences at the LYS2 locus, sequences encompassing LYS2, including 420bp upstream and 50bp downstream, were amplified using primers AJM838 and AJM839, and inserted at the SmaI site of pRS315 to create pAM191. An XbaI site was engineered into the reverse primer AJM839 so that XbaI sites flank the HO cassette in pAM191. A step-wise PCR was used to generate PSPO13–HO. First, SP013 promoter sequence was amplified from genomic DNA using primers AJM763 and AJM764. AJM764 has sequence that overlaps with the beginning of HO. Second, HO endonuclease ORF sequence (ATG through 200bp downstream of the STOP) were amplified from a plasmid carrying PGAL–HO (JENSEN AND HERSKOWITZ 1984), using primers AJM765 and AJM766. Finally, the two overlapping DNA fragments were “stitched” in an amplification using primers AJM763 and AJM766. The PSPO13–HO fragment was inserted at the SnaBI site of pAM191 to create pAM200. Strains in
which $P_{\text{spo13}}$–$HO$ successfully integrated at the $LYS2$ locus were identified first by using counterselection against $LYS2$ on alpha-aminoadipate plates and then by PCR.

To create a template containing the $HO$ cut site sequence ($HO\ cs$) linked to a genetic marker, a 100bp HindIII fragment containing the $HO\ cs$ sequence was excised from pAR134 (RAY et al. 1988) and inserted at the HindIII site of pAG25, which carries $natMX$ (GOLDSTEIN AND MCCUSKER 1999) to create pAM159. $HO\ cs$ sequences were amplified from pAM159 using primers with sequence homology to various locations on chromosome IV (see Table S5). $HO\ cs$ sequences were integrated at the chromosome IV coordinates indicated in Figure 1A; except for $cs1$ and $cs2$, each of these chromosomal positions have been identified as regions of frequent cleavage by Spo11 (BLITZBLAU et al. 2007; PAN et al. 2011; THACKER et al. 2014; MARKOWITZ et al. 2017). The chromosomal position of $cs2$, which is 250 nucleotides from $CENIV$, has been found to be enriched for the meiosis-specific cohesin subunit Rec8 but not for the meiosis-specific axis proteins Red1 and Hop1 (PANIZZA et al. 2011). For technical reasons, two consecutive PCRs were performed for creating DNA unique to each cut site location on chromosome IV. The first PCR was carried out using AJM760, a forward primer common to all integration site cassettes, in conjunction with a reverse primer carrying homology to both pAM159 and the specific integration site on chromosome IV. The second PCR was performed using the same reverse primer in conjunction with a site-specific forward primer carrying homology to the template and the specific chromosome IV integration site. The specific forward and reverse primers used for PCR2 are: $cs1$ (AJM750 and
AJM751), cs2 (AJM752 and AJM753), cs4 (AJM756 and AJM757), cs5 (AJM975 and AJM976), cs6 (AJM1137 and AJM1138), cs7 (AJM1141 and AJM1142), cs8 (AJM1145 and AJM1146), cs9 (AJM1255 and AJM1256) and cs10 (AJM1259 and AJM1260). The position of the HO cs in each strain was confirmed by sequence analysis.

To generate strains with genetic markers flanking HO cs2, the LEU2 gene cassette was amplified using primers AJM1702 and AJM1698 and inserted at position 447 kb (2.8 kb left of CEN4). THR1 was PCR amplified with AJM1241 and AJM1242 and inserted at 1,416 kb on chromosome IV. To generate strains with genetic markers flanking all other HO cs loci, LEU2 was integrated 705 nucleotides to the right of CEN4 (described below). THR1 was inserted as described for strains carrying HO cs2.

To build strains for pairing analysis, a lacO array was integrated 705bp to the right of CEN4 by digesting pJBN156 (BACHANT et al. 2002) with NheI, and a tetO array was integrated at coordinate 1,242 kb of chromosome IV by digesting pAM152 (kindly donated by Karen Voelkel-Meiman) with EcoR1. pAM152 was generated as follows: First, a 920bp fragment encompassing the sequences at position 1,242 kb on chromosome IV was amplified from genomic DNA using primers AJM650 and AJM651, and cloned into the HindIII/SphI site of p306tetO224 (MICHAELIS et al. 1997), which contains 224 tandem tetO (11.2 kb) sequence repeats in a pRS306 vector (SIKORSKI AND HIETER 1989); this created pAM145. Next, the THR1 gene was
amplified using primers AJM666 and AJM667 and cloned between AatII and SacI sites of pAM145, creating pAM152.

*spo11*-Y135F::kanMX4 was integrated to replace the *SPO11* gene by digesting pJY20 (a kind gift of Dr. Scott Keeney, Memorial Sloan Kettering Cancer Center) with SalI and SacII. pJY20 contains *spo11*-Y135F::kanMX4 inserted into the multicloning site of pRS316 (Sikorski and Hieter 1989). Dr. Douglas Bishop (University of Chicago) kindly provided a *rad51-II3A* strain, which was used to amplify the *rad51-II3A* allele sequence that we introduced into our strain background.

The 2µ-RED1-HOP1, pNH219 (Hollingsworth and Ponte 1997) were a kind gift of Dr. Nancy Hollingsworth. A 2µ-REC8-MYC (pAM356) plasmid was constructed using gap repair to replace the *HOP1* ORF in pDW72 with *REC8-MYC*, which was amplified from strain LY893 (Table S4) with primers AJM2164 and AJM2165 (Table S5). The viability of spores from a strain homozygous for the *REC8-MYC* sequence that is carried by LY893 is 97% (n= 52), similar to the wild-type value (95%, n=52).

*Assay for mitotic HO-mediated MAT recombination*

A single culture of each diploid strain used for the genetic analysis of crossover recombination (LY407, LY208, LY555, LY324, LY322 and LY207), which express *P_{spo11}-HO* or no HO endonuclease (control) was grown overnight in liquid rich medium at 30°C. Cells were then diluted and transferred to rich medium plates at a density of 100-200 colonies per plate. After 2 days of incubation, all colonies were
assessed for mating type using a complementation-based assay in which only those cells that mate with a tester \textit{MAT}a or \textit{MAT}a strain are capable of growth on minimal media. Zero colonies from strains without \textit{P}_{\text{SPO13}}-\textit{HO} exhibited any capacity to mate with either the \textit{MAT}a or \textit{MAT}a tester strain, as assessed by growth on minimal media (n = 421). A low frequency of plated colonies (0.7\%, 0.7\%, 1.5\% and 1.2\% in four independent tests involving >400 colonies) from strains carrying \textit{P}_{\text{SPO13}}-\textit{HO} exhibited papilla of growth on minimal media after mixing with either the \textit{MAT}a or \textit{MAT}a tester strain, indicating a low level of \textit{HO} activity during vegetative growth.

\textit{Southern Blots}

Genomic DNA was prepared at 0, 12, 18 and 24 hours of sporulation from strains, LY491, LY456, LY492, LY459, LY481, LY457 and LY458, which are homozygous for a rad51 null mutation and thus severely delayed in completing DNA repair using homologous recombination. After digestion with the appropriate restriction enzyme (EcoRV for strains carrying \textit{HO} cs1, cs5 or cs7, XhoI for strains carrying \textit{HO} cs2, PvuII and XhoI for strains carrying \textit{HO} cs6 and SpeI for strains carrying \textit{HO} cs4), genomic DNA was separated on a 0.8\% TAE-buffered agarose gel and transferred to Hybond-Nylon membrane (KLAPHOLZ AND ESPOSITO). A 500bp probe homologous to \textit{natMX4} sequences, prepared using a DIG High Prime DNA Labeling and Detection kit was hybridized to the membranes in order to detect genomic DNA fragments containing \textit{HO} cs sequences that were either cut or intact. A Syngene G:Box was used to detect chemiluminescence of the hybridized probe; intensity profiles for each
lane were generated using the Syngene Gene Tools program. The percentage of \textit{HO cs} DNA that was cut was calculated by dividing the intensity of the cut \textit{HO cs}–containing fragment by the sum of the cut and uncut fragments. The average of two experiments is presented for each strain.

\textit{Western blots}

Protein was precipitated from 5 ml meiotic cultures at 24 hours of sporulation by trichloroacetic acid precipitation as previously described (\textit{Voelkel-Meiman et al.} 2016). Precipitated protein was dissolved in 2x Laemmli sample buffer (\textit{Laemmli} 1970), supplemented with 30mM DTT. Samples were heated for 10 minutes at 65°, then centrifuged at top speed before the concentration of soluble protein was determined using a NanoDrop (Thermo Fisher). 25-50 µg of protein was loaded on an 8% polyacrylamide/SDS gel and separated at 200 volts for one hour. Separated proteins were transferred to 0.2 µm nitrocellulose membranes (Amersham) after equilibrating the membrane and gel in Towbin buffer (\textit{Towbin et al.} 1979) for 15 minutes. Protein transfer was performed at 60 volts for one hour at room temperature, using a stir bar and ice pack. Ponceau S (Sigma) was used to confirm protein transfer to the membrane. Mouse anti-MYC (clone 9E10, Invitrogen), mouse anti-Hop1 (gift from S. Roeder, Yale) and rat anti-Tubulin (Santa Cruz) were used at 1:2500, 1:1000 and 1:5000 dilutions, respectively. Primary antibody incubations were performed overnight at 4°. HRP-conjugated goat anti-mouse (Jackson ImmunoResearch) and donkey anti-rat (Santa Cruz) were applied at 1:5000 for 2 hours at room temperature.
Amersham ECL Prime Western Blotting Detection Reagent was used to visualize bands. A Syngene “G” box was used to detect chemiluminescence and the Syngene Tools program was used to analyze the data. Normalization was achieved by dividing the intensity of anti-Hop1 signal by the intensity of anti-Tubulin signal in each lane. The fold change of Hop1 abundance in strains with Hop1 overexpression was calculated by dividing the normalized Hop1 intensity in the overexpression strains by the normalized Hop1 intensity in the corresponding control strains. The same approach was used to quantify Rec8-MYC overexpression. The average of 3 experiments is shown.

Cytology
Preparations of surface-spread meiotic chromosomes, their immunostaining and imaging were performed as previously described (Voelkel-Meiman et al. 2016). In a subset of experiments (noted in text), phleomycin (Invivogen) was added to the sporulation medium at a final concentration of 0, 30, 100 and 120 µg/ml. Primary antibodies were applied for 16 hours at 4°C in a humid chamber. The following primary antibodies were used at the indicated dilutions: chicken anti-GFP (1:100) (Abcam), rabbit anti-mCherry (1:100) (Abcam), mouse anti-Hop1 (1:100) (a gift from S. Roeder, Yale), rabbit anti-Rad51 (1:100) (Calbiochem), mouse anti-MYC (1:100) (clone 9E10, Invitrogen), affinity purified rabbit anti-Zip1(1:100) (YenZym Antibodies; raised against a C-terminal fragment of Zip1 as described (SYM et al. 1993), and a polyclonal antibody raised (ProSci Inc.) against a mixed population of
partial Emc11 and Gmc2 proteins (partial proteins kindly provided by Dr. Owen Davies, New Castle University). Fluorophore-conjugated secondary antibodies (Jackson ImmunoResearch) were applied at a 1:200 dilution for 2 hours at room temperature. Following antibody staining, samples were mounted in Vectashield medium (Vector Laboratories) supplemented with 1 µg/ml 4,6-diamidino-2-phenylindole (DAPI). Imaging was carried out using a Deltavision RT imaging system (Applied Precision/GE) adapted to an Olympus (IX71) microscope, and processed using Softworx software (GE). The 3-dimensional distances between the centers of GFP and mCherry foci within each surface-spread nucleus were measured using the Softworx Measure Distance Tool. Distances less than or equal to 0.5 µm were considered paired.

**Results**

*Creating strains in which HO endonuclease is the sole initiator of meiotic recombination*

We created strains in which HO endonuclease is the sole source of meiotic DSB activity by integrating a DNA cassette, $P_{SPO13}^{HO}$, which contains the $HO$ ORF downstream of promoter sequences for the meiosis-specific $SPO13$ gene, as in a prior study to assess HO endonuclease-mediated meiotic DSBs (MALKOVA et al. 1996b). We integrated $P_{SPO13}^{HO}$ at the $LYS2$ locus in strains missing both endogenous $HO$ activity and $SPO11$ (Figure 1A).
A spo13 mutation was used to facilitate analysis of interhomolog recombination in a spo11 strain background. The spo13 mutation allows the isolation of viable spore products from diploid cells that fail to initiate or complete recombination, because spo11 spo13 meiotic cells undergo a single equational division to produce two diploid (dyad) spores instead of four haploid spores (KLAPHOLZ AND ESPOSITO 1980). Genetic markers can be inspected in the diploid spores to determine if interhomolog recombination has occurred.

Robust HO endonuclease activity was detectable in \( P_{SPO13-HO} \)-containing meiotic cells, as meiotic spore products frequently carried a chromosome III that had undergone gene conversion at the MAT locus, which carries the sequence that HO endonuclease targets (HO cs) (HABER 2012). In the absence of meiotic recombination at MAT, a spo11 spo13 (MATa/MAT\( \alpha \) ) meiotic cell will produce two MATa/MAT\( \alpha \) diploid spores (Figure 1B). Indeed, almost all of the dyad spores that result from control spo11 spo13 diploids (with no HO endonuclease expression) fail to mate with either MATa or MAT\( \alpha \) cells, as predicted for the MATa/MAT\( \alpha \) genotype (Table 1). By contrast, approximately 40\% of dyads from strains carrying \( P_{SPO13-HO} \) carry at least one spore that is phenotypically either MATa or MAT\( \alpha \) (Table1), reflecting meiotic gene conversion at MAT. These data are similar to the findings of (MALKOVA et al. 1996b), where 29\% of otherwise recombination-less (rad50 mutant) meioses involving \( P_{SPO13-HO} \) expression and a single cleavable MAT locus displayed gene conversion at MAT; this study also determined that every HO-associated meiotic gene
conversion event arose from interhomolog recombination, versus intra-chromosomal recombination using one of the two silent mating type loci.

A

**Chromosome II**

![lys2::P\textit{SPO13}::HO](image)

Positions of HO cut sites on Chromosome IV

<table>
<thead>
<tr>
<th>cs9</th>
<th>cs10</th>
<th>cs1</th>
<th>cs2</th>
<th>cs5</th>
<th>cs7</th>
<th>cs6</th>
<th>cs4</th>
<th>cs8</th>
</tr>
</thead>
<tbody>
<tr>
<td>302,127bp</td>
<td>385,670bp</td>
<td>448,700bp</td>
<td>449,461bp</td>
<td>635,791bp</td>
<td>1,056,152bp</td>
<td>1,162,006bp</td>
<td>1,241,060bp</td>
<td>1,417,114bp</td>
</tr>
</tbody>
</table>

1,532kb
Figure 1. Creating strains in which HO endonuclease is the sole source of meiotic DSBs. (A) Illustration indicates the chromosomal positions of a meiosis-specific HO endonuclease gene cassette and various HO cut sites (HO cs). HO endonuclease, driven by a meiotic promoter $P_{spo13}$, interrupts the $LYS2$ locus on chromosome II. HO cs sequences together with the $natMX4$ drug marker were targeted to the indicated chromosome IV coordinates. Centromere IV (solid circle) corresponds to coordinates 449,711-449,821bp (Saccharomyces Genome Database). (B) Illustration depicts genotypic and phenotypic MAT locus outcomes of $spo11$ spo13 meiotic nuclei with or without meiotic expression of HO endonuclease. Meiotic cells undergo a single
equational division in *spo11 spo13* strains producing a dyad with diploid spores. In the absence of HO-mediated recombination, each dyad spore receives one copy of *MATa* and one copy of *MATα*, resulting in two non-mating spores (nm). In the presence of HO endonuclease, interhomolog or intrachromosomal recombination at the *MAT* locus on any or all of the chromatids (1, 2, 3 or 4) can produce homozygous *MATa* or *MATα* spores, which will be phenotypically a or α “maters”.

Table 1. *HO* endonuclease-mediated meiotic recombination at the *MAT* locus.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total dyads</th>
</tr>
</thead>
<tbody>
<tr>
<td>ho</td>
<td>583</td>
</tr>
<tr>
<td>P<em>spo11</em>HO</td>
<td>543</td>
</tr>
<tr>
<td>P<em>apo11</em>HO + c5, c6, and c7</td>
<td>2897</td>
</tr>
</tbody>
</table>

Chromosome segregation is predominantly equational during *spo11 spo13* meiosis, resulting in dyads that are diploid and transheterozygous for *MATa* and *MATα* (the parental genotype) and are thus non-maters (nm). HO-mediated interhomolog recombination at *MAT* locus during *spo11 spo13* meiosis can result in mating-capable spores, homozygous for *MATa* or *MATα* (Figure 1B). Shown is the percentage of two-spore-viable dyads that carry spores of a specific phenotype (bold), and inferred genotype (unbold), from the diploid strains (LY407, LY208, LY555, LY207, LY322 and LY324) listed. Companion spores within a dyad are separated by a vertical line. The total number of two-spore-viable dyads in the analysis is given in the final column. The proportion of dyads carrying two non-mater (nm) spores is similar
between strains carrying different \( HO \) \( cs \) loci: \( LY555 = 66.3\% \), \( LY207 = 58.3\% \), \( LY322 = 53.3\% \) and \( LY324 = 67.3\% \) (\( n>500 \) for each of these strains).

We observed similar levels of meiotic recombination at \( MAT \) in each of four \( P_{SPO13}^\prime-HO \) strains containing chromosome IV-targeted \( HO \) \( cs \) loci (generated for experiments discussed below; Table 1). Diploid strains carrying \( P_{SPO13}^\prime-HO \) display very little mating type switching during vegetative growth (see Materials and Methods), indicating that \( HO \) endonuclease activity in our \( P_{SPO13}^\prime-HO \) strains is nearly completely meiosis-specific.

**\( HO \) forms double strand breaks at \( HO \) \( cs \) loci on chromosome IV during \( spo11 \) meiosis**

\( P_{SPO13}^\prime-HO \) strains were used to investigate whether the position of a meiotic DSB along the chromosome is a variable in assuring homologous centromere pairing and SC assembly. Toward this end, we integrated an \( HO \) \( cs \) at nine distinct locations on chromosome IV, including a site positioned 250 nucleotides to the left of \( CENIV \) (\( HO \) \( cs2 \); Figure 1A; Materials and Methods). Except for \( cs1 \) and \( cs2 \), the chromosomal position of each of our \( HO \) \( cs \) integration sites is associated with a high probability of DSB formation by Spo11 during wild-type meiosis (Blitzblau et al. 2007; Pan et al. 2011). Neither \( cs1 \) nor \( cs2 \) are positioned at sites that have been found to be strongly enriched for meiosis-specific axis proteins but the position of \( cs2 \) has been
found to be strongly enriched for the meiosis-specific cohesin subunit, Rec8 (PANIZZA et al. 2011).

A Southern blot was used to evaluate HO-mediated DSB formation in six spo11 null strains, each homozygous for a different chromosome IV HO cs (Figure 2). These strains were additionally homozygous for a rad51 null mutation, in order to slow or abolish the completion of DNA repair and thereby allow us to evaluate the maximal level of HO-mediated DSB formation at these chromosomal sites during meiosis. Genomic DNA was isolated from HO cs-containing strains at 0, 12, 18 and 24 hours of sporulation; prior studies using this strain background indicate that most cells at the 24 hour time point have reached or progressed beyond late meiotic prophase (VOELKEL-MEIMAN et al. 2012). Genomic DNA was digested using restriction enzymes that create a 2-7 kb DNA fragment encompassing the HO cs, and probed for HO cs-associated sequences using a Southern blot.

In strains devoid of P_SPO13\_HO, or at the zero-hour time point in strains carrying P_SPO13\_HO, a single DNA fragment of predicted size was detected (Figure 2A). In strains carrying P_SPO13\_HO and chromosome IV-targeted HO cs1, cs2, cs4, cs5 or cs7, a faster migrating fragment, corresponding to the product of an HO endonuclease-mediated DSB, was also detected (Figure 2A). In these five strains, the maximal percentage of DNA cut by HO endonuclease ranged from ~20%-60% (Figure 2B). These numbers suggest that, in our strains, HO endonuclease maximally cuts one or two out of the four available chromatids in a given meiotic nucleus.
Unexpectedly, DSB formation at \( HO \) \( cs6 \) was not detected in Southern blotting experiments with either of two different restriction enzymes (Figure 2A, B; Materials and Methods). \( HO \) endonuclease is active in the \( cs6 \) strain, based on observed meiotic recombination at the \( MAT \) locus that was comparable to strains carrying the other \( HO \) \( cs \) loci (Table 1). The position of \( cs6 \) on chromosome IV has been previously classified as a frequent target of Spo11 (BLITZBLAU et al. 2007; PAN et al. 2011), but aspects of our strains’ genetic background may affect the accessibility of \( cs6 \) DNA to the \( HO \) endonuclease.
Figure 2. *HO* endonuclease-mediated DSBs at *HO* cs sequences on chromosome IV in *spo11* *rad51* meiosis. (A) Southern blot analysis shows DNA cleavage by HO endonuclease at *HO* cs sequences on chromosome IV in *spo11 spo13 rad51* diploid strains (LY491, LY456, LY492, LY481, LY459, LY457 and LY458; see Figure 1 for *HO* cs positions, Table S4 for strain genotypes). Samples were collected and processed at 0, 12, 18 and 24 hr after placement in sporulation medium. Genomic DNA was digested with restriction enzymes that target sites flanking each of the *HO* cs loci within a 10 kb region; DNA fragment sizes (kb) are displayed next to blots. Fragments were visualized using a probe that hybridizes to the *natMX4* sequence adjacent to each *HO* cs. In the absence of HO and before entry into meiosis in the presence of HO (0 hr of sporulation), the probe detects a single large fragment that corresponds to an intact fragment of DNA containing the *HO* cs. In the presence of HO endonuclease, a faster migrating (smaller) fragment is also seen for each of the cut sites beginning at 12hr, except for cs6. The absence of HO-mediated DSBs at cs6
was verified using two different restriction enzymes (See Materials and Methods). (B) Bar graph shows the percent of DNA cut by HO endonuclease within $P_{spo13-HO}$ spo11 spo13 rad51 meiotic nuclei at the indicated time points. Values were calculated by dividing the intensity of the smaller fragment with the sum of the intensities of the smaller and larger fragment. Bars depict the range given by two experiments.

**HO promotes interhomolog recombination on chromosome IV in spo11 meiotic nuclei**

Meiotic DSBs preferentially engage the homolog over the sister chromatid for repair (Schwacha and Kleckner 1994; Schwacha and Kleckner 1997; Hong et al. 2013). To ask whether HO-mediated meiotic DSBs on chromosome IV engage the homologous chromosome for repair when Spo11 is absent, we assessed interhomolog crossing over between genetic markers that flank an HO cs (Figure 3A). The LEU2 gene was introduced near to the centromere on one copy of chromosome IV, either 705 bases to the right of CEN4 in spo11 spo13 strains homozygous for HO cs5, cs6 or cs7 or 2.8 kilobases to left of CEN4 in analogous strains homozygous for HO cs2. In all strains, the THR1 gene was inserted at coordinate 1,416,000 on the right arm of the chromosome IV carrying LEU2+. The LEU2 and THR1 genetic markers thus allowed us to measure the frequency of HO endonuclease-mediated crossing over.

In the absence of HO endonuclease, spo11 spo13 dyads heterozygous for LEU2 near the centromere and THR1 on the arm of chromosome IV would be expected to
undergo equational division to give diploid dyads containing spores that are each heterozygous for the LEU2 and THR1 insertions (Figure 3A, “No Recombination” outcome). An HO endonuclease-mediated, interhomolog crossover recombination event at an HO cs to the right of the centromere and between the LEU2 and THR1 markers, however, can result in a situation where both chromosomes IV carry one chromatid that is devoid of the THR1 insertion (Figure 3A, “Recombination” outcome). Half of the diploid dyads arising from meioses involving such recombinant chromosomes IV will carry a spore that is phenotypically Leu+ Thr-. Figure 3A illustrates the phenotypes of each spore in such recombinant dyads. Note that a reciprocal crossover at cs2, which is positioned to the left of centromere IV, can result in dyads that contain a Leu-, Thr+ spore in which both chromosomes IV lack the LEU2 insertion.

We measured percent of meioses with an apparent reciprocal crossover event by dividing twice the number of “recombinant” dyads (dyads containing a Leu+, Thr- spore for non-cs2 strains and containing a Leu-, Thr+ spore for the cs2 strain) by the total number of dyads analyzed (Figure 3B, Table 2). Based on this calculation, HO-mediated, interhomolog crossover recombination apparently occurred in ~21% of meioses for HO cs5, 18% of meioses for HO cs7, and 19% of meioses for HO cs2 (closest to centromere IV) (Table 2, Figure 3B). Consistent with the absence of detectable DSBs at HO cs6, interhomolog crossover recombination could be detected at this cut site in only 1.3% of meioses (Table 2, Figure 3B).
The Leu+, Thr- spores that we have presumed to be due to interhomolog crossover recombination could instead arise from an interhomolog non-reciprocal recombination event in which sequences encompassing $THRI$ are converted without an accompanying crossover. In the case of a gene conversion without a reciprocal crossover, the Leu+, Thr+ sister spore in the recombinant dyad is expected to carry one chromatid devoid of the $THRI$ insert. Whereas in the case of a reciprocal crossover, both chromatids in the Leu+, Thr+ spore within a recombinant dyad is expected to contain the $THRI$ insert. We used PCR to detect the presence or absence of the $THRI$ insert in Thr+ spores of 44 recombinant dyads for $cs5$-carrying strains, and to detect the presence or absence of the $LEU2$ insert in Leu+ spores of 54 recombinant dyads from $cs2$-carrying strains. We found that the 71% (31 out of 44) of Thr+ spores from $cs5$ recombinant dyads carried only $THRI$ insert-carrying chromosomes IV, indicating that 71% of recombinant dyads in this strain result from a reciprocal crossover event. ~30% of Thr+ spores were heterozygous for $THRI$ insert-carrying chromosome IV, indicating that the recombinant spore in these dyads resulted from a gene conversion event instead of a reciprocal crossover. In an analogous analysis for $cs2$, we found that both chromosomes IV carried the $LEU2$ insert in only 52% (28 out of 54) of Leu+ spores from $cs2$ recombinant dyads. Thus, about half of the recombinant dyads from the $cs2$ strain result from a reciprocal crossover event, while the remainder derive from a gene conversion event instead of a reciprocal crossover. The higher fraction of interhomolog gene conversion without reciprocal crossing over in $cs2$ versus $cs5$ strains may be due to the proximity of the
LEU2 marker to the DSB site: the LEU2 marker is approximately 2.5 kilobases from cs2, whereas the THR1 insert is ~580 kilobases from cs5. On the other hand, it is remarkable that ~30% of interhomolog recombination events in cs5 strains involve a gene conversion event that encompasses sequences ~580 kilobases from the DSB site. Such remarkably long gene conversion tracts have been previously associated with HO-mediated DSB repair in spo11 meiotic nuclei (Malkova et al. 1996b; Malkova et al. 2000) and have been proposed to arise through a Break-Induced Replication (BIR) process whereby a strand invasion intermediate is extended by replication through the end of the chromosome instead of undergoing second-end capture (Malkova et al. 1996a; Kraus et al. 2001). While the extremely long gene conversion tracts observed here support the idea that a BIR-like mechanism repairs a subset of meiotic HO-mediated DSBs when Spo11 is absent, it is noteworthy that, unlike the mitotic BIR mechanism that has been described (Signon et al. 2001), Rad51 is required for most if not all interhomolog repair of HO DSBs in spo11 meiosis, even those repair events with long conversion tracts (see below).

In sum, as was reported in (Malkova et al. 2000) for the endogenous HO cs on chromosome III, our data suggest that HO endonuclease-mediated meiotic DSBs on chromosome IV can engage a non-sister chromatid for their repair. Furthermore, our data indicate that a non-sister chromatid is utilized for HO-mediated meiotic DSB repair in at least 20% of meioses, and that a substantial fraction (~30%) of HO-mediated interhomolog events involve extremely long gene conversion tracts.
A

spo11 spo13

No Recombination

CO Recombination

\[
\begin{align*}
\text{Leu}^+ & \quad \text{Thr}^+ & \quad \text{Leu}^+ & \quad \text{Thr}^+ & \quad \text{Leu}^+ & \quad \text{Thr}^+ & \quad \text{Leu}^+ & \quad \text{Thr}^+ & \quad \text{Leu}^+ & \quad \text{Thr}^+ & \quad \\
\text{OR} & \quad \text{Leu}^+ & \quad \text{Thr}^+ & \quad \text{Leu}^+ & \quad \text{Thr}^+ & \quad \text{Leu}^+ & \quad \text{Thr}^+ & \quad \\
\text{OR} & \quad \text{Leu}^+ & \quad \text{Thr}^+ & \quad \text{Leu}^+ & \quad \text{Thr}^+ & \quad \text{Leu}^+ & \quad \text{Thr}^+ & \\
\end{align*}
\]

= iLEU2

= iTHR1

= HO cut site
Figure 3. *HO* endonuclease mediated interhomolog recombination during meiosis.

(A) Assay for interhomolog recombination at *HO* cs loci on chromosome IV in *spo11*
spo13 diploids. LEU2 is inserted at 450 kb (705 bp to the right of CEN4) and THR1 is inserted at 1,416 kb on chromosome IV for all strains, except for cs2 where LEU2 is at 447 kb (2.8 kb to the left of CEN4). A single HO cs is integrated between LEU2 and THR1. spo11 spo13 diploid cells undergo a single equational division during meiosis, resulting in spores carrying a chromatid from each parental homolog. In the absence of HO, each spore receives one LEU2 THR1 chromatid and one chromatid with neither marker (“No Recombination” column). In the presence of HO mediated DSBs, interhomolog reciprocal crossover recombination can result in a spore lacking the THR1 marker on chromosome IV (accompanied by a sister spore with two THR1 markers; upper right dyad). Because half of the crossover events will be invisible by this assay (lower right dyad), the percentage of apparent reciprocal crossovers were calculated by dividing twice the number of observed Thr- spores by the number of total 2-spore viable dyads (Table 2 and Table 4). (B) The upper bar graph plots the percentage of apparent crossing over calculated as described in (A) (n > 100 2-spore viable dyads assayed; Table 2) in various strains that carry no chromosome IV HO cs (LY208), distinct chromosome IV HO cs locations, or HO cs5 with rad51 and/or dmc1 null mutations (left to right: LY208, LY555, LY324, LY322, LY207, LY459 and LY393). The lower bar graph plots the percentage of apparent crossing over in mutant strains carrying HO cs5 (left to right: LY207, LY290, LY904, LY939, LY922, LY935 and LY910). Vegetative cultures of control expressing no HO nor HO cs (LY407) as well as LY208, LY555, LY324, LY322 and LY207 were independently evaluated for the uniform presence of the LEU2 and THR1 marker by
Table 2. HO endonuclease-stimulated meiotic recombination in strains with HO cut sites on Chromosome IV

<table>
<thead>
<tr>
<th>Genotype</th>
<th>(Strain)</th>
<th>Phenotype of spo11 spo13 dyads</th>
<th>2x observed apparent CD events</th>
<th>% apparent CD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>no cs</td>
<td>(LY208)</td>
<td>Leu+</td>
<td>Leu+</td>
<td>Leu+</td>
<td>Leu+</td>
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<tr>
<td>cs2</td>
<td>(LY555)</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cs7</td>
<td>(LY524)</td>
<td>46</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cs6</td>
<td>(LY322)</td>
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<td>1</td>
<td>601</td>
<td>0</td>
</tr>
<tr>
<td>cs5</td>
<td>(LY207)</td>
<td>182</td>
<td>10</td>
<td>1384</td>
<td>0</td>
</tr>
<tr>
<td>cs5 rad51</td>
<td>(LY459)</td>
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<td>0</td>
<td>104</td>
<td>0</td>
</tr>
<tr>
<td>cs5 rad51 dmc1</td>
<td>(LY393)</td>
<td>1</td>
<td>0</td>
<td>138</td>
<td>0</td>
</tr>
<tr>
<td>cs5 dmc1</td>
<td>(LY290)</td>
<td>32</td>
<td>1</td>
<td>372</td>
<td>0</td>
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<td>cs5 mek1</td>
<td>(LY904)</td>
<td>29</td>
<td>1</td>
<td>247</td>
<td>0</td>
</tr>
<tr>
<td>cs5 red1</td>
<td>(LY939)</td>
<td>36</td>
<td>0</td>
<td>289</td>
<td>0</td>
</tr>
<tr>
<td>cs5 rad54</td>
<td>(LY922)</td>
<td>13</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>cs5 rad51-I3A</td>
<td>(LY935)</td>
<td>14</td>
<td>0</td>
<td>164</td>
<td>0</td>
</tr>
<tr>
<td>cs5 xrs2</td>
<td>(LY910)</td>
<td>5</td>
<td>0</td>
<td>17</td>
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</table>
Table 2. *HO* endonuclease stimulated meiotic recombination in strains with *HO* cs loci on Chromosome IV. The number of dyads with particular spore phenotypes was used to calculate interhomolog crossover recombination in *spo11 spo13* meiotic cells with *HO*-endonuclease mediated DSB formation at a chromosome IV *HO* cs location (See Figure 3A). *LEU2* is at chromosome IV coordinate 450,000bp, to the immediate right of *CEN4*, except for the cs2 strain where *LEU2* is positioned at 447,000 bp (to the left of *CENIV*); *THR1* is positioned at chromosome IV coordinate 1,416,000. The percentage of apparent crossing over in all strains except LY555 (cs2) was calculated by first doubling the number of dyads carrying a single Leu+, Thr- spore along with a Leu+, Thr+ spore (the first phenotype column), in order to account for the fraction of events not observed, and then by dividing the product by the total number of two-spore-viable dyads (n). The percentage of apparent crossing over in LY555 was calculated by doubling the number of dyads carrying a single Leu-, Thr+ spore along with a Leu+, Thr+ spore (the sixth phenotype column) and dividing this product by the total number of two-spore-viable dyads. The minor population of dyads from LY555 that carry one Leu+, Thr+ spore and one Leu+, Thr- spore could be explained by a reductional chromosome segregation event, may be associated with recombination nearby to the centromere (Simchen and Hugerat 1993). The minor population of dyad spores with loss of both *THR1* markers in some strains is possibly due to two interhomolog recombination events (involving all 4 chromatids). The minor class dyads were not included in the recombination calculation. A Fisher’s Exact test was used to determine whether values differ significantly from the LY207
value. 400 or more colonies from vegetative cultures of LY208, LY555, LY324, LY322 and LY207 were independently evaluated for the uniform presence of the LEU2 and THR1 markers among all cells in the population by plating on selective media. All colonies evaluated were found to be Leu+, Thr+.

Dmc1 is dispensable for HO-mediated interhomolog recombination in meiotic cells

We next asked whether HO-mediated interhomolog recombination events rely on meiosis-specific strand exchange machinery. In rad51 single or rad51 dmc1 double mutants, the viability of diploid dyad spores is dramatically decreased, from 74% in the spo11 spo13 control meiocytes expressing HO, to 38% in the analogous strain missing Rad51, and to 34% in the combined absence of Rad51 and Dmc1 (Table 3). Genetic removal of HO endonuclease from the rad51 single or rad51 dmc1 double mutant strain restored dyad spore viability to 76% and 77%, respectively (Table 3), indicating that the spore lethality observed in our HO endonuclease-expressing, rad51 strains is due to a failure to repair HO-mediated DSBs.

We found that HO endonuclease-mediated interhomolog crossovers at cs5 are diminished in the absence of Rad51. Apparent crossovers between the LEU2 and THR1 markers flanking HO cs5 decreased from 20.8% observed among two-spore viable dyads from spo11 spo13 control meiocytes expressing HO, to 0% and 1.4% observed among two-spore viable dyads from spo11 spo13 rad51 and spo11 spo13 rad51 dmc1 strains expressing HO, respectively (Figure 3B, Table 2). HO-mediated
meiotic gene conversion at the MAT locus also disappeared in spo11 spo13 rad51 meiotic cells expressing HO: 99.0% and 96.4% of two-spore viable dyads from spo11 spo13 rad51 and spo11 spo13 rad51 dmc1 strains, respectively, exhibited two non-mating spores (n=105; Table S1A), similar to the proportion of non-mating spores from spo11 spo13 strains that altogether lack meiotic HO expression (Table 1). Furthermore, only 2 out of 181 (1.1%; Table S1B) one-spore viable dyads from \( P_{\text{SPO13-HO}} \) spo11 spo13 rad51 meiotic cells and 5 out of 363 (1.4%; Table S1B) one-spore viable dyads from \( P_{\text{SPO13-HO}} \) spo11 spo13 rad51 dmc1 meiotic cells exhibited the capacity to mate, indicating that they are homozygous at the MAT locus; these few MAT homozygotes may have arisen as a consequence of meiotic gene conversion or chromosome loss. Similarly, only 4 out of 181 (2.2%) and 1 out of 363 (0.3%) one spore viable dyads from \( P_{\text{SPO13-HO}} \) spo11 spo13 rad51 strains and \( P_{\text{SPO13-HO}} \) spo11 spo13 rad51 dmc1 strains, respectively, exhibited a nonparental configuration of the LEU2 and THR1 markers that flank cs5 on chromosome IV.

The low spore viability and absence of interhomolog recombination when Rad51 is missing from HO-expressing, spo11 spo13 meiocytes indicate that Rad51 is essential for robust repair of meiotic DSBs by homologous recombination during meiosis, which aligns with the expectation that strand exchange is a requirement for homologous recombination. In addition, these data indicate that any Rad51-independent mechanism responsible for repair of HO-mediated DSBs in \( spo11 \) spo13 rad51 meiosis does not lead to gene conversion. Most strikingly, in contrast to Spo11-mediated meiotic DSBs, which are capable of utilizing a Dmc1-only pathway
for repair by homologous recombination when Rad51 is absent (Bishop et al. 1992; Shinohara et al. 1997a), our data reveal that HO-mediated meiotic DSBs are incapable of undergoing homologous recombination using a Dmc1-only pathway.

In contrast to the Rad51-deficient context, no decrease in spore viability is associated with HO DSB activity at cs5 in the absence of Dmc1 (75% spore viability in spo11 spo13 dmc1 versus 74% in spo11 spo13 DMC1 strains respectively; Table 3) and apparent crossovers at HO cs5 are only slightly decreased in spo11 spo13 meiotic cells missing Dmc1 (15.8% in dmc1 versus 20.8% in DMC1 strains; Figure 3; Table 2). Furthermore, HO-mediated interhomolog crossovers in spo11 spo13 meiotic cells appear unaffected by the individual removal of two factors that promote the use of the Dmc1 recombinase (Schwacha and Kleckner 1997; Callender et al. 2016): HO-mediated interhomolog crossover levels are unchanged in spo11 spo13 meiotic cells missing the meiosis-specific kinase, Mek1 (20.8% in both spo11 spo13 control and spo11 spo13 mek1 meiotic cells; Figure 3, Table 2), and in the absence of the meiosis-specific chromosomal protein, Red1 (22.4% in spo11 spo13 red1 strains; Figure 3, Table 2).

Thus, in contrast to wild-type meiosis where diminished Dmc1 activity causes a strong defect in programmed DSB repair, a slowing of the cell cycle, and a dramatic reduction interhomolog crossing over (Bishop et al. 1992; Shinohara et al. 1997a; Bishop et al. 1999), HO-mediated, interhomolog DSB repair in spo11 meiotic cells is not constrained to use Dmc1 but instead can access a “Rad51-only” recombinase pathway.
Table S1. HO-mediated meiotic recombination at the MAT locus in two-spore- and one-spore-viable dyad progeny of select mutants

A.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% Mating phenotype and genotype of spo11 spo13 two-spore-viable dyads</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>nm</td>
</tr>
<tr>
<td>ho</td>
<td>99.6</td>
</tr>
<tr>
<td>Fspo13-1 HO</td>
<td>60.2</td>
</tr>
<tr>
<td>Fspo13-1 HO cs5 rad51</td>
<td>99.0</td>
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</tr>
<tr>
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</tr>
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<td>53.8</td>
</tr>
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<td>Fspo13-1 HO cs5 xrs2</td>
<td>68.5</td>
</tr>
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</table>

B.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% Mating phenotype and genotype of spo11 spo13 one-spore-viable dyads</th>
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<tbody>
<tr>
<td></td>
<td>dead</td>
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<tr>
<td>Fspo13-1 HO cs5 rad51</td>
<td>99.9</td>
</tr>
<tr>
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<td>99.9</td>
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</tr>
<tr>
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<td>55.0</td>
</tr>
<tr>
<td>Fspo13-1 HO cs5 xrs2</td>
<td>84.0</td>
</tr>
</tbody>
</table>
one-spore-viable dyad progeny of select mutants. HO-mediated interhomolog recombination at the MAT locus during spo11 spo13 meiosis can result in spores homozygous for MATa or MATα (Figure 1). The table in (A) gives the percentage of two-spore-viable dyads carrying spores of a specific phenotype (bold) and inferred genotype (unbold) from select spo11 spo13 strains carrying mutations that confer low spore viability (genotypes listed in column 1); companion spores in a dyad are separated by a vertical line. The total number of two-spore-viable dyads in the analysis is given in the final column. The table in (B) lists the percentage of one-spore-viable dyads whose viable spore exhibits a specific phenotype (bold) and inferred genotype (unbold) from the spo11 spo13 strains with low spore viability; the viable spore is indicated to the right of its dead companion (separated by a vertical line). The total number of one-spore-viable dyads in the analysis is given in the final column. *One-spore-viable dyads carrying a spore with the capacity to mate could reflect either a meiotic gene conversion or chromosome loss; in the case of chromosome loss the strains carry only a single chromosome III.

Can HO-mediated interhomolog recombination in meiotic cells utilize Dmc1 when Rad51 recombinase activity is absent?

The data presented above establish a requirement for Rad51 and the lack of a requirement for Dmc1 in the interhomolog repair of HO-mediated DSBs in meiosis. We used two distinct Rad51 separation-of-function genetic contexts to determine if
the Dmc1 recombinase functions redundantly with Rad51 recombinase activity, or whether Dmc1 is completely unavailable to the repair of HO-mediated meiotic DSBs in spo11 mutants.

We first examined mutants missing the Rad54 DNA motor protein. In rad54 mutants Rad51’s recombinase activity is nearly absent and meiotic recombination is forced to rely on Dmc1’s recombinase activity in conjunction with non-recombinase functions of Rad51 for the success of most events (SHINOHARA et al. 1997b; CLOUD et al. 2012). We observed no decrease in HO-mediated crossing over (23%) in spo11 spo13 rad54, relative to 20.8% in spo11 spo13 control meiotic cells (Figure 3; Table 2). Together with the data presented above, these results suggest that HO-mediated meiotic DSBs are capable of utilizing a repair pathway in which Dmc1 provides recombinase activity so long as Rad51 is also present.

Despite the capacity to generate meiotic interhomolog recombinants, the poor overall spore viability of the spo11 spo13 rad54 strain expressing meiotic HO (38% versus 74% for the control; Table 3) resembles that of spo11 spo13 rad51 meiotic cells that express HO. This result indicates that Dmc1 is only partially capable of rescuing HO-mediated meiotic DSB repair when Rad51 recombinase activity is diminished. Consistent with this interpretation, we observe evidence of Dmc1-mediated repair even in meioses that presumably also sustained unrepaired HO DSBs due to the absence of Rad51 activity: ~28% (n=173) of one-spore-viable dyads from rad54 spo11 spo13 strains expressing $P_{\text{SPO13-HO}}$ contain a spore that is homozygous at the MAT locus (likely due to interhomolog recombination at MAT), in contrast to
rad51 and rad51 dmc1 spo11 spo13 strains expressing $P_{SPO13}$-HO where only ~1% of one-spore viable dyads carry a spore that is homozygous at $MAT$ (Table S1B).

Second, we analyzed HO-mediated meiotic DSB outcomes in the context of the rad51-II3A separation-of-function allele (a kind gift of D. Bishop; (CLOUD et al. 2012)). rad51-II3A encodes a protein that lacks strand exchange activity and thus supports a Dmc1-Rad51 joint recombinase pathway but not a Rad51-only pathway. We observed robust HO-mediated interhomolog recombination in spo11 spo13 meiotic cells homozygous for rad51-II3A (15.7%; Figure 3, Table 2). Similar to spo11 spo13 rad51 and spo11 spo13 rad54 strains, spo11 spo13 rad51-II3A meiotic cells that express $P_{SPO13}$-HO exhibit diminished spore viability relative to control (RAD51 RAD54) strains (52% in rad51-II3A versus 74% in the control; Table 3), again indicating that Dmc1 recombinase activity only partially compensates for Rad51 in repairing HO-mediated, meiotic DSBs.

Taken together, our analysis of HO-mediated meiotic DSB repair in rad51, rad54 and rad51-II3A mutants supports the conclusion that HO-mediated meiotic DSBs are capable of accessing either a Rad51-only pathway, or a Dmc1-Rad51 joint repair pathway in which Dmc1 provides recombinase activity.
Table 3. Meiotic HO-mediated double strand breaks lead to spore death in the absence of Rad51 activity or Mre11

<table>
<thead>
<tr>
<th>Genotype</th>
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<th>dyads dissected</th>
<th>% Distribution of dyads types</th>
<th>% Spore viability</th>
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<td>spo11 spo13</td>
<td>(LY407)</td>
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<td>81 16 3</td>
<td>89</td>
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<td>624</td>
<td>87 10 3</td>
<td>92</td>
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<td>15 39 46</td>
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<tr>
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<td>75</td>
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<tr>
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<td>59 24 17</td>
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<td>22 33 45</td>
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<tr>
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<td>75 14 11</td>
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<tr>
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<td>29 42 29</td>
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<tr>
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<td>35 40 26</td>
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<tr>
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<td>(LY913)</td>
<td>104</td>
<td>50 31 19</td>
<td>65</td>
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Table 3. Meiotic HO-mediated DSBs lead to spore death in the absence of Rad51 activity or Mre11.

The percentage of total dyads (n) from spo11 spo13 diploids homozygous for various mutant alleles carrying two viable spores (2-sv), one viable spore (1-sv) and 0 viable spores (0-sv) is shown. The percentage of the total number of spores (nx2) that are viable is given for each strain in the final column (% Spore viability). Asterisks indicate a significant difference between spore viabilities for indicated strains, as determined by a Fisher’s Exact test ($P \leq 0.01$). Note that LY481 and LY500 were created by replacing lys2::PSPO13-HO in LY459, and LY393, respectively, with LYS2 DNA sequence.

HO-mediated DSB repair in meiotic cells is influenced by Mre11 and Xrs2

The Mre11 nuclease, Rad50 ATPase, and FHA-containing Xrs2 protein can form a complex (“MRX”) that functions in DSB repair in mitotic cells, and has been implicated in both DSB formation and repair during meiosis (BORDE 2007; GOBBINI et al. 2016). We investigated the role of this complex in the repair of HO-mediated, meiotic DSBs in spo11 mutants by examining interhomolog recombination in spo11 spo13 mre11 and spo11 spo13 xrs2 meiotic cells that carry PSPO13-HO and the HO cs5. We found that, even in the absence of HO expression, the mre11 and xrs2 mutations alone confer a substantial decrease in spore viability to spo11 spo13 strains (50% and 65%, respectively, versus 89% for the spo11 spo13 control; Table 3). This
data indicates that Mre11 and Xrs2 activities are critical for maintaining genomic integrity during a spo11 spo13 meiotic cell cycle even in the absence of meiotic DSBs, and is consistent with a prior finding that diminished Mre11 activity reduces the spore viability of a spo13 strain (AjiMura et al. 1993). In strains carrying \( P_{\text{SPO13}}^-\)HO, we observed that the mre11 mutation confers a further reduction in spore viability (from 50% in the absence of \( P_{\text{SPO13}}^-\)HO to 21% in the presence of \( P_{\text{SPO13}}^-\)HO; Table 3), indicating that Mre11 is also important for the repair of HO-mediated meiotic DSBs. Zero out of thirteen two-spore viable dyads and only 3 out of 60 (5%) one-spore-viable dyads produced by the spo11 spo13 mre11 mutant exhibited a Leu+Thr- marker configuration, but this dataset is too small to conclude that HO-mediated interhomolog and/or crossover repair is dependent on Mre11 in spo11 spo13 meiotic cells. The strikingly low spore viability might lead one to conclude that HO-mediated meiotic DSBs uniformly fail to repair in spo11 spo13 mre11 meiocytes expressing \( P_{\text{SPO13}}^-\)HO. However, 46% of two spore viable dyads and 45% of one spore viable dyads from spo11 spo13 mre11 mutants were found to exhibit at least one mating capable spore (Table S1). While diploid spores homozygous for \( \text{MAT} \) can arise from a spo11 spo13 meiosis either as a consequence of meiotic gene conversion at \( \text{MAT} \) or chromosome loss, the near absence of such \( \text{MAT} \) homozygotes among two-spore-viable and one-spore-viable dyads from spo11 spo13 rad51 strains suggests that at least some HO-mediated DSBs in spo11 spo13 mre11 meiotic cells are repaired via interhomolog recombination. Thus, we conclude that Mre11 is not uniformly required
for the interhomolog repair of HO-mediated DSBs during meiosis, but that a subset of HO-mediated meiotic DSBs cause inviable meiotic products when Mre11 is absent.

Similar to Mre11, Xrs2 is not required for the interhomolog repair of HO-mediated meiotic DSBs, as is evident by the robust HO-mediated gene conversion previously observed at MAT in SPO11 spo13 xrs2 strains (Malkova et al. 1996b). Indeed, similar to spo11 spo13 control strains that express P<sub>SPO13</sub>-HO (Table 1), we found that sporulated P<sub>SPO13</sub>-HO spo11 spo13 xrs2 strains give rise to a large fraction (31.5%; Table S1A) of two spore viable dyads in which one or both spores is mating-capable, indicative of robust meiotic gene conversion at the MAT locus. In contrast to spo11 spo13 mre11 strains, however, meiotic expression of HO in spo11 spo13 xrs2 strains is associated with only a slight reduction in spore viability (from 65% in the absence of HO, to 55% in the presence of HO; Table 3), indicating that Xrs2 is less critical than Mre11 for the repair of that putative subset of HO-mediated DSBs that, left unrepaired, cause the low spore viability observed for spo11 spo13 mre11 strains.

Interestingly, the level of HO-mediated, apparent meiotic crossovers at HO cs5 is also significantly reduced when Xrs2 is absent, from 20.8% in the spo11 spo13 control to 5.5% in spo11 spo13 xrs2 meiotic cells (P = 0.0001; Figure 3, Table 2). Thus, while Xrs2 (and Mre11) are not essential per se for the interhomolog repair of HO-mediated meiotic DSBs, the strong diminishment in apparent meiotic crossover events in spo11 spo13 xrs2 strains may reflect a capacity of Xrs2 to drive HO-initiated interhomolog repair intermediates toward a crossover outcome.
Does HO-mediated, meiotic DSB repair in the absence of Spo11 rely on canonical meiotic recombination proteins that act downstream of strand exchange?

We asked whether the Spo11-independent repair of HO endonuclease-mediated DSBs utilize meiosis-specific recombination proteins that act downstream of strand exchange. Removal of the SC associated crossover-promoting factors Zip1, Zip2, Zip4, Mer3, Zip3, Msh4-Msh5 or Mlh3 during otherwise wild-type meiosis leads to a ~50-70% reduction in crossing over in otherwise wild-type meiotic cells (SYM et al. 1993; ROSS-MACDONALD AND ROEDER 1994; HOLLINGSWORTH et al. 1995; HUNTER AND BORTS 1997; AGARWAL AND ROEDER 2000; NOVAK et al. 2001; BORNER et al. 2004; TSUBOUCHI AND ROEDER 2006; VOELKEL-MEIMAN et al. 2016). Of these factors, Zip1, Zip2 and Zip4 are known to be absolutely essential for SC assembly, while at least some assembled SC has been observed in mutants missing Zip3 or Msh4-5 complexes (AGARWAL AND ROEDER 2000; NOVAK et al. 2001). We found that removal of Zip1 does not reduce the level of HO endonuclease-mediated interhomolog crossovers observed at HO cs5 in spo11 spo13 meiotic cells (21% in zip1 versus 20.8% in the control), and removal of Zip2 conferred only a mild (~16%; P = 0.13) reduction in HO endonuclease-mediated interhomolog crossovers observed at HO cs5 (Figure 4, Table 4). However, removal of Zip3, Msh4, Mlh3, or Mer3 resulted in a significant reduction of HO endonuclease-mediated interhomolog crossovers in spo11 spo13 meioses, by approximately 30%-43% (P < 0.0001 for zip3, msh4, mlh3; P=0.0095 for mer3; Figure 4, Table 4), suggesting that these meiosis-specific factors do influence the repair of a subset of HO-mediated recombination
events. Taken together, these results suggest that HO meiotic recombination intermediates may be accessible to a select subset of SC-associated recombination factors but not those that are absolutely critical for SC assembly.

We note that the observed reduction in crossing over in zip3, msh4, mlh3 and mer3 mutants could reflect a selective diminishment of non-reciprocal events that involve extremely long gene conversion tract lengths, instead of a diminishment in reciprocal crossovers. Under this scenario, the effect of Zip3, Msh4, Mlh3 and Mer3 meiotic crossover-associated proteins on HO-initiated meiotic recombination intermediates would be to promote and/or stabilize longer conversion tracts. Consistent with this idea, strains missing Zip3 exhibited a 43% reduction in apparent interhomolog crossovers at HO cs5, which is positioned approximately 580 kb from the THR1 insertion on IV, but only a 22% reduction in crossovers at HO cs7, which is positioned approximately 359 kb from THR1. Given the distance of cs5 from the THR1 insertion on IV, non-reciprocal THR1 gene conversions due to DSBs at cs5 (versus DSBs at cs7) would be expected to be more sensitive to a mild shortening in tract length.

Factors that process joint molecule recombination intermediates independent of an SC-associated pathway include Mms4, the Slx1/Slx4 complex, and Yen1 (DE LOS SANTOS et al. 2003; FRICKE AND BRILL 2003; IP et al. 2008; JESSOP AND LICHTEN 2008). To assess if HO DSBs on chromosome IV rely on any of these so-called “class II” recombination factors, we examined HO endonuclease-mediated crossing over at HO cs5 in spo11 strains deficient in meiotic Mms4 and Yen1 (P_{CLB2-MMS4} and P_{CLB2-}
MMS4 yen1 double mutants). We found that HO-associated crossovers were not significantly diminished in the absence of these proteins (Figure 4; Table 4). Finally, we created a strain in which both SC-associated and SC-independent classes of recombination factors are missing. Together, Mlh3, Yen1, Mms4 and S1x1/S1x4 proteins have been implicated in ensuring the resolution of most Spo11-mediated crossovers during wild-type meiosis (Zakharyevich et al. 2012). Simultaneous removal of these four factors did not, however, significantly reduce HO endonuclease-mediated crossing over at cs5, nor cs7 in our spo11 spo13 meiotic cells (Figure 4, Table 4).
**Figure 4.** *HO*-mediated recombination in the absence of Spo11 does not rely heavily on canonical meiotic recombination factors. Bar graph shows the frequency of apparent crossovers in *spo11 spo13* strains carrying $P_{SPO17\text{-}HO}$, an *HO* cs on chromosome IV, and mutant alleles of various recombination factors. Calculations were performed as described in Figure 3 (n > 250; precise values and strain names are reported in Table 4). Left part of graph shows data for strains carrying *HO* cs5, and the right 3 strains carry *HO* cs7. Significant deviations, relative to the wild-type value, were determined using Fisher’s Exact test (*P*-value ≤ 0.05, **P*-value ≤ 0.01, ***P*-value ≤ 0.001).
Table 4. HO-mediated meiotic recombination in the absence of canonical meiotic recombination factors.

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<th>% apparent CO</th>
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<td>Leu-</td>
<td>Thr+</td>
<td>Thr-</td>
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Table 4. *HO*-mediated recombination in the absence of canonical meiotic recombination factors. The number of dyads with particular spore phenotypes was used to calculate apparent meiotic interhomolog crossover recombination in *spo11 spo13* strains carrying mutant alleles of various meiotic genes and *HO*-mediated DSB formation at a chromosome IV *HO cs* location. Genetic markers and calculation of apparent crossover frequency among the total number of two-spore-viable dyads (n) are as described in Table 2 and Figure 3. A Fisher’s Exact test was used to determine whether values differ significantly from the control for the *HO cs5* or the *HO cs7* strains.

The Sgs1 helicase has both pro- and anti-crossover activity and its absence allows multi-chromatid exchange events during meiosis (ROCKMILL et al. 2003; JESSOP et al. 2006; DE MUYT et al. 2012). Expression of *sgs1-C795*, a meiotic null allele (MULLEN et al. 2000; ROCKMILL et al. 2003), results in a mild increase in *HO* endonuclease-mediated crossover recombination at *HO cs5*, from 20.8% to 25.9% $P = 0.008$; Figure 4, Table 4). Thus, Sgs1 may have a mild negative influence on *HO*-mediated interhomolog crossover recombination in *spo11* mutant meiotic cells.

These data, however, need to be considered in light of the unexpected result that *mlh3 msh4* double mutants and our *slx1 mlh3 yen1 P CLR2-MMS4* quadruple mutant exhibit no significant decrease in *HO* endonuclease-mediated crossing over, despite the reduction in crossing over observed in *msh4* and *mlh3* single mutants. While this
observation could reflect *bona fide* genetic interactions between crossover promoting factors, it may instead indicate that non-specific strain background effects may modulate the recombination phenotype.

Altogether, our data suggest that in Spo11’s absence, HO endonuclease-mediated meiotic interhomolog recombination intermediates might engage with a subset but do not rely on the full cohort of SC associated (class I) crossover factors. Furthermore, HO endonuclease-mediated meiotic DSBs are capable of undergoing interhomolog repair in *spo11* meiosis even when normally critical class I and class II resolution factors are simultaneously absent.

*An HO-mediated DSB located proximal or distal to the centromere is not sufficient to promote stable pairwise associations between homologous centromeres during meiosis*

We targeted HO-mediated recombination to budding yeast’s longest chromosome in order ask whether the position of an interhomolog recombination event is a determining factor in the success of processes that reinforce homologous pairing. One such process is the recombination-dependent transition from homology-independent centromere “coupling” to homologous centromere pairing (Tsubouchi and Roeder 2005; Stewart and Dawson 2008). We used lacO-associated GFP-LacI and tetO-associated TetR-mCherry to assess whether HO-mediated recombination proximal or distal to centromere IV can promote homologous centromere pairing in *spo11* mutant meiosis.
lacO DNA sequences were integrated 705 nucleotides to the right of centromere IV, and tetO DNA sequences were introduced at coordinate 1,242 kb on chromosome IV (See Materials and Methods). The lacO and tetO DNA sequences are visualized on surface spread nuclei by lacO- or tetO-binding proteins GFP-LacI or TetR-mCherry, expressed in trans within the same strains. These cytological tools were built into diploid strains carrying HO cs2, cs4, or cs5 and into a strain carrying eight chromosome IV HO cs loci (Figure 5A). Diploid strains were sporulated for ~15 hours before preparing surface-spread nuclei for immunofluorescence, and an antibody that targets the meiosis-specific chromosomal protein Hop1 was utilized to verify that nuclei had progressed into meiosis.

We found that among meiotic nuclei from control spo11 strains lacking HO endonuclease, 25% exhibited homologous pairing between the centromere regions of chromosome IV (Figure 5B, C). This frequency of association is higher than expected if pairwise centromere associations between 32 individual chromosomes are random and completely independent of homology in spo11 mutants, thus we also examined pairing between the centromere regions of chromosomes III and V, and between centromeres III, V, and IV, in spo11 mutant meiotic cells. Consistent with previous studies that found a preferential association between chromosomes of similar size (LEFRANÇOIS et al. 2016), centromeres III and V, which exhibit a smaller differential in size, were paired in 22% of surface spread nuclei (n >100) compared to the 15% pairing frequency (n > 100) of centromeres III and IV, which have a greater size differential (Figure 5D).
We determined that an HO-mediated DSB does not stimulate homologous centromere IV pairing in spo11 mutant meiotic cells. spo11 mutants carrying a single HO cs in conjunction with HO endonuclease exhibited a similar frequency of paired centromeres IV as spo11 cells devoid of HO endonuclease. Homologous centromeres IV were paired in 22%, 29% and 24% of meiotic cells at 15 hours of sporulation from strains carrying cs2, cs4, or cs5, respectively (n= 300; Figure 5B, C). Furthermore, homologous centromeres IV were paired in 21% of meiotic cells from strains carrying 8 chromosome IV HO cs loci (cs2, cs4, cs5, cs6, cs7, cs8, cs9 and cs10; n= 300; Figure 5B, C).

Similarly, pairing at a chromosome IV arm location, detected in about 8% of spo11 control cells devoid of HO endonuclease, was detected at approximately the same frequency in spo11 cells carrying single or multiple HO cs loci and meiotic HO endonuclease: Chromosome IV arm pairing was observed in 9%, 15%, and 11% of meiotic cells from HO endonuclease-expressing strains carrying cs2, cs4 and cs5, respectively, and in 10% of the meiotic cells from a strain carrying 8 chromosome IV HO cs loci (Figure 5B, C).

We conclude that a single HO endonuclease-mediated recombination event, while capable of promoting interhomolog crossovers in ~20% of meioses, is not sufficient to promote stable pairwise associations between homologous centromeres, even when positioned 250 nucleotides from the centromere.
A

ChrIV

cs2         cs7         cs4 cs8

cs9 cs10      cs5  cs6

B

SPO11

spo11-Y135Fcs5 HO

DAPI/ GFP/ mCherry
Figure 5. A centromere-proximal or distal HO DSB is not sufficient to pair homologous chromosomes. (A) Cartoons show the locations of lacO DNA sequences 705bp to the right of centromere IV (green) and tetO DNA sequences at 1,242 kb on chromosome IV in strains used for cytology experiments. GFP-LacI and TetR-
mCherry, expressed in trans, bind lacO and tetO respectively. Strains homozygous for a single HO cs (top) or carrying eight HO cs loci (bottom) were utilized in cytological experiments. (B) Images show surface spread meiotic nuclei from wild-type strains (LY42; left column), and from spo11-Y135F strains expressing meiosis-specific HO endonuclease and carrying HO cs5 (LY887; center and right columns) at 15 hrs. of sporulation. The presence of Hop1 (not shown) was used to select meiotic nuclei for pairing analysis. The distance between foci corresponding to LacI-GFP bound to lacO sequences near CEN IV (green), and TetR-mCherry bound to tetO sequences on the arm of chromosome IV were considered paired if foci center to foci center < 0.5 µm apart. Bar, 1µm. (C) Bar graphs display the average frequency of CEN IV pairing or chromosome IV arm pairing at 15 hr of sporulation in control (LY176) and chromosome IV HO cs – carrying strains (left to right: LY176, LY173, LY174, LY175, LY331 and LY887). 100 meiotic nuclei per genotype were analyzed in triplicate (n = 300 total per genotype). Bars depict standard error of the mean. (D) Homologous and non-homologous centromere pairing between centromeres indicated on the x axis was assessed in spo11 mutant strains (left to right: LY303, LY176, LY356, LY357 and LY358) at 15 hr of sporulation (n > 100).
An HO-mediated meiotic DSB fails to promote SC assembly in the context of a null or catalytically inactive spo11 allele even when meiotic axis proteins are overexpressed

Initial steps in meiotic recombination are required for the assembly of SC in budding yeast, which, at early time points, occurs predominantly from centromere regions (Tsubouchi et al. 2008). An earlier study reported that HO endonuclease-mediated recombination between homologous chromosomes III was unaccompanied by SC assembly in spo11 meiotic cells (Malkova et al. 2000), but the small size of chromosome III leaves open the possibility that a partial SC assembly event was missed in this prior experiment. We thus asked whether SC structure(s) assemble on the largest yeast chromosome (IV) in response to an HO endonuclease-mediated DSB, and furthermore whether the distance from the centromere of the HO-mediated recombination event matters.

Surface-spread meiotic nuclei from HO-expressing and control spo11 strains at 15 hours of sporulation were labeled with antibodies against the SC transverse filament protein Zip1 to visualize SC, and with antibodies against the meiosis-specific chromosomal protein Hop1 to verify that nuclei had progressed into meiosis (Figure 6A). In spo11 meiotic nuclei, Zip1 localizes diffusely on surface-spread chromosomes, with several brighter foci likely corresponding to centromeres (Tsubouchi and Roeder 2005). A large fraction of spo11 control nuclei also display an aggregate of Zip1 called a polycomplex. Zip1’s distribution pattern on meiotic chromatin from spo11 strains expressing HO endonuclease and carrying 8 HO cs loci
(including cs2, positioned immediately adjacent to centromere IV) appeared indistinguishable from spo11 control meiotic nuclei (Figure 6A, B). The absence of linear Zip1 structures on meiotic chromatin indicates that HO endonuclease-mediated recombination is incapable of interfacing with and/or successfully activating the molecular pathway(s) that facilitate SC assembly in spo11 meiotic nuclei.

Spo11 requires 9 additional accessory proteins to initiate recombination during meiosis (LAM AND KEENEY 2014). A catalytically inactive mutant version of Spo11, Spo11-Y135F, is capable of localizing to chromatin during meiosis (PRIELER et al. 2005). With the hope of targeting Spo11 partners to an HO-mediated DSB, we introduced a gene fusion between Spo11-Y135F and the N terminus of HO endonuclease into our HO cs-containing spo11 cells. However, the chimeric protein did not exhibit HO endonuclease activity, as indicated by the absence of meiotic recombination at the MAT locus in strains carrying this fusion. We next analyzed strains sustaining HO-mediated, meiotic DSBs with Spo11-Y135F generated in trans. We found no evidence of homologous centromere pairing nor SC assembly in meiotic nuclei carrying meiotic HO-mediated DSBs at cs5, nor in strains carrying eight cs loci and homozygous for spo11-Y135F (Figures 5, 6A), indicating that the Spo11-Y135F protein cannot confer to HO-mediated DSBs the capacity to promote meiotic chromosome pairing processes.

SC assembly requires the meiosis-specific cohesin, Rec8 (KLEIN et al. 1999). It was previously reported that artificial DSBs, applied to spo11 mutant meiotic cells that overexpress Rec8, lead to Zip1 linear assemblies that were interpreted to be
tripartite SC (BRAR et al. 2009). This result led us to wonder whether the overexpression of a meiosis-specific cohesion or another chromosome axis protein would enable HO endonuclease-mediated DSBs to promote SC assembly. To address this question, we introduced a 2-micron plasmid carrying REC8-MYC driven by the HOP1 promoter in order to overexpress REC8-MYC in HO endonuclease-expressing spo11 meiotic cells carrying no HO cs, a single HO cs, or 8 HO cs loci. A western blot demonstrated that Rec8-MYC is approximately five times more abundant in our REC8-MYC overexpression strains relative to the level produced by two chromosomal copies of REC8-MYC (Figure 6C). However, Zip1’s distribution on surface-spread chromosomes from these meiotic cells appeared dotty-diffuse regardless of whether the strain expressed higher than wild-type levels of Rec8-MYC (Figure 6A, B). Thus, the level of Rec8-MYC overexpression achieved in this experiment fails to bestow HO endonuclease-mediated DSBs with the capacity to promote SC assembly.
Figure 6. Synaptonemal complex does not assemble in response to an HO-mediated meiotic DSB. (A) Representative surface spread meiotic nuclei from SPO11 (top row; YAM424), or spo11 null strains carrying eight HO cs loci (cs2, cs4, cs5, cs6, cs7, cs8, cs9, cs10; LY371) at 15 hours of sporulation (upper panel, second and third row). Lower panel displays a representative nucleus from the spo11-Y135F strain carrying HO cs5 and overexpressing Rec8 (LY890; lower panel, top row) and two representative nuclei from the spo11 null strain carrying eight HO cs and overexpressing Rec8 (LY892, lower panel, bottom two rows) at 24 hours of sporulation in ndt80 strains. Zip1 (green) binds diffusely to and also assembles some bright foci on DAPI-stained meiotic chromatin (blue) from these spo11 strains, regardless of HO-induced meiotic DSBs; polycomplex aggregates of Zip1 (white arrowheads) are often observed. The presence of the meiosis-specific Hop1 protein or Rec8-MYC (red) is displayed in the third column. Bar, 1µm. (B) The proportions of nuclei (n=50) with different Zip1 and Rec8-MYC distribution phenotypes at 24 hours of sporulation are plotted for a control strain missing P_{SPO13-HO} (LY893), the spo11 null strain carrying eight HO cs loci (LY371), a Rec8-overexpression control spo11-Y135F strain with no chromosome IV HO cs (LY891), the spo11-Y135F strain carrying HO cs5 and overexpressing Rec8 (LY890) and the spo11 null strain carrying eight HO cs loci and overexpressing Rec8 (LY892). Immunoblot in (C) shows Rec8-MYC levels in meiotic cells at 24 hours of sporulation from a control SPO11 ndt80 strain in which REC8 is untagged and which happens to also be homozygous for P_{GAL-HOP1} (LY769), a control spo11-Y135F ndt80 strain homozygous for REC8-MYC.
(LY893), followed by REC8-overexpressing LY890, LY891 and LY892 strains. The same cultures were used to prepare meiotic surface spread nuclei analyzed at the 24 hour time point. Molecular weight indicators are given (kDa) to the left. (D) Graph plots Rec8-MYC protein levels from a strain carrying two endogenous copies of REC8-MYC and strains carrying 2µ-REC8-MYC. Tubulin levels were used to normalize Rec8-MYC levels across samples. The average of 3 replicates is plotted; bars give standard error of the mean.

We also asked whether the overexpression of the meiosis-specific chromosome axis proteins Hop1 and Red1 results in HO endonuclease-mediated SC assembly in spo11 meiotic nuclei. A western blot confirmed that Hop1 is overexpressed at least nine-fold relative to control cells in spo11 cells carrying a HOP1-RED1 2-micron plasmid (a kind gift of N. Hollingsworth; (HOLLINGSWORTH AND PONTE 1997) (Figure S1). Immunofluorescence on surface-spread nuclei indicated that, with or without HO endonuclease-mediated DSBs, spo11 cells carrying the HOP1-RED1 2-micron plasmid exhibited a dotty-diffuse Zip1 distribution on chromatin and/or a Zip1 polycomplex, with no linear SC-like structures (Figure S1).
Figure S1. Overexpression of chromosome axis proteins does not facilitate SC assembly in response to an HO-mediated meiotic DSB.

The Western blot in (A) indicates Hop1 protein levels in a $P_{Gal}^{+} HOP1 ndt80$ strain (LY769; lane 1), a $HOP1 spo11^{	ext{Y135F}}$ strain homozygous for $HO$ $cs5$ (LY841; lane 2), the LY841 strain carrying a $2\mu$-$HOP1$-$RED1$ plasmid (LY864; lane 3 and 4), a strain without a chromosome IV $HO$ $cs$ (LY846; lane 5), and this LY846 strain carrying a $2\mu$-$HOP1$-$RED1$ plasmid (LY865; lanes 6, 7). Numbers at the left indicate...
molecular weight (kDa). The average of 3 Western experiments to detect Hop1 levels are plotted on the graph in (B), with strains listed in the same order as in (A). Tubulin levels were used to normalize Hop1 levels across samples; bars indicate standard error of the mean. (C) Representative surface spread meiotic nuclei from a SPO11 ndt80 strain (YAM424; top row), and spo11-Y135F ndt80 strains carrying cs5 with and without a 2µ-HOP1-RED1 plasmid (LY841 and LY864) at 24 hours of sporulation. The ndt80 null allele ensures the strains will not progress beyond the pachytene stage of meiosis. Percentage of nuclei exhibiting various indicated Zip1 and Hop1 phenotypes for control and 2µ-HOP1-RED1-carrying strains (left to right: LY846, LY841, LY865, LY864) are plotted in (D); Each bar represents a total of 50 nuclei.

Artificial DSBs supplied en masse by exposure to phleomycin influence SC protein distribution but fail to promote robust SC assembly in spo11 meiotic nuclei

To explore whether the failure of our meiotic HO-mediated DSBs to support centromere pairing or SC assembly is due to the fact that these processes need a minimal threshold level of DSBs in order to progress, we examined pairing and SC protein distribution in meiotic nuclei from spo11 mutants that had sustained multiple DSBs, due to phleomycin exposure. A significant increase in Rad51 foci was observed in spo11 meiotic nuclei exposed to an increasing series of phleomycin doses ($P \leq 0.001$; Figure S2A), reflecting the phleomycin-dependent presence and repair of
DSBs. We observed a modest but statistically insignificant increase in homologous pairing at centromeres and arm regions of chromosome IV in strains that experienced the highest dose of phleomycin ($P = 0.378$; Figure S2B). The vast majority of meiotic nuclei exposed to high doses of phleomycin displayed a “dotty-diffuse” distribution of SC structural proteins Zip1 and Ecm11 (similar to that seen in the control). However, nuclei from cells that had been exposed to high doses of phleomycin rarely displayed the Zip1 polycomplex structure that was frequently observed in control nuclei, and a small fraction (~10%) of these nuclei displayed multiple short linear assemblies of coincident Zip1 and Ecm11 (Figure S2C, D). Taken together, these results indicate that DSBs supplied by phleomycin exposure do not trigger robust homologous synapsis mechanisms but may infrequently facilitate homologous associations between chromosomes, counteract the tendency of Zip1 to assemble a polycomplex structure, and support some minimal assembly of SC structures.
A

Phleomycin

100 µg/ml  120 µg/ml

DAPI
Rad51

B

% Nuclei with Paired CEN IV

0  30  100  120

Phleomycin (µg/ml)

% Nuclei with Paired Arm IV

0  30  100  120

Phleomycin (µg/ml)
**Figure S2.** DSBs generated via phleomycin exposure alter SC protein distribution but do not promote robust SC assembly in spo11 meiotic nuclei.
(A) Image displays representative surface-spread meiotic nuclei from a \( spo11 \ ndt80 \) strain (LY471) exposed to 100 \( \mu g/mL \) (left) or 120 \( \mu g/mL \) phleomycin; DNA is labeled by DAPI (blue) and Rad51 targeted by an antibody (red). Graph plots the number of Rad51 foci measured per nucleus in LY471 meiotic cells sporulated for 24 hours with different doses of Phleomycin (n= 20). Graphs in (B) plot the frequency of \( CEN \ IV \) pairing or chromosome IV arm pairing in a \( spo11 \) strain (LY176) with increasing concentration of Phleomycin (n= 50). Cells were assessed at 15 hours of sporulation. \( (P > 0.1 \) for all strains relative to the control) (C) Representative surface spread meiotic nuclei from a \( spo11 \ ECM11\-MYC \ ndt80 \) strain (LY471), exposed to 0, 30, 100 or 120\( \mu g/ml \) phleomycin at t = 0 of meiosis. Top two rows give representative images of the Zip1 and Ecm11 ‘dotty-diffuse’ phenotype, while the bottom two rows give representative images of the less frequent ‘dotty-linear’ phenotype where Zip1 and Ecm11 co-assemble short linear structures. The proportion of nuclei with different Zip1 and Ecm11 distribution phenotypes are plotted in (D); n= 20-50 for each concentration of phleomycin (indicated on x axis).

Discussion

The current study was motivated by an interest in the molecular mechanism that connects meiotic recombination to processes that generate and reinforce homolog pairing in budding yeast. The HO endonuclease was found to promote interhomolog crossover recombination in \( spo11 \) yeast meiosis at higher frequency than in mitotic
cells (Malkova et al. 1996b; Malkova et al. 2000), consistent with the idea that Spo11-independent features of the meiotic nucleus might allow DSBs to engage with chromosome pairing and/or recombination pathways that promote favorable outcomes for meiosis; an example of such a mechanism might be the interhomolog bias-promoting activity of meiosis-specific chromosome axis proteins like Red1 and Rec8, and/or meiosis-specific recombinase machinery (Hong et al. 2013). However, the presence of Spo11 has also been found to have a positive effect on the capacity of artificially-supplied meiotic DSBs to repair as interhomolog crossovers (Malkova et al. 1996b; Malkova et al. 2000; Neale et al. 2002; Medhi et al. 2016). The extent to which Spo11 activity is uniquely engaged with and capable of driving meiotic chromosome pairing processes remains obscure.

We began our investigation into how meiotic DSB repair is coordinated with chromosome pairing in meiotic cells bearing several questions in mind: Does the position of a DSB relative to the centromere affect early homologous pairing? Does a threshold level of DSBs need to be met in order to ensure homolog alignment pairing or SC assembly? Are Spo11 DSBs specialized in their capacity to promote homolog pairing events? In this study, we asked whether HO-mediated DSBs, many positioned at chromosome IV locations that are frequently cut by Spo11, are capable of facilitating any of the meiotic chromosome pairing processes that normally accompany programmed DSBs. We furthermore asked the same question of DSBs supplied en masse by exposure to phleomycin.
HO-mediated meiotic DSBs on chromosome IV may lack robust repair template bias

Successful homolog segregation in meiosis relies on recombination-based associations between non-sister chromatids; it follows that meiotic recombination is strongly biased toward utilizing the homolog as repair template (SCHWACHA AND KLECKNER 1994; SCHWACHA AND KLECKNER 1997; HUNTER AND KLECKNER 2001; HONG et al. 2013). By contrast, homologous recombination mechanisms in mitotic cells almost exclusively use the sister chromatid (KADYK AND HARTWELL 1992). One question that our experiments highlight is to what extent is a Spo11-associated DSB specialized in its capacity to preferentially engage the homolog?

One can explore this question by asking whether HO-mediated, meiotic DSBs exhibit a meiotic-like or mitotic-like preference in repair template choice. An earlier study demonstrated that HO DSBs frequently access the non-sister chromatid for repair (MALKOVA et al. 2000), and our observation of interhomolog repair of meiotic HO DSBs on chromosome IV is consistent with the idea that HO DSB repair in meiotic cells has a more “meiotic” versus “mitotic” repair template bias.

However, considering the frequency of HO-mediated DSB formation in our system, we suggest that HO-mediated DSB repair in spo11 meiotic cells exhibit a modest to strong intersister repair template bias. Southern blot analysis of total HO-mediated DSBs in our system suggests that 20-60% of total DNA is cleaved in strains homozygous for a given HO cut site, suggesting that, on average, one or two chromatids are cut by HO in 100% of meioses. Presuming a random resolution
process that does not favor a crossover nor noncrossover outcome, the detected ~20% HO-mediated interhomolog crossover events per meiosis suggests that ~40% of meioses involved an HO meiotic DSB that repaired off of the homolog (20% resulting in a crossover, and 20% resulting in a non-crossover). In fact, the aforementioned study that analyzed HO-mediated meiotic DSB repair determined that in the absence of Spo11, interhomolog repair may be slightly biased toward a non-crossover outcome: Only 27% of total HO–mediated interhomolog recombination events gave a crossover outcome in the absence of Spo11 (versus 52% in the presence of Spo11) (MALKOVA et al. 2000). Taking this into consideration, perhaps ~54% of meioses involved interhomolog repair of an HO break. Assuming ~1.5 HO-mediated DNA breaks per meiotic cell in our spo11 populations, these estimations suggest that HO-mediated meiotic DSBs utilize the sister chromatid with a higher probability than would be expected if no bias existed (no bias corresponds to an interhomolog:intersister repair ratio of 2:1).

In fact, the interhomolog to intersister repair template bias we infer for HO-mediated DSBs in spo11 meiosis is likely to be similar to the repair template bias associated with Spo11-mediated DSBs in the absence of Red1, Dmc1 or Mek1 proteins (SCHWACHA AND KLECKNER 1997; HONG et al. 2013), since HO-mediated meiotic interhomolog crossovers remain robust in red1, dmc1, and mek1 mutants. The repair of Spo11-mediated DSBs in the absence of Red1, Dmc1 or Mek1 proteins has been reported to be strongly biased toward the sister chromatid, perhaps even as strong as the intersister bias observed in mitotic cells (HONG et al. 2013).
Our results are consistent with the idea that while Spo11-independent factors may generate a context within the meiotic nucleus that allows a DNA break to engage the homolog for repair more often than is permitted in a mitotic nucleus, Spo11-associated DNA breaks are uniquely capable of engaging in repair that is strongly biased toward utilizing the homolog as repair template.

**Distinct roles for MRX complex components in the repair of a non-Spo11 meiotic DSB**

The Mre11, Rad50, and Xrs2 proteins have been implicated in assembling a complex (MRX) which facilitates the formation and repair of Spo11-mediated DSBs (Alani et al. 1990; Johzuka and Ogawa 1995; Tsubouchi and Ogawa 1998; Bressan et al. 1999). Separation-of-function mre11 and rad50 alleles indicate that these factors have evolved distinct activities that are independently critical for Spo11 DSB formation and repair (Alani et al. 1990; Tsubouchi and Ogawa 1998); on the other hand, bypass of xrs2 null meiotic phenotypes by artificially localizing Mre11 to the nucleus suggests that Xrs2 is indirectly involved in Spo11 DSB formation and repair through its singular capacity to recruit Mre11 (Oh et al. 2016).

One conclusion we draw from our analysis of mre11 and xrs2 mutants is that MRX components are differentially relied upon for the repair of at least a subset of HO-mediated DSBs during meiosis. The HO-associated spore inviability phenotype observed in spo11 spo13 mre11 is absent from spo11 spo13 xrs2 strains, suggesting that a subset of HO-mediated meiotic DSBs critically depend upon Mre11 but not
Xrs2 for their repair. In light of the idea that Xrs2’s role in MRX function during meiosis is thought to be through its capacity to recruit Mre11 to the nucleus, the successful repair of HO-mediated meiotic DSBs in spo11 spo13 xrs2 meiocytes implies the existence of an Xrs2-independent mechanism for recruiting Mre11 to the meiotic nucleus.

Second, we observed robust meiotic gene conversion at MAT in spo11 spo13 mre11 and spo11 spo13 xrs2 strains expressing \( P_{SPO13-HO} \), suggesting that, while a subset of HO-mediated meiotic DSBs critically depend upon Mre11 for their repair, neither Mre11 nor Xrs2 is essential per se for the interhomolog repair of HO-mediated meiotic DSBs. A prior study’s finding that Xrs2 and Rad50 are dispensable for HO-mediated meiotic DSB repair at MAT (Malkova et al. 1996b) both corroborates our finding and furthermore indicates that each of the MRX components are dispensable for the interhomolog repair of HO-mediated meiotic DSBs when Spo11 is absent. This is in contrast to Spo11-mediated meiotic DSBs, which rely on at least Mre11 and Rad50, and possibly Xrs2, for their repair during meiosis.

Interestingly, our data suggests that while dispensable for repair per se, Xrs2 nevertheless influences the repair outcome of meiotic HO DSBs, at least those formed at \( HO_{cs5} \). Crossover levels on chromosome IV diminished by 74% at \( HO_{cs5} \) in spo11 spo13 mutants devoid of Xrs2. The fact that HO-mediated gene conversions at the MAT locus remained unchanged in the xrs2 mutant relative to the control (XRS2) strain leads us to suggest that Xrs2 activity may engage with HO-mediated interhomolog recombination intermediates in a manner that promotes a crossover
outcome. However, we note that data from earlier studies, while not completely conclusive, indicates little effect of Rad50 or Xrs2 on the likelihood of a crossover outcome associated with HO-mediated meiotic gene conversions at the \textit{MAT} locus (if one compares HO-mediated crossover outcomes at the \textit{MAT} locus determined for \textit{rad50} mutants and inferred for \textit{xrs2} mutants (Malkova \textit{et al.} 1996b), with crossover outcomes at the \textit{LEU2} locus determined for \textit{spo11} mutants (Malkova \textit{et al.} 2000)). Taking these earlier results into consideration, diminished HO-mediated crossing over at \textit{cs5} among diploid dyads from \textit{spo11 spo13 xrs2} strains may not be due to Xrs2’s involvement in the crossover/noncrossover decision, but instead may reflect a role for Xrs2 in promoting interhomolog (over intersister) repair at certain select DSB locations in the genome, for example at \textit{cs5} but not at \textit{MAT}.

\textbf{Artificial DSBs are a poor substitute for yeast \textit{Spo11} in promoting homologous pairing or synapsis from centromeres}

We are particularly interested in the molecular criteria associated with meiotic DSBs that allows or facilitates the coordination between recombination initiation and homologous centromere pairing. Centromeres may be the first regions of meiotic chromosomes to undergo stable homologous alignment in response to recombination since SC assembly initiates earliest from centromere regions (Tsubouchi \textit{et al.} 2008), but centromere pairing is also independent of SC assembly, as it remains intact even in mutants that are missing certain building block components of the SC, such as Ecm11 and Gmc2 (Kurdzo \textit{et al.} 2017). Our data indicate that artificial DSBs in
*spo11* meioses, generated close to or far from a centromere, and either delivered as singular events by the HO endonuclease or *en masse* by the radiomimetic drug phleomycin, are unable to promote stable homologous pairing even between centromeres. Our chromosome pairing analysis points to the conclusion that *Spo11*-associated recombination events are uniquely specialized to facilitate the homologous centromere pairing process.

A second conclusion that is supported by our study as well as by a prior analysis of meiotic HO DSBs (Malkova et al. 2000) is that *Spo11*-mediated recombination has a unique capacity to promote SC assembly (synapsis) during budding yeast meiosis. Synapsis normally initiates from centromere regions as well as a subset of interhomolog recombination events along chromosome arms (Chua and Roeder 1998; Agarwal and Roeder 2000; Henderson et al. 2004; Tsubouchi et al. 2008). Although HO DSBs in *spo11* meiotic cells are capable of interhomolog recombination, we observed no evidence of even partial SC assembly on the long chromosome IV in response to one or more HO DSBs positioned at *Spo11* hotspot locations close to or far from the centromere, and only a low frequency of partial SC assembly in response to high doses of phleomycin.

We did observe a slight increase in homologous centromere pairing and a low frequency appearance of coincident Zip1 and Ecm11 linear assemblies in meiotic nuclei that had been exposed to high dose of phleomycin. We interpret these observations to mean that while *Spo11*-associated recombination has a specialized capacity to efficiently trigger timely homologous synapsis, a large number of artificial
DSBs is capable of engaging with pathways that may promote weak pairing events and influence SC protein dynamics.

We conclude that Spo11-associated DSB machinery has a specialized capacity to promote homologous centromere pairing and synapsis from centromeres. Although we point out that centromere pairing is an independent process from SC assembly, it remains possible that SC assembly normally relies on centromere pairing. Thus the possibility exists that the failure to assemble SC in response to HO meiotic DSBs is due to a failure of those DNA breaks to facilitate homologous centromere pairing interactions.

In an earlier study we demonstrated that, so long as the Zip1 regulators Fpr3 and Zip3 are also absent, robust SC assembly can occur from centromeres even in the absence of the recombination protein, Zip2, which is normally required both for stabilizing a large number of interhomolog recombination intermediates and for SC assembly from any site on the chromosome (MacQueen and Roeder 2009). This result argues that SC initiations at centromeres do not require the nucleating presence of a recombination intermediate, and instead DSBs somehow are able to activate SC assembly at centromeres through a trans acting signal. We conclude, based on experiments reported here, that such a putative trans signal is not robustly supplied by HO-mediated or phleomycin-associated meiotic DSBs. However, it remains unknown whether Spo11-associated events themselves have varying capacity (based on their position on the chromosome and/or proximity to the centromere) to activate mechanisms that promote homologous centromere pairing or centromere-associated
SC assembly. It also remains unclear whether a threshold level of Spo11 DSBs is required for initial SC assembly to occur from any site on the chromosomes during normal meiosis.

**What is the molecular basis for the differential ability of programmed meiotic DSBs to promote homolog pairing processes?**

Although the mechanism that drives a transition from non-homologous to homologous centromere pairing during budding yeast meiosis is not well understood, this process may be critically regulated by the phosphorylation of Zip1 by the Mec1 kinase. Prior to meiotic recombination initiation in budding yeast meiosis, Zip1 mediates the pairwise association of centromeres without regard to homology. Falk et al. (2010) found that Zip1’s capacity to mediate centromere associations is abolished by phosphorylation of serine residue 75, and proposed that Mec1-mediated phosphorylation of Zip1 might serve to promote the dissolution of centromere coupling events in coordination with recombination initiation, facilitating the subsequent formation of homologous centromere interactions. The failure of meiotic DSBs initiated by HO or phleomycin to robustly promote homologous centromere pairing may not be due to the absence of Mec1 kinase activity *per se*, however, as both artificial and Spo11 DSBs during meiosis activate Mec1 and Tel1 kinases (Lydall et al. 1996; Usui et al. 2001; Cartagena-Lirola et al. 2006) and phosphorylate the meiosis-specific kinase, Mek1 (Wan et al. 2004; Carballo et al. 2008), although artificially-supplied DNA breaks also lead to phosphorylation Rad53
effector kinase in meiotic cells while Spo11-mediated DSBs do not (LYDALL et al. 1996; CARTAGENA-LIROLA et al. 2008). Instead, it may be that the presence of activated Mec1 in the specialized chromosomal context of a Spo11-mediated DSB is uniquely connected to a network of factors, perhaps associated with the chromosome axis, that communicate functional information to centromeres. Alternatively, or in addition, perhaps Spo11-mediated events are specialized in activating a second regulatory factor required in addition to activated Mec1 for the dissolution of non-homologous centromere associations, or for the re-establishment of centromere pairing between homologs.

A number of observations indicate that SC assembly normally occurs downstream of a discrete intermediate step in the homologous recombination process (ZICKLER AND KLECKNER 2015). How might Spo11-associated DSB machinery be uniquely capable of facilitating synapsis? Interestingly, we found that a subset of the so-called “class I” proteins which are dually required for the stabilization of Spo11-initiated double Holliday junction intermediates and for promoting SC assembly (BORNER et al. 2004) influence a substantial fraction (~40%) of HO-mediated meiotic interhomolog repair events even when Spo11 is absent. Malkova et al. (2000) showed that approximately half of HO-mediated meiotic interhomolog repair events in SPO11+ cells rely on the MutSγ protein Msh4 and that HO-mediated meiotic interhomolog events are significantly more likely to become crossovers when Spo11 is present. In light of this, our data suggest that additional factors, apart from access
of the HO-recombination intermediate to MutS\(\gamma\) per se, must be involved in Spo11’s capacity to increase the probability of a crossover outcome in trans.

Our findings further allow for the possibility that one of the factors determining whether or not a meiotic DSB engages with crossover-promoting mechanisms is proper access to the other class I recombination proteins, including Zip1, Zip2, Zip4 and Spo16. Importantly, while Zip1, Zip2, Zip3 and Msh4-Msh5 act in the same (class I) crossover recombination pathway during wild-type meiosis, Zip1 and Zip2 play a more critical role in SC assembly than Zip3 and Msh4, and (unlike Zip3 and Msh4) neither Zip1 nor Zip2 were found to have a significant influence on HO-mediated interhomolog repair events when Spo11 is absent. Thus, while perhaps some meiosis-specific recombination factors (such as Zip3, Mer3, Msh4 and Mlh3) can access and appear to influence HO-mediated recombination intermediates, these factors are not likely acting on HO recombination intermediates in their normal “class I” manner since other factors in the same recombination pathway, such as Zip1 and Zip2, do not engage with HO-mediated recombination intermediates (at least when Spo11 is absent). We suggest that the success of a meiotic DSB to promote meiotic-like chromosomal pairing pathways depends upon its early engagement with recombination complexes that properly coordinate Zip3 and Msh4 with the other SC-associated crossover proteins such as Zip1, Zip2, Zip4 and Spo16. The engagement of a meiotic DSB with this sort of “complete” class I recombination pathway could dually ensure that 1) the ensuing recombination intermediate is channeled into a stable crossover-associated configuration, and 2) that global alignment of
homologous chromosomes, via the promotion of processes like centromere pairing and assembly of tripartite SC, is achieved.

Perhaps HO-mediated recombination intermediates and Spo11-mediated recombination intermediates engage with meiotic crossover maturation factors differently because of how they interface with the chromosome axis. Meiosis-specific factors that localize to chromosome axes, such as Hop1, Red1, and the meiosis-specific cohesin subunit Rec8, appear to be important for coupling DSBs to homolog bias and/or pairing reinforcement mechanisms; mutants carrying separation-of-function alleles for either Hop1 or Rec8 that are proficient in meiotic DSB formation are nevertheless defective in homologous synapsis (CARBALLO et al. 2008). Furthermore, DNA that is most frequently cleaved by Spo11 lies in chromosome “loop” regions, away from axis locations where the meiosis-specific Hop1 and Red1 proteins are enriched (GERTON et al. 2000; BLAT et al. 2002; BLITZBLAU et al. 2007; PAN et al. 2011). However, the axis localization of several of Spo11’s accessory proteins (PANIZZA et al. 2011) and the reliance of meiotic DSB formation on Hop1 and Red1 (MAO-DRAAYER et al. 1996; SCHWACHA AND KLECKNER 1997; XU et al. 1997) suggests that Spo11 DSB sites are physically close to chromosome axes during break formation and subsequent repair steps (PANIZZA et al. 2011). The axis-association of DNA sequences undergoing a DSB is furthermore consistent with the localization of “recombination nodules” (structures associated with ongoing DNA repair visualized by electron microscopy) between intimately-aligned homologous chromosome axes (ZICKLER AND KLECKNER 1999).
The possibility that axis proximity is a critical prerequisite for an early DSB to engage with meiotic-like repair pathways is supported by a recent finding that repair of a VDE-mediated DNA break during meiosis utilizes SC associated repair factors more frequently when the break is positioned close to the axis, versus when the break is positioned far from the axis (Medhi et al. 2016). Importantly, this effect was found to be dependent on the presence of Spo11 in trans. This result raises the possibility that the key feature of Spo11’s capacity to promote meiotic chromosomal dynamics is its capacity to ensure (even in trans) that DSBs are processed in the context of axis protein-regulated structures. Furthermore, in their classic study, Thorne and Byers noted a role for the meiotic chromosome axis-associated protein, Hop1, in allowing artificial DSBs to rescue the chromosome segregation functions of Spo11 in meiotic cells (Thorne LW 1993).

In support of the idea that programmed DSBs engage with chromosome axis proteins to promote homolog alignment and synapsis, we found that interhomolog repair of HO-mediated meiotic DSBs in spo11 meiocytes, which fail to promote homolog pairing or synapsis, occurs independently of the meiotic axis protein Red1, and independently of the Mek1 kinase (which regulates the activity of meiosis-specific axis proteins).

On the other hand, a prior study indicated that artificial DSBs rescue Spo11’s SC assembly function if the Rec8 cohesin protein is overexpressed (Brar et al. 2009). While this result would suggest the intriguing possibility that a direct interaction between DSBs and Rec8 facilitate the initiation of SC assembly, we were unable to
observe the analogous result for phleomycin or HO-mediated DSBs in our strain background: SC assembly, as measured by coincident linear stretches of Zip1 and Ecm11 proteins on surface-spread meiotic nuclei, remained undetectable among meiotic nuclei with HO- or phleomycin-supplied DSBs, even when Rec8 (or axis proteins Red1 and Hop1) were overexpressed. Taking previously-published and our own data into account, we conclude that while the proximity of a DSB to the chromosome axis may be important, DSB-independent activity of Spo11-associated machinery appears to be uniquely capable of ensuring that homologous synapsis properly accompanies DSB repair.

**Spo11 DSB machinery may trigger global chromosome dynamics through a specialized interface with Dmc1-associated factors.**

An intriguing outcome from our analysis is the indication that a unique property of Spo11 DSBs is their constraint to repair using the Dmc1 recombinase. Schwacha and Kleckner (1997) characterized Red1 and Dmc1 as important core features of an “interhomolog only” pathway for DSBs, in which axis-associated Red1 is responsible for coupling the maturation of recombination intermediates to the Dmc1 recombinase. In the absence of Red1, remnant meiotic DSBs do not engage with this “interhomolog only” pathway and instead undergo repair without interhomolog template bias. Similarly, the interhomolog repair of an HO DSB during meiosis does not rely on Red1 nor Dmc1, nor Mek1 (which targets multiple molecular pathways that coordinately ensure the use of the Dmc1 recombinase by Spo11-associated meiotic
DSBs), and appears to exhibit low interhomolog repair template bias (as mentioned above). However, artificially supplied DSBs in \textit{spo11} meiotic cells have been shown to induce Mek1 phosphorylation (\textsc{Cartagena-Lirola et al.} 2008), indicating that events apart from Mek1 activation are required for artificially-supplied DSBs to properly engage with an “interhomolog only” meiotic recombination pathway defined by Red1 and Dmc1.

Interestingly, Smith and colleagues found that the repair of a set of non-canonical DSBs in fission yeast meiosis also occurs independently of Dmc1, but is dependent on Rad51, unlike programmed meiotic DSBs in fission yeast meiocytes (\textsc{Farah et al.} 2005).

Altogether, these data point to the idea that that Spo11-associated DSBs differ from HO-associated DSBs in the manner by which they progress into the strand exchange stage of meiotic recombination. As is particularly evident in strains that progress through meiotic prophase more slowly (such as those of the BR background), the absence of Dmc1 diminishes but does not abolish homologous synapsis, indicating that Dmc1 itself is not an essential requirement for DSB-dependent SC assembly (\textsc{Rockmill et al.} 1995). Importantly, our \textit{rad51} separation-of-function experiments also indicate that HO-mediated meiotic DSBs are not prohibited from utilizing the Dmc1 recombinase, and that forcing phleomycin-induced or HO-induced meiotic DNA breaks to repair using the Dmc1 recombinase (through use of the \textit{rad51-II3A} allele) does not confer a robust capacity to facilitate synapsis (Figure S3). Thus, the critical difference between programmed and artificial
meiotic DSBs in promoting homologous synapsis may not be their use of the Dmc1 recombinase *per se*. We speculate that the unique capacity of Spo11 DSBs to promote “meiotic-like” outcomes, such as a high probability of crossing over, homologous centromere coupling, and SC assembly, may arise in part from their early engagement with the mechanism that censors their access to recombinase activity (constrains their repair to Dmc1).
Figure S3. Forced use of the Dmc1 recombinase does not lead to SC assembly triggered by HO-mediated or phleomycin DSBs during meiosis.

(A) Image displays a representative surface-spread meiotic nucleus from a spo11 spo13 rad51-II3A strain (LY935) or a spo11 spo13 rad54 strain (LY922) sporulated for 20 hours with 120µg/ml phleomycin (n=20); DNA is labeled by DAPI (blue) and
Rad51 targeted by an antibody (red). Graph plots the number of Rad51 foci measured per nucleus in each strain.

(B) Representative surface spread nuclei from each strain (indicated) immunostained to show Zip1 (green) and Ecm11/Gmc2 (red); DNA is labeled by DAPI (blue). (C) The percentage of nuclei displaying indicated distributions of Zip1 and Ecm11/Gmc2 is plotted for rad51-II3A (LY935) and rad54 (LY922) strains, with and without the 120µg/ml phleomycin treatment (n=20 per strain and condition).

Additional Tables

Table S2. Sporulation efficiency of strains used to assess recombination. Strains were sporulated on plates for 5 days at 30°C and the frequency of dyad spores was evaluated by light microscopy (n >1000).
Table S2. Sporulation efficiency of strains used to assess recombination

<table>
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<tr>
<th>Genotype</th>
<th>Strain</th>
<th>% Sporulation efficiency (n)</th>
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<td>spo11 spo13</td>
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<td>33 (1068)</td>
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<td>25 (1030)</td>
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<tr>
<td>LY208 cs2</td>
<td>LY555</td>
<td>18 (1025)</td>
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</tr>
<tr>
<td>LY208 cs5</td>
<td>LY207</td>
<td>26 (1063)</td>
</tr>
<tr>
<td>LY207 rad51</td>
<td>LY459</td>
<td>25 (1087)</td>
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<tr>
<td>LY207 rad51 dmc1</td>
<td>LY393</td>
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<tr>
<td>LY207 dmc1</td>
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<td>LY299</td>
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<td>LY413</td>
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</tr>
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Table S3. Spore viability of various strains used to assess recombination. The percentage of total dyads (n) from spo11 spo13 diploids homozygous for various mutant alleles carrying two viable spores (2-sv), one viable spore (1-sv) and 0 viable spores (0-sv) is shown. The percentage of the total number of spores (nx2) that are viable is given for each strain in the final column (% Spore viability).

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<th>Genotype</th>
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<th>n</th>
<th>% Distribution of dyad types</th>
<th>% Spore viability</th>
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Table S4. Strains used in this study. All strains are derived from a BR1919-8B background (ROCKMILL AND ROEDER 1998).

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<td>his4-260,519 leu2-3,112 MATα ho trp1-289 ura3-1 thr1-4 ade2-1</td>
</tr>
<tr>
<td></td>
<td>ade2-1</td>
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<tr>
<td></td>
<td>Description</td>
</tr>
<tr>
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| LY208 | BR1919  
lys2::P<sup>SPO13-HO</sup> lacO::LEU2@450kb  
lys2::P<sup>SPO13-HO</sup> CENIV  
iTHR1<sup>i</sup>@1,416kb  
spo11::ADE2 spo13::URA3  
1,416kb  
spo11::ADE2 spo13::URA3 |
| LY207 | LY208 homozygous  
<sup>cs5::natMX4@836kb</sup> ChrIV |
| LY322 | LY208 homozygous  
<sup>cs6::natMX4@1,162kb</sup> ChrIV |
| LY324 | LY208 homozygous  
<sup>cs7::natMX4@1,056 kb</sup> ChrIV |
| LY555 | LY208  
447kb  
<sup>cs2::natMX4@449kb</sup> CENIV  
LEU2@ChrIV  
<sup>cs2::natMX4@449kb</sup> CENIV |
| LY407 | BR1919  
<sup>spo11::ADE2 spo13::URA3</sup>  
<sup>spo11::ADE2 spo13::URA3</sup> |
| LY299 | LY207 homozygous  
<sup>msh4::kanMX4</sup> |
| LY291 | LY207 homozygous  
<sup>kanMX4-P<sub>CLB2</sub>-MMS4</sup> |
| LY363 | LY207 homozygous  
<sup>mlh3::kanMX4</sup> |
| LY288 | LY207 homozygous  
<sup>zip1::kanMX4</sup> |
| LY289 | LY207 homozygous  
<sup>zip2::kanMX4</sup> |
| LY341 | LY207 homozygous  
<sup>zip3::kanMX4</sup> |
| LY376 | LY207 homozygous  
<sup>mer3::kanMX4</sup> |
| LY290 | LY207 homozygous  
<sup>dmcl1::kanMX4</sup> |
| LY459 | LY207 homozygous  
<sup>rad51::kanMX4</sup> |
| LY393 | LY207 homozygous  
<sup>dmcl1::kanMX4 rad51::hphMX4</sup> |
| LY904 | LY207 homozygous  
<sup>mek1::kanMX4</sup> |
| LY907 | LY407 homozygous  
<sup>mek1::kanMX4</sup> |
| LY939 | LY207 homozygous  
<sup>red1::kanMX4</sup> |
| LY922 | LY207 homozygous  
<sup>rad54::kanMX4</sup> |
| LY925 | LY407 homozygous  
<sup>rad54::kanMX4</sup> |
| LY935 | LY207 homozygous  
<sup>rad51-IJ3A::kanMX4</sup> |
| LY910 | LY207 homozygous  
<sup>xrs2::kanMX4</sup> |
| LY913 | LY407 homozygous  
<sup>xrs2::kanMX4</sup> |
| LY916 | LY207 homozygous  
<sup>mre11::kanMX4</sup> |
| LY919 | LY407 homozygous  
<sup>mre11::kanMX4</sup> |
| LY410 | LY207 homozygous  
<sup>mlh3::kanMX4 msh4::hphMX4</sup> |
| LY413 | LY207 homozygous  
<sup>mlh3::kanMX4 zip3::hphMX4</sup> |
| LY388 | LY207 homozygous  
<sup>yen1::hphMX4 kanMX4-P<sub>CLB2</sub>-MMS4</sup> |
| LY850 | LY207 homozygous  
<sup>sgs1-ΔC795</sup> |
| LY868 | LY207 homozygous  
<sup>yen1::hphMX4 kanMX4-P<sub>CLB2</sub>-MMS4</sup>  
<sup>slx1::hphMX4 mlh3::kanMX4</sup> |
| LY871 | LY324 homozygous  
<sup>yen1::hphMX4 kanMX4-P<sub>CLB2</sub>-MMS4</sup>  
<sup>slx1::hphMX4 mlh3::kanMX4</sup> |
| LY382 | LY324 homozygous  
<sup>zip3::kanMX4</sup> |
| LY458 | LY324 homozygous  
<sup>rad51::kanMX4</sup> |
| LY457 | LY322 homozygous  
<sup>rad51::kanMX4</sup> |
| LY481 | LY459 homozygous  
<sup>LYS2</sup> (LYS2 replaces P<sup>SPO13-HO</sup>) |
| LY492 | LY208 homozygous  
<sup>cs4::natMX4@1,241kb</sup> ChrIV  
<sup>rad51::kanMX4</sup> |
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Table S5. Primers used in this study. Underlined letters indicate nucleotides on plasmids, short underlined letters indicate a restriction site and non-underlined letters indicate nucleotides on chromosomes.
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| AJM753 | TTTTTATATTCAAAAACAAGAAACAAAAAGGAAAGAACATATGTCATG |
| AJM756 | TAGAAGATAGAAACACGGGACGAGGGGCCGGTTCAACAACTATCAT |
| AJM1137 | TTTAATAATATGATAACGTTACGTTTAGACATAGTCTTTTTTTTCATATCACACTATG |
| AJM1138 | TTTTATATTCAAAAACAAGAAACAAAAAGGAAAGAACATATGTCATG |
| AJM1141 | TTCTCTATCTCTTCTTCTCGACAGCTTAATAAATCTTTTCGACCTCC |
| AJM1142 | GCGCGACTGCCGGAAGAAGGACGCGGGCGCGACTCC |
| AJM1145 | CGCCATCCTAGGAGGCTAAAGATGTAAGCTAGTGTATGATGATTAG |
| AJM1146 | GCTTGACATATCTCAAATTTTATGTGTACCTGTGTATGATGAC |
| AJM1255 | ATCATCAAGTGTTCGTGCTGAGCTGCTGAGCAGTTCAACAGAA |
| AJM1256 | CGTGCTCAAAAGTGTAATGTGTCATGACAAATTTGACCAAGC |
| AJM1259 | TTTGTCATCATTCATAGTCTCGAGCTGAATTTGGTGAAGGACG |
| AJM1260 | AGGTATCCACATCTTCTTATAAAGAAAACACTACTAATATCTATG |
| AJM1702 | TGGTACATTAGCAGCCAGAGAACTATTAGTCAGGTGTTGAGCAAGGAG |
| AJM1698 | GCTTTATCCGAAATTTGTCATTAAAAATTCATAGATAAAAAAGTGGCCTCCTCCCTTGTCC |
| AJM2164 | ACACCTTTAAACGCTTTTCTCAGAAAAGTCAGGAATATGTCACCTTCTTCGTTGAAC |

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### Acknowledgements

We are extremely grateful to Scott Holmes, Jim Haber, Doug Bishop, Scott Keeney and Nancy Hollingsworth for sharing reagents critical for experiments reported in this study. We are indebted to Karen Voelkel-Meiman for her valuable input on experiments and preparation of the manuscript. This work was supported by National Institutes of Health grants R00 GM084293, and R15 GM104827 (to A.J.M.).

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Literature Cited


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*Saccharomyces cerevisiae* recA homologues RAD51 and DMC1 have both distinct and overlapping roles in meiotic recombination. Genes Cells 2: 615-629.


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Chapter 3

*Defining functional residues in the Zip1 spacer region: A scalpel approach*
Introduction

Meiotic cell division is necessary to create haploid gametes from diploid parents. Meiosis involves two divisions during which chromosomes must segregate accurately to create viable reproductive cells that carry a single complete set of chromosomes. The first division, which halves chromosome ploidy, requires physical associations between homologous partner chromosomes that are usually generated through homologous DNA recombination. A second conserved feature of meiosis that contributes to homologous chromosome alignment is the assembly of a proteinaceous, tripartite structure called the synaptonemal complex (SC) (PAGE AND HAWLEY 2004; CAHOON AND HAWLEY 2016).

The SC consists of structural proteins that form a lattice between homologous chromosomes during meiosis (PAGE AND HAWLEY 2004; CAHOON AND HAWLEY 2016). The axes of meiotic chromosomes are themselves protein-rich macromolecular structures that develop through the action of cohesin proteins and meiosis-specific factors, such as the Red1 and Hop1 proteins in budding yeast, which link sister chromatids and organize chromatin into a series of tethered loops (HOLLINGSWORTH et al. 1990; SMITH AND ROEDER 1997). The budding yeast SC structural protein, Zip1, assembles the rod-like transverse filaments that bridge the aligned axes of homologous chromosomes homologous axes (referred to as lateral elements in the context of the SC structure) (SYM et al. 1993). The central ~500 residues of Zip1 are predicted to form an extensive coiled-coil structure which is part of the basis for expecting Zip1 to assemble a rod-like dimer (Figure 1A, B) (PAGE AND HAWLEY
Additional evidence for the rod-like nature of Zip1 is the finding that Zip1’s N termini localize to the midline of the 100 nm wide SC structure, away from chromosome axes, while Zip1’s C termini localize near aligned homologous chromosome axes (Dong and Roeder 2000). A set of more recently identified proteins, including Ecm11, Gmc2 and SUMO, assemble the third component of the tripartite SC structure, the central element, at the midline of the SC (coincident with Zip1’s N termini) (Humphries et al. 2013; Voelkel-Meiman et al. 2013).

The functional role of the SC during meiosis appears to be multi-faceted. The assembly of SC is dependent on meiotic recombination initiation, indicating a coordination between early steps in recombination and synapsis (Alani et al. 1990; Loidl et al. 1994). Furthermore, a variety of mutants missing different proteins required for SC assembly (in budding yeast as well as other organisms) display diminished meiotic crossovers, suggesting that the SC structure, once assembled, could be mechanistically involved in promoting crossovers. In budding yeast, this class of mutants includes strains missing the structural SC protein Zip1, as well as several proteins like Zip2, Zip3, Zip4, Spo16, Mer3 and Msh4/Msh5 (Sym et al. 1993; Agarwal and Roeder 2000; Borner et al. 2004; Tsubouchi et al. 2006; Shinohara et al. 2008). However, the recent discovery that the SC building block proteins Ecm11 and Gmc2 are dispensable for crossing over in budding yeast indicates that the SC structure itself is not mechanistically integral to crossover recombination; in fact the ecm11 and gmc2 mutant phenotype suggests that the SC structure plays a negative regulatory role, in constraining the number and resolution
pathways that crossover recombination intermediates can access (Voelkel-Meiman et al. 2016). Partial depletion of SYP-1, an SC transverse filament protein in C. elegans, also results in excess crossing over, suggesting that a role for the SC structure in down-regulating crossovers, may be conserved (Libuda et al. 2013).

The fact that SC-deficient ecm11 and gmc2 mutants display excess crossing over while SC-deficient zip1 mutants display a severe decrease in crossovers indicates that the Zip1 protein has two distinct and separable roles in meiosis: to assemble SC and to promote crossover recombination (Storzazzi et al. 1996; Voelkel-Meiman et al. 2015). We still have little understanding of the molecular features of the Zip1 protein that correspond to its different meiotic activities.
Figure 1. Transverse filament proteins. (A) Diagram shows the predicted secondary structures of known transverse filament proteins in various organisms. Red cylinders show the predicted coiled-coil region and black shows non coiled-coil regions (Image...
from Page and Hawley, 2004). (B) Top is cartoon of *S. cerevisiae* Zip1 with coiled-coil regions shown in black and non-coiled coil region in grey (not drawn to scale). Bottom shows the coiled-coil program prediction output for Zip1 protein. The x-axis show the position of Zip1 amino acid residue and the y-axis show the probability of coiled-coil structure, with a scale of 1 showing the highest probability for coiled-coil structure. (C) A list of *zip1* mutants from Tung and Roeder (1998) study is shown. The name of each allele is listed (left) with a cartoon of the deleted residues (center) and the amino acids deleted shown (right). (D) The summary of phenotypes for the *zip1* alleles in (C).

Structure function analysis in the MacQueen lab has begun to hone in on regions of Zip1 that are important for its capacity to serve as a structural building block of the SC and/or for its capacity to promote crossover recombination. For example, Voelkel-Meiman and colleagues (2016) showed that strains expressing *zip1*-N1, an allele that encodes a version of Zip1 missing residues 21-163, are incapable of synapsis but exhibit high levels (more than wild-type) of Zip1-mediated crossover recombination, similar to mutants missing the SC central element proteins Ecm11 or Gmc2, and mutants in which SUMO is depleted during meiosis (Voelkel-Meiman et al. 2013; Voelkel-Meiman et al. 2016). Tung and Roeder (1998) created a large internal deletion allele, *zip1*-M1 (Figure 1C, D), which encompasses residues 244 to 511, corresponding to much of a continuous coiled-coil region as well as a predicted
“interruption” in the coiled-coil of about 96 residues (this study) (Figure 2A). Tung and Roeder reported abnormal SC assembly and reduced spore viability in the zip1-M1 mutant, indicating that this large deletion encompasses residues important for Zip1’s capacity to assemble SC, and suggesting that these residues may also contribute to Zip1’s capacity to promote recombination (TUNG AND ROEDER 1998).

To specifically investigate the functional role of residues within the coiled-coil interruption in the Zip1 protein, I continued a study that was initially begun by a former graduate student in the lab, Samantha Hughes. In this study, a series of zip1 alleles (zip1-A; zip1-B; zip1-C; zip1-D; zip1-E) were created that encode versions of Zip1 missing consecutive ~10-20 residue regions between amino acid coordinates 258 and 354 (Figure 2). S. Hughes had performed a preliminary analysis of SC assembly for zip1-M1, zip1-B, zip1-C and zip1-D and spore viability for zip1-B, zip1-C, zip1-D and zip1-E, which showed that zip1-C and zip1-D fail to assemble proper SC, while zip1-B and zip1-E form apparently normal SC, at least at a late time point. Additionally, it was found that zip1-C and zip1-E exhibit a severe spore viability defect, potentially indicating a defect in crossover recombination in these mutants. I have carried out an in-depth recombination analysis of the zip1A-E and zip1-M1 alleles. My data refines and corrects S. Hughes’ initial spore viability data, and implicates Zip1’s residues 279-296, in a crossover-constraining function of Zip1 that potentially is the molecular basis for the crossover-constraining function that we have previously ascribed to tripartite SC (VOELKEL-MEIMAN et al. 2016).
Materials and Methods

Strains

All strains are isogenic to the BR1919-8B (Rockmill and Roeder 1998). Genotypes of strains used in this study are listed in Table 1. zip1 mutants were verified by sequencing to confirm the deletion of amino acids and additionally, to verify that the rest of ZIP1 open reading frame sequence was correct. After sequence confirmation, standard yeast genetics crosses and manipulation was used to generate strains that bear markers on chromosome III and chromosome VIII to be used for recombination analysis (tetrad analysis) in S. cerevisiae. Each diploid strain is heterozygous for each marker and the position of the markers is depicted in Figure 3. The following changes are present on chromosome III or chromosome VIII: HIS4 is mutated to his4-260,519; hphMX4 is inserted near centromere III; ADE2 is inserted near RAD18; and natMX4 is inserted near HMR on chromosome III. TRP1MX4 is inserted near SPO11; URA3 replaces SPO13; THR1 is mutated to thr1-4, and LYS2 is inserted at 210kb on chromosome VIII.

Table 1. Strains used in this study.

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**Tetrad analysis**

Diploid strains were grown on YEPADU solid media at 30°C. Cells were transferred to minimal solid media and sporulated for 5 days at 30°C to produce tetrads. A small amount of sporulated cell was incubated in 1:10 dilution of Glusulase for 15 minutes to digest the outer-membrane (asci) that encapsulates the 4-spores of each tetrad.

Tetrads were dissected using a micro-manipulating needle fitted on a light microscope and grown on YEPADU plate for 48 hours. Dissected spores were replica-plated on to various plates to determine the genotype of each spore. Synthetic complete media plates lacking an auxotroph marker (SC-HIS, SC-ADE, SC-TRP, SC-URA, SC-THR or SC-LYS), antibiotic plates (YEPADU + hygromycin B, YEPADU + G418, YEPADU + nourseothricin) and minimal media plates for mating type complementation test were used to determine the genotype at chromosome III and chromosome VIII utilizing a total of nine locus.
Spore viability was calculated for the total tetrads dissected for each strain analyzed for recombination. Genotypes of tetrads were scored twice using Microsoft Excel. The numbers of PD, NPD and TT were tallied and utilized to calculate the genetic map distance (centiMorgans, cM) using an Excel Linkage Macro program, created by Jonathan Green and donated by Eva Hoffmann (University of Copenhagen). Map distances were confirmed and standard errors were calculated using Stahl online tools.

Results and Discussion

Deletion of residues in the Zip1 “spacer” region does not disrupt meiotic chromosome segregation

The Zip1 protein is predicted to form an extensive, relatively continuous coiled-coil between residues 175-750 of the 875 amino acid protein, but there is a notable interruption in coiled-coil compatible sequence between residues 258 and 401 (Figure 1). Strains expressing the zip1-M1 allele, encoding a version of Zip1 missing residues 244-511 was reported to have a defect in SC assembly as well as a reduction in spore viability (Figure 1D) (TUNG AND ROEDER 1998), suggesting that residues 244-511 of Zip1 are important for accurate meiotic chromosome segregation.

To investigate the function of the “spacer” residues that interrupt Zip1’s coiled-coil, we assessed the spore viability phenotype of diploids carrying zip1-M1, and strains expressing the smaller in-frame deletion alleles that had been created in our
lab, which correspond to residues 258-354 (zip1-A, zip1-B, zip1-C, zip1-D and zip1-
E; Figure 2). Consistent with the Tung and Roeder study, the zip1-M1 strain exhibited
a reduced spore viability, from 97% to 72% (Table 2, Figure 2). The spore viability of
zip1-A, zip1-B, zip1-C, zip1-D and zip1-E diploids are 97%, 89%, 84%, 81% and
90%, respectively. The spore viability in zip1-C and zip1-D are mildly reduced as
compared to wild-type. However, these reductions in spore viability are not as severe
as the larger deletion zip1-M1 (72%). Thus, these residues appear proficient in
successful meiotic chromosome segregation.
Figure 2. Spore viability of Zip1 spacer mutants. (A) Top: Diagram shows the full-length Zip1 and the secondary structure prediction for Zip1. Below: Cartoons show the Zip1 mutant proteins in this study. The alleles include zip1-M1, zip1-A, zip1-B, zip1-C, zip1-D and zip1-E. (B) Graph plots the spore viability of the various mutants. The percentage of viable spores out of total dissected spores is plotted.

Interhomolog crossovers are moderately reduced in zip1-C and zip1-D, but form in excess in zip1-B

As budding yeast has multiple pathways for crossover recombination, a deficiency in one of the pathways does not always result in a strong defect in spore viability. Thus, we examined interhomolog recombination across seven genetic intervals in 4-spore viable tetrad products of zip1-M1, zip1-A, zip1-B, zip1-C, zip1-D and zip1-E strains (n
Chromosome III and chromosome VIII were used for this analysis (Figure 3A).

An absence of Zip1-mediated crossing over altogether is expected to reduce chromosome III and VIII genetic maps by 50% (Voelkel-Meiman et al. 2015; Voelkel-Meiman et al. 2016). zip1-M1 strains display only a mild reduction in crossovers on chromosome III (84% of wild-type), and a slight increase in crossing over on chromosome VIII (119% of wild-type) in our strain background. This data indicates that the Zip1-M1 protein, despite the absence of much of its coiled-coil and inability to assemble SC, supports some level of Zip1’s crossover function during yeast meiosis. Similarly, strains carrying the zip1-A allele exhibited normal crossover levels on III and VIII. However, strains carrying zip1-C and zip1-D alleles exhibited a stronger reduction in crossover levels than strains carrying the zip1-M1 deletion (67% of wild-type’s chromosome III cumulative map length; 60% of wild-type’s chromosome VIII cumulative map length in the zip1-C mutant strain, for example). The fact that the smaller zip1-C deletion makes strains worse off for crossover recombination than strains expressing the large zip1-M1 deletion allele suggests that the region deleted in Zip1-M1 carries both positive and negative elements that regulate Zip1’s capacity to promote crossing over.
Figure 3. zip1-B shows excess interhomolog crossovers while zip1-C and zip1-D show reduction in interhomolog crossovers. (A) The position of markers used to mark
genetic intervals on chromosome III and chromosome VIII is shown. These markers were used in tetrad analysis to assess recombination in various zip1 mutants. (B) The percentage of map distance for each chromosome is shown for wild-type and all zip1 mutants analyzed in this study (LY674, LY582, LY579, LY583, LY584 and LY625). Wild-type ZIP1 (K842) is shown for comparison (Adapted from Voelkel-Meiman, 2016). (C) The percentage of map distance is displayed for each interval on chromosome III or chromosome VIII for all zip1 mutants.

Interestingly, strains expressing the zip1-B internal deletion allele exhibit a strong increase in recombination on both chromosome III and VIII (150% and 154%, respectively, of the wild-type map distance). Closer examination shows a strong increase in crossing over in all seven genetic intervals of the zip1-B strain (Table 3, Figure 3). The increase in crossing over observed in zip1-B mutants appears similar to the increase in crossovers observed in SC central element – deficient mutants ecm11, gmc2, and zip1-N1. By contrast with these latter mutants, however, zip1-B strains appear to assemble normal SC. It is possible that SC assembly is delayed in the zip1-B mutant strain, which could allow Zip1-B protein to promote Msh4-Msh5 (SC-associated) crossovers in the absence of negative regulation from an SC structure, similar to what we envision is occurring in ecm11, gmc2 and zip1-N1 mutants. On the other hand, perhaps Zip1-B protein assembles SC with normal kinetics but the structure of the SC is deficient in its capacity to negatively regulate crossover.
recombination. We can test these models by assessing a time course of SC assembly in the zip1-B strain, and by asking whether overexpression of the Zip1-B protein, which should rescue a delayed SC assembly phenotype, restores normal crossover levels to the zip1-B expressing strain.

If SC assembles with normal kinetics, the excess recombination in zip1-B implies the existence of an “island” at the center of Zip1 which may independently or together with the N-terminus enforce robust crossover homeostasis. As Zip1 builds transverse filaments that span a 100nm length between two aligned chromosomes, the existence of a regulatory region that interrupts the long coiled-coil regions may provide molecular network that connect Zip1’s central region to the lateral regions (axis sites) of chromosomes.

Further, if we reconcile the crossover analysis of zip1 mutants with spore viability measurements, we see the mild reduction of spore viability correlates with the reduction in interhomolog recombination in zip1-C and zip1-D. First, a simple explanation is that global reduction in interhomolog recombination results in some chromosomes not receiving enough crossovers to segregate accurately. Another explanation can be that viability defect resulted from smaller chromosomes not receiving enough crossovers to hold homologous chromosomes together rather than larger chromosomes. Evidence for this is that zip1-M1 and zip1-C have more reduction in interhomolog recombination across intervals on chromosome III as compared to chromosome VIII (Figure 3B). Thirdly, the mild reduction in viability may be due to impaired Zip1 function in centromere tethering. Zip1 is necessary to
pair centromeres in the absence of recombination between chromosomes, as previously shown for non-exchange chromosomes in yeast (Newnham et al. 2010). Therefore, in the absence of robust recombination, in zip1-M1, Zip1-C or zip1-D, some chromosomes may rely on Zip1’s tethering function for accurate segregation, which may not be robust in these mutants.

*Crossover interference indicates that the Zip1-M1 protein may ectopically promote class II crossovers*

The distribution of crossovers along the length of a chromosome is non-random and the presence of one crossover (reciprocal recombination event) prevents the formation of another one nearby in a process called crossover interference (Muller 1916). Budding yeast carry two major pathways for generating crossovers, called “class I” and “class II”. SC-associated crossovers belong to the class I pathway and display interference, while the class II crossover pathway, which generates the residual crossovers observed in zip1 or msh4 mutants, generates crossovers that are randomly distributed. The level of interference can be calculated from the ratio of the frequency of observed double crossovers to that of the expected crossovers, termed coefficient of coincidence (c.o.c) (Papazian 1952; Snow 1979; Griffiths 2015). Cells that have normal levels of interfering crossovers thus show interference values that are less than one.

Using the coefficient of coincidence measurements of recombinants from tetrad dissected in the zip1 mutants, we found that while crossovers are at close to wild-type
levels in \textit{zip1-M1} strains, interference is drastically reduced across most intervals (Table 3). This is in contrast to the \textit{zip1} null mutant, which displays a reduction in interference but a severe reduction in crossing over as well. These data suggest that the Zip1-M1 protein actually promotes class II crossovers, which is a novel sort of \textit{S. cerevisiae zip1} allele. We propose that the Zip1-M1 residues are important for ensuring that early recombination intermediates that are stabilized by Zip1 are protected from being processed by class II recombination machinery. We can test the idea that Zip1-M1 promotes class II crossovers by asking whether \textit{zip1-M1} crossovers reduce when Msh4 is removed.

\textit{zip1-B} mutants exhibit relatively strong crossover interference, suggesting that like the phenotypically-similar \textit{ecm11}, \textit{gmc2} and \textit{zip1-N1} mutants, the excess crossovers generated in this strain are likely class I (SC-associated and Msh4-dependent). \textit{zip1-C}, \textit{zip1-D} and \textit{zip1-E} strains exhibit a moderate, interval-dependent reductions in interference, suggesting that the intermediate reduction in crossing over in these strains corresponds to a parallel intermediate reduction in class I crossovers. The level of class II crossing over will be analyzed for all of these \textit{zip1} strains by assessing crossover recombination in the double mutants that are missing Msh4.
Table 2: Spore viability of tetrads.

<table>
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<tr>
<th>Genotype</th>
<th>Strain</th>
<th># Tetrads</th>
<th>% 4 Spore viable</th>
<th>% 3 Spore viable</th>
<th>% 2 Spore viable</th>
<th>% 1 Spore viable</th>
<th>% 0 Spore viable</th>
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<td>3</td>
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<td>0</td>
<td>0</td>
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Table 3. Map distance from 4-spore viable tetrads carrying various S. cerevisiae zip1 alleles.

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<th>tt</th>
<th>npd</th>
<th>total</th>
<th>cm (± se)</th>
<th>%wt</th>
<th>cm (± se)</th>
<th>%wt</th>
<th>NPDobs/NPDexp (± se)</th>
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<td>100</td>
<td>106.0 (III)</td>
<td>100</td>
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<td>(Voelkel-Neiman, 2016)</td>
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Literature Cited


Chapter 4

Questions raised and future directions
A long-standing question in organisms, from yeast to mammals has been to know how recombination initiation is coupled to chromosome pairing and synapsis. To start addressing this question, I examined the capacity of artificial breaks introduced near and far from the centromere to promote chromosome pairing, DNA recombination and synapsis in spo11 cells. In wild-type cells, all of these processes rely on the initiation of recombination by Spo11 (Keeney et al. 1997). We introduced artificial DSBs by a site-specific HO endonuclease or chemically, using phleomycin treatment in spo11 cells. The advantage of using HO endonuclease is that we were able to direct DSBs to specific sites, proximal to the centromere or distal to centromere, and to sites that are usually broken by Spo11. With phleomycin, we were able to introduce a high number of artificial breaks to test if the number of breaks was a factor in chromosome pairing and synapsis.

Our investigation led to several interesting insights into meiotic DNA recombination process. We found that artificial breaks were able to promote recombination between homologous chromosomes, but these interhomolog interactions were not sufficient to promote centromere pairing or synapsis. This suggests that Spo11’s breaks are unique in their capacity to pair chromosomes. We also found that large numbers of breaks introduced by phleomycin are able to promote synapsis in some nuclei. This could have resulted due to a number of reasons. First, it could be that a threshold of breaks is necessary to promote synapsis regardless of the source of break. Spo11 normally forms ~200 breaks in every cell. Thus, in our experimental population a fraction of cells may have achieved the level
of breaks necessary to activate Tel1/Mec1 signaling and recruitment of meiotic
synapsis initiating factors. Second, it is possible that most cells receive many breaks,
but only a fraction of cells receive enough phleomycin breaks at Spo11 hotspot
locations. In this scenario, both the number of breaks and the location would be two
important factors necessary to promote SC. Spo11 forms breaks frequently in regions
labelled hotspots and these hotspots are conserved across numerous yeast species
(GERTON et al. 2000; BLITZBLAU et al. 2007; LAM AND KEENEY 2015). Thus, if both
number of breaks and location are important factors, it explains our observations that
many cells had dotty (discontinuous) Zip1, while small fraction of nuclei had Zip1
stretches that were coincident with Ecm11, suggesting that in the latter case, some
partial SC was able to successfully assemble.

**Differential use of recombinase enzymes by Spo11**

Our examination of HO-induced recombination and its dependency on various
recombination factors revealed an interesting difference between the repair of an
artificial break from Spo11 break. We observed that Spo11 DSBs are unique in their
capacity to establish Dmc1, the meiosis specific recombinase, as the primary strand
exchange protein. In *dmc1* cells, Spo11 breaks remain unrepaired and cells get
arrested due to checkpoint activation (BISHOP et al. 1992). Proper repair of Spo11
breaks requires both Dmc1 and Rad51, and the absence of either strand exchange
protein causes accumulation of unrepaired DSBs (SHINOHARA et al. 1997). On the
other hand, we observed that repair of HO breaks on chromosome IV progresses with
comparable efficiency, in the presence and absence of Dmc1. This suggests that the repair of exogenous breaks differs as early as the strand exchange step during meiosis. The strand exchange activity of Rad51 has been shown to be dispensable during meiosis, but the protein functions as an accessory for Dmc1 function (CLOUD et al. 2012). Thus, Rad51 is functionally important in both mitosis and meiosis in wild-type cells, but the capacity through which it is involved in homologous recombination in mitosis varies from meiosis. Dmc1 protein together with axis proteins, has been shown to establish an interhomolog-only pathway (SCHWACHA AND KLECKNER 1997). Our study shows that Dmc1 is dispensable to repair an HO break, but not Rad51, which demonstrates that an early mechanism for a primarily “interhomolog pathway” is lost at artificial breaks sites.

It was interesting to find that a separation-of-function allele, rad51-II3A could promote HO recombination. This Rad51 allele has a defective strand exchange activity. This means that in the absence of Rad51 strand exchange activity, Dmc1 is available to repair an HO break. This idea is supported by a study that showed that both Dmc1 and Rad51 localize at sites of an artificial break formed by VDE using chromatin immuno-precipitation (ChIP) (FUKUDA et al. 2003). Similar to the Malkova (2000) study, Fukuda et al conclude that artificial breaks are repaired through a “Spo11-like” manner based on localization of Dmc1 and Rad51 at the break sites. However, our study shows that although both strand exchange proteins appear to be accessible, there is a distinct difference in the way Dmc1 and Rad51 is
used at an artificial break compared to a Spo11 DSB, specifically the independence from Dmc1 use.

The differential use of Dmc1 to repair HO DSBs raises several interesting questions. Is Dmc1 utilized just as a Rad51 protein in the context of an HO break repair? Does rad51-II3A allele provide Dmc1 protein the capacity to be more “Rad51-like”? Rad51 and Dmc1 proteins have 30% and 26% amino acid identity with E. coli RecA protein, while Rad51 and Dmc1 are 45% similar to one another (BROWN AND BISHOP 2014). We can address these questions experimentally by replacing Dmc1 with E. coli RecA protein in rad51-II3A spo11 cells and measuring HO recombination. A previous study has shown that yeast Rad52 which is a functional partner of Rad51 could be substituted by RecA protein (DUDAS et al. 2003). Thus, this will allow us to further characterize the role of Dmc1 in repairing an HO break and further bolster the idea that Spo11 uniquely activates Dmc1 and this activity functionally differentiates it from other Rad51-like activity.

Why are Spo11 breaks unique?

Our study reveals that Spo11 breaks are not only unique in their capabilities to utilize Dmc1 and Rad51, but also to efficiently promote centromere associations and synapsis in between homologous chromosomes. The differences in DSB initiation between Spo11 and other artificial sources such as irradiation or site-specific endonucleases may bestow Spo11 the ability to promote homologous pairing or synapsis.
First, Spo11 has preferred regions for DSB formation on each chromosome known as hotspots which most often occur in promoter regions of genes, and are less frequent at the centromere and telomeres (Gerton et al. 2000). It is possible that these chromatin regions have evolved to accommodate the architecture of the meiosis factors that are recruited to a DSB site. In mammals, DSBs are enriched at H3 lysine 4 (H3K4) trimethylation marks and a sequence specific protein PRDM9, a histone H3 methyltransferase, determines sites of hotspots (Baudat et al. 2010; Brick et al. 2012). It is possible that yeast have a similar factor that has yet to be discovered, that targets DSBs in specific chromatin regions. This targeting could be functionally important for early recombination repair, such as recruiting recombination enzymes. The fact that hotspots are conserved among different species of yeast (Lam and Keene 2015), supports the idea that forming DSBs in the right locations on the chromosome may be functionally important.

Second, meiotic recombination initiation requires Spo11 and nine other accessory proteins to initiate DSBs on chromosomes in yeast (Lam and Keene 2014). Additionally, Spo11 remains covalently linked to broken ends of DNA soon after DSB initiation (see Figure 1, below). Among the accessory proteins, Mre11, Rad50, and Xrs2 (MRX) are also required for resection of the broken DNA ends in subsequent recombination repair steps. Thus, the basis for the unique properties of Spo11 may be due to 1) this early coupling of recombination initiation with strand resection which may promote the recombination efficiency of Spo11 breaks as compared to artificial breaks, (see Figure 1, Model), and 2) the association of Spo11
to the ends of DNA which suggests that Spo11 accessory proteins may stay at breaks sites and influence break repair or synapsis after their initial DSB initiation function. In support of this idea, one of the accessory proteins for Spo11, Ski8 is seen to associate with chromatin in meiotic chromosome spreads as late as the pachytene stage of meiosis (ARORA et al. 2004). This suggests that the Ski8 may have additional function after DSB initiation on chromosomes. Ski8 may promote synapsis between chromosomes. Additionally, in spo11 cells, Ski8 fails to localize on meiotic chromosomes and Ski8 remains in the cytoplasm (ARORA et al. 2004). This raises the question: Does recruitment of Spo11 accessory factors to artificial break sites promote homologous chromosome synapsis in the presence of an artificial break? In our study, we fused the catalytically inactive Spo11Y135F to the N-terminus of HO endonuclease in order to target Spo11’s interacting partners to the break sites. However, this fusion incapacitated HO endonuclease from cutting DNA. Alternatively, we can try to recruit the accessory factors by globally increasing their expression using over expression plasmids in yeast.

Third, non-Spoll breaks may be incapable of promoting adequate signaling to process breaks similar to Spo11. We reported in Chapter 2 that HO breaks contained long gene conversion tracks that were up to ~580kb away from the break sites. Artificial breaks formed by VDE were shown to have an extended resection tract in the absence of Spo11, suggesting a role for Spo11 in trans to influence any break formed inside the nucleus (NEALE et al. 2002). Even though Mec1/Tel1 has been shown to be stimulated by artificial breaks formed by phleomycin (CARTAGENA-
LIROLA et al. 2008), the signaling cascade, such as levels of phosphorylation of target proteins or subsequent dephosphorylation of substrates may be a critical factor to promote synapsis.

**Are ZMM proteins recruited to artificial DSB sites?**

Although the specific functions of ZMM proteins has yet to be well-defined, mutation analysis show that the ZMMs stabilize recombination intermediates such as crossovers during DSB repair (BORNERT et al. 2004). We observe a partial reduction in HO recombination in the absence of ZMM proteins such as Zip3 and Mer3, suggesting HO recombination may partially depend on these proteins. We also see a small subset of nuclei that have SCs after phleomycin-induced DSBs, raising the possibility that ZMM proteins may be at numerous recombination sites. Do the Zip proteins, Mer3 or Msh4/5 localize to recombination sites that arose from artificial DSBs? To test this, we can look at the recruitment of these proteins on meiotic chromosomes through cytology. For example, if Zip3 or Zip4 protein is present at HO DSBs on chromosomes, it will indicate they have a role, and explain the partial reduction in HO-mediated CO we observe in single and double zmm mutants. In addition to cytology, we can utilize chromatin immno-precipitation (ChIP) of any of the ZMM proteins to find out their enrichment at eight of our HO DSBs on chromosome IV in spo11 cells. Rad51 has been shown to interact with Zip3 in a yeast-2-hybrid study in SPO11 cells (AGARWAL AND ROEDER 2000). We can ask if Zip3 and Rad51 interact in our phleomycin-treated spo11 cells. Additionally, we can
also use cytology to ask if any of the ZMMs are recruited to chromosomes as a result of DSBs by phleomycin.

**Is Zip1 phosphorylated as a result of HO or phleomycin breaks?**

Zip1 is phosphorylated in a Spo11-dependent manner in wild-type cells (Falk *et al.* 2010), and this is thought to modulate Zip1’s early function in centromere coupling and centromere pairing. Artificial DSB formation by phleomycin leads to phosphorylation of Mek1 in yeast (Cartagena-Lirola *et al.* 2008). Thus, it will be interesting to know if Zip1 is also phosphorylated as a result of artificial DSBs. This can be done by removing the phosphatase, Pph3, and examining modification of Zip1 in the presence of HO or phleomycin DSBs in spo11 background using western blot analysis.

If Zip1 protein is phosphorylated as a result of an HO break, it would mean that a single DSB in spo11 cells is sensed and this break can trigger phosphorylation of Zip1. Is this break activating the Mec1/Tel1 kinases? We can verify that this phosphorylation is dependent on Mec1 or Tel1 by mutating these kinases and showing that Zip1 phosphorylation can be abolished. Alternatively, multiple breaks introduced by phleomycin may promote Zip1, but not a single HO break. First, this could mean that a certain threshold of DSBs is necessary for Mec1/Tel1 kinase to be activated. This result would also be consistent with the previous observation of phosphorylation of Mek1 through numerous breaks formed by phleomycin (Cartagena-Lirola *et al.* 2008). Second, the phosphorylation of Zip1 due to DSBs
formed by phleomycin means that exogenous DSBs were sufficient to achieve Zip1 phosphorylation. Zip1 phosphorylation at serine 75, as a result of Spo11 DSBs has been proposed to be the key step that dissociates non-homologous centromere coupling by destabilizing the Zip1 that holds the two non-homologous centromeres together (Falk et al. 2010). Thus, this process mediates a homology search step. The phosphorylation of Zip1 as a result of phleomycin breaks would suggest that an early homology search mechanism is activated. However, we did not see a statistically significant increase in centromere pairing as a result of phleomycin in spo11 cells. This could mean that non-homologous interactions are destabilized, but there were not sufficient centromere pairing factors that followed this initial step to reinforce pairing. These pairing factors can include sufficient dephosphorylation of Zip1 by Pph3 to stabilize centromere association or sufficient interhomolog recombination events along the length of a chromosome.

**zip1 alleles reveal regions of Zip1 that are important for crossover regulation**

In Chapter 3, I characterized zip1 deletion alleles that remove amino acids within the interruption in between Zip1’s coiled-coil region. Various mutants were analyzed for their spore viability, recombination and crossover interference. One interesting outcome of this analysis is identification of a region in Zip1 (279-296 amino acids, zip1-B) that appears to have a crossover constraining ability. The zip1-B showed an elevated crossing over as compared to wild-type, but showed normal interference. On the other hand, zip1-M1 had a reduced crossover level, but showed loss of
interference. The decrease in interference suggests that the region of Zip1 deleted in the \textit{zip1-M1} is crucial for maintaining interference. The M1 region may interface with ZMM proteins and other recombination factors. Hence, it will be interesting to know 1) if the observed recombination in \textit{zip1-M1} are Msh4-independent, thus formed through class II pathway. 2) if the residues in this region interact with the ZMM machinery. To address these questions, we can mutate Msh4 protein in \textit{zip1-M1} and measure recombination. We can also test protein-protein interaction of the M1 region with candidate proteins with yeast-2-hybrid technique.

\textit{Does zip1-B cause a delay in SC assembly or assemble aberrant SC?}

Determining if there is a delay in SC assembly of these \textit{zip1} mutants, especially \textit{zip1-B} which causes an elevated level of recombination will be insightful to determine if the absence of SC is leading to elevation in recombination. Time-course experiments to compare the levels of Zip1, Ecm11-Gmc2 or SUMO localization on chromosomes would address this. Although Hughes did not report any SC defect in a preliminary SC analysis at a late time point, a super-resolution microscopy imaging of chromosomes in \textit{zip1-B} background may be necessary to see if SC is not able to assemble well or if chromosome axis sites are not aligned as expected. Further, the number of recombination sites can be quantified per chromosome by assessing the level of Zip3 foci that are present in mutants with an elevated crossover level. This could even allow us to determine if long chromosomes behave differently than small chromosomes with regards to additional recombination nodules, thereby giving us a
cytological read out of interference in various mutant backgrounds and across various chromosome sizes. However, if the number of SC-associated recombination nodules has not increased in \textit{zip1-B} more than wild-type (\textit{ZIP1}), it will inform us that a class II pathway, which does not involve SC-associated proteins, causes the excess crossover recombination.

\textit{Do specific residues in the Zip1 spacer region mediate interaction with another recombination factor?}

Another interesting idea is the identification of the molecular interactions that are perturbed with the mutations analyzed in this study. Do the deletions in \textit{zip1-B} or \textit{zip1-C} change the interacting factors that regulate crossovers? Single residues within “B” can be mutated to Alanine to narrow down the residues responsible for crossover regulation. Mutants obtained in this way can be analyzed in a number of ways. First, we can assess alanine replacement mutants for recombination and ask if the mutants recapitulate the \textit{zip1-B} phenotype. Second, the interaction of the mutant with other meiotic proteins such as Zip3 or Msh4 can be assessed using a yeast-2-hybrid system. Or alternatively, co-immunoprecipitation methods can be used to determine if the deletion interferes with any of the proteins important for crossover formation, such as Zip3 protein.
Figure 1. Model depicts outcomes of a Spo11-initiated DSB and an artificial break (irradiation, HO or VDE) in parallel. Spo11 and its partners, a total of 10 proteins, initiate DSBs on chromosomes during meiosis (Left). Spo11 remains attached to the break ends. The MRX complex which is part of the Spo11 partner group is required to form breaks, but its functional role during break initiation remains unclear. The requirement of MRX for break initiation could be a way to tether MRX near a DSB site. Thus, most Spo11 DSBs will be sensed by MRX efficiently. This can lead to
robust Mec1/Tel1 activation which in turn activates Mek1 kinase. Mek1 ensures interhomolog repair by down-regulating Rad51 and establishing Dmc1 as the primary recombinase. On the other hand, an HO break or other artificial breaks may not recruit the MRX sufficiently, leading to a poor Mec1/Tel1 signaling (Right). This may not recruit meiosis-specific proteins that are interhomolog promoting factors. Thus, artificial breaks sites will not discriminate between Dmc1 or Rad51 for repair.
Literature Cited


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