The Molecular Structures of Recombination Intermediates in Yeast

by

Katrina Mitchel

Department of Molecular Genetics and Microbiology
Duke University

Date:_______________________

Approved:

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Sue Jinks-Robertson, Supervisor

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Tom Petes

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Kenneth Kreuzer

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John McCusker

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J. Andrew Alspaugh

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of
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ABSTRACT

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Abstract

The genetic information necessary for the survival and propagation of a species is contained within a physical structure, DNA. However, this molecule is sensitive to damage arising from both exogenous and endogenous sources. DNA damage can prevent metabolic processes such as replication and transcription; thus, systems to bypass or repair DNA lesions are essential. One type of lesion in particular - the double strand break (DSB) - is extremely dangerous as inappropriate repair of DSBs can lead to deletions, mutations and rearrangements. Homologous recombination (HR) uses a template with sequence homology to the region near the DSB to restore the damaged molecule. However, this high-fidelity pathway can contribute to genome instability when recombination occurs between diverged substrates. To further our understanding of the regulation of HR during vegetative growth, we have used the budding yeast *Saccharomyces cerevisiae* as a model system and a plasmid-based assay to model repair of a DSB. In the first part of this work, the molecular structures of noncrossover (NCO) and crossover (CO) products of recombination were examined. While the majority of NCOs had regions of heteroduplex DNA (hDNA) on one side of the gap in the repaired allele and no change to the donor allele, most COs had two tracts of hDNA. They were present on opposite sides of the gap, one in each allele. Our results suggest that the majority of NCOs are generated through synthesis-dependent strand annealing (SDSA), and COs
are the result of constrained cleavage of a Holliday junction (HJ) intermediate. To clarify the mechanisms regulating NCO production, the effects of three DNA helicases - Mph1, Sgs1 and Srs2 - on the structures of NCO events were examined. All three helicases promote NCO formation by SDSA, but Sgs1 and Srs2 also assist in NCO formation arising from an HJ-containing intermediate, consistent with HJ-dissolution. To study how CO products are generated, we have investigated the contribution of the following candidate HJ resolvases to the structures of CO events: Mus81, Yen1 and Rad1. The results suggest that Rad1 is important to normal CO formation in this assay, but Mus81 and Yen1 are largely dispensable. Together, this work advances our knowledge of how the NCO versus CO outcome is determined during HR, expanding our understanding of how mitotic recombination is regulated.
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The faithful transmission of genetic information between generations is crucial to maintaining viability. However, genomes are under constant threat from both exogenous and endogenous sources of damage. DNA is a molecule, and, as such, spontaneous reactions within the aqueous conditions of a cell can lead to deamination of bases or the creation of abasic sites (Lindahl, 1993). Reactive oxygen and nitrogen species are generated by cellular metabolism and can also lead to single-strand breaks. Exogenous physical and chemical agents can damage DNA as well, leading to lesions such as pyrimidine dimers, intra or interstrand crosslinks and DNA double-strand breaks (DSBs). DNA damage can be mutagenic and/or cytotoxic, and the accumulation of DNA damage is linked to both aging and the development of cancer (reviewed in Hoeijmakers, 2009). Thus, multiple DNA repair systems have evolved to promote genome stability.

While DNA damage can take many forms, one of the most cytotoxic lesions is the DSB. As a single unrepaired DSB can lead to cell death (Frankenberg-Schwager, 1990; Resnick and Martin, 1976), mechanisms that repair DSBs are of particular importance. These mechanisms include “error-prone” nonhomologous end joining (NHEJ), single-strand annealing (SSA), which repairs a break by annealing two repeated sequences but results in deletion of one of the repeats and the information between them, and “error-free” homologous recombination (HR) (Figure 1).
DSBs can be repaired through nonhomologous end joining (NHEJ), single-strand annealing (SSA) or homologous recombination, which includes break-induced replication (BIR), synthesis-dependent strand annealing (SDSA) or double-strand break repair (DSBR) going through the double Holliday junction (dHJ) intermediate. Solid lines represent one strand of DNA, and arrowheads indicate the 3’ end of each strand. Dashed lines symbolize newly synthesized strands and are in the color of the template DNA strand. Figure adapted from Krogh and Symington, 2004.

HR can be initiated in mitosis as a result of damage to both strands of DNA (by sources such as ionizing radiation (IR)), single-strand gaps, a collapsed replication fork or endonucleolytic cleavage of both strands. Restoration of the damaged molecule without additional changes is crucial, but HR can also lead to large-scale genome rearrangements, sequence duplication, loss of heterozygosity or other types of genome
instability. These outcomes can have devastating phenotypic consequences, and
disregulation of HR is linked to both developmental abnormalities and tumorigenesis in
humans (reviewed in Moynahan and Jasin, 2010). However, some mitotic DSBs are
programmed as well. One well-studied example is that of the HO endonuclease, which
generates a DSB at the mating-type locus MAT in *Saccharomyces cerevisiae* (reviewed in
Haber, 2012). Using one of the two epigenetically silenced HM cassettes as a template,
repair of the break within the MAT allele will result in mating-type switching of a
haploid yeast. During meiosis, programmed DSBs are formed throughout the genome in
a reaction catalyzed by Spo11, a meiosis-specific protein with homology to the type IIB
topoisomerase family (reviewed in Keeney, 2001). Repair of these breaks by HR during
prophase of the first meiotic division allows for accurate segregation of the homologous
chromosomes and is a mechanism for the creation of genetic diversity amongst spores
(or gametes in higher organisms).

The primary pathway for mitotic DSB repair in budding yeast *S. cerevisiae*, HR
uses an intact donor sequence with homology to the region near the break to restore the
damaged molecule (reviewed in Paques and Haber, 1999a). This process can lead to the
reciprocal exchange of the sequences flanking the break on the template and the
recipient molecules, a crossover (CO) product. Alternatively, repair of the break by HR
can yield a noncrossover (NCO), where the damage is repaired, but there is no change to
flanking linkages. In light of its potential as a source of genome instability, control of HR
is necessary to prevent deletions, duplications, and chromosome rearrangements. Below, I review background information concerning the mechanism and regulation of HR, including the relationship between DNA helicases and NCO formation and the roles of structure specific endonucleases in CO formation.

1.1 Repair of DNA Damage by HR

Repair by HR can be broken down into three major pathways: DSB repair (DSBR), synthesis-dependent strand annealing (SDSA) and break-induced replication (BIR). In the model described by (Szostak et al., 1983), a DSB is the relevant lesion. The DSBR model is a modification of existing concepts outlined by Robin Holliday and by Meselson and Radding, where recombination was initiated by a nick on both chromatids (Holliday, 1964) or a single nick on one chromatid, respectively (Meselson and Radding, 1975). Both of these models involved a single Holliday Junction (HJ), a four-stranded structure formed where the duplexes exchange strands. Formation and movement of the HJ intermediate by branch migration results in heteroduplex DNA (hDNA), and the processing of hDNA leads to gene conversion, which is defined as the unidirectional transfer of information between chromosomes. The HJ intermediate can be cleaved to separate the duplexes and yield either a CO or NCO. These models were thus consistent with the observation that crossing over often accompanied regions of gene conversion. However, neither model provided an explanation for how multiple regions of gene
conversions could be produced within a single recombinant, a phenomenon that could be explained by two HJs in the DSBR model, one on either side of the initiating break.

Many groups have contributed additional molecular details to the DSBR model. Following 5’ to 3’ resection of the ends of the DSB (reviewed in Mimitou and Symington, 2009a), the 3’ single-stranded DNA (ssDNA) tail is coated with replication protein A (RPA), the eukaryotic ssDNA binding protein. The displacement of RPA and subsequent loading and polymerization of the Rad51 protein onto the ssDNA creates a recombinogenic nucleoprotein filament (reviewed in San Filippo et al., 2008b). Once coated with Rad51, the 3’ end conducts a homology search and invades an intact duplex molecule, pairing with the complementary strand and forming a displacement loop (D loop) structure (Sung, 1994). The invaded DNA duplex serves as the “donor” of information to repair the break, and the DNA duplex with the break is defined as the “recipient”. New DNA synthesis is primed off the invading 3’ end in a reaction dependent on proliferating cell nuclear antigen (PCNA) and DNA polymerase δ (Li et al., 2009), and the D loop is enlarged. Eventually, the expansion of the D loop exposes a region of homology to the other end of the break, and second-end capture can occur (Figure 1). A second round of DNA synthesis primed from the captured end and ligation of nicks results in a double Holliday junction (dHJ) intermediate. Resolution of the dHJ by symmetrical cleavage of two of the four strands can yield either a CO or a NCO. In theory, the HJ is symmetrical structure, and cleavage should give rise to equal numbers
of COs and NCOs. However, numerous studies in multiple organisms have found that NCO production is more frequent than CO production, especially in mitosis, suggesting additional levels of regulation in this process and/or the existence of alternative mechanisms that generate only NCOs (Esposito, 1978; Ira et al., 2003; Johnson and Jasin, 2000; Malkova et al., 2000; Nassif et al., 1994b; Virgin et al., 2001).

If synthesis proceeds off the invading 3’ end but the D loop collapses before second-end capture can occur, the newly extended 3’ end can anneal to the resected non-invading end of the break (Figure 1). Annealing will provide a template for synthesis of the other strand of the broken molecule, and the damaged sequence will be restored. Initially coined in experiments in *Drosophila melanogaster*, this process is called synthesis-dependent strand annealing (SDSA) (Nassif et al., 1994b). SDSA is a mechanism that only produces NCOs, and no change is expected to the donor molecule. As SDSA does not involve an HJ-containing intermediate, it has been proposed as the “earliest” pathway for repair of a DSB in meiosis and mitosis (Allers and Lichten, 2001; Ira et al., 2003).

The third HR pathway is BIR, in which invasion by the 3’ end creates a replication fork that extends to the end of the invaded chromosome (Malkova et al., 1996). The product of BIR has been described as a “half-crossover”, due to its similarity to one of the recombinant products of a true CO (Figure 1). BIR is a Rad52-dependent and Rad51-dependent process (Smith et al., 2009). Unlike DSBR or SDSA, BIR is
suppressed by the presence of the other end of the break (Jain et al., 2009). Specifically, the repression of BIR lasts for ~4-6 hours after the formation of an HO-endonuclease induced DSB, ample time for repair leading to a gene conversion, which can be detected within ~1-2 hours (Malkova et al., 2005; Sugawara et al., 2003). As BIR involves extensive synthesis, this process is dependent on POL32, which encodes a subunit of the Polδ replication complex (Lydeard et al., 2007).

1.2 Processing of hDNA and Repair by HR

Recombination most often occurs between sister chromatids, but the repair template can be found in homologous chromosomes in diploid cells or repetitive sequences located elsewhere in the genome. Regions of hDNA are the result of invasion or annealing reactions during HR, and hDNA can contain mismatches if the donor and recipient are not identical. Mismatches in regions of hDNA are processed by the mismatch repair (MMR) DNA repair pathway, the same process that corrects mismatched bases that result from replication errors introduced by DNA polymerases (Kunkel and Erie, 2005). Base-base mismatches and 1-2 bp insertion/deletion loops are recognized by Msh2-Msh6, the MutSα heterodimer. In the case of larger insertions or deletions (>2 bp), these errors are recognized by the MutSβ Msh2-Msh3 heterodimer. Binding by either heterodimer then recruits the MutLα complex (a Mlh1 and Pms1 heterodimer), which signals for further processing. During HR, removal of one of the strands and synthesis using the intact strand as a template results in the phenomenon of
gene conversion. If mismatches are not corrected, the next round of DNA replication will lead to a sectored colony as the mismatched strands segregate to separate cells.

In addition to providing the substrate for gene conversion, mismatches in regions of hDNA contribute to regulation of HR by triggering MMR-mediated anti-recombination (Evans and Alani, 2000). Indeed, a single mismatch has been shown to reduce the efficiency of HR significantly (Datta et al., 1997). Though the exact mechanisms are still unclear, the DNA helicase Sgs1 contributes to preventing repair off non-identical templates, presumably through an hDNA rejection process (Myung et al., 2001a; Spell and Jinks-Robertson, 2004a). Whether this control involves removal of the invading strand, destruction of the intermediate or prevention of extension of the invading 3’ end, the end result is the same: decreased repair in the presence of mismatches between donor and recipient molecules. Previous work with a plasmid-based gap-repair system demonstrated that total repair decreased when 2% sequence divergence was introduced and that CO products were decreased more than NCOs (Welz-Voegele and Jinks-Robertson, 2008). Genes implicated in the regulation of recombination fidelity were the helicases Sgs1 and Srs2, as well as the MMR components Msh2, Msh6, Mlh1 and Pms1. The Rad1 protein, a component of the Rad1-Rad10 endonuclease important for nucleotide excision repair (NER), was not necessary for regulating recombination between diverged sequences. However, Rad1 was required for ~90% of COs generated in this system. Thus, numerous levels of regulation exist to
prevent recombination between diverged sequences, and NCOs and COs are treated differently.

1.3 The Choice between NHEJ or HR is Cell-cycle Regulated through Control of End Resection

In addition to MMR-mediated antirecombination, another way that recombination between diverged sequences is prevented is by limiting the initiation of HR to when a template such as a sister chromatid, which is less likely to generate chromosomal changes, is present. In the G1 phase of the cell cycle, the genome has not yet been replicated, and NHEJ is favored (Clerici et al., 2008; Frank-Vaillant and Marcand, 2002; Mimitou and Symington, 2011). Correspondingly, in the S and G2 phases when a sister chromatid is available, HR is active (Aylon et al., 2004; Ira et al., 2004). While NHEJ can operate throughout other stages of the cell cycle, one mechanism of signaling that promotes NHEJ over HR during G1 is the regulation of end resection (Figure 2).

In yeast, the ends of a DSB are immediately bound by the highly conserved complex of Mre11-Rad50-Xrs2 (MRX) (Lisby et al., 2004). In fission yeast and mammals, this complex consists of the Mre11, Rad50 and Nbs1 proteins (the MRN complex). The MRX complex is involved in multiple processes including telomere maintenance, damage signaling, end tethering and end resection (reviewed in Stracker et al., 2004). Mutations disabling components of MRX or the DNA endonuclease Sae2 result in
sensitivity to IR, the accumulation of unprocessed meiotic DSBs and mitotic recombination defects (reviewed in Krogh and Symington, 2004).

**Figure 2: Regulation of end resection in yeast during G1 versus G2 stages of the cell cycle. (Figure adapted from Mimitou and Symington, 2009b)**

To limit HR in G1, resection of broken DNA ends is prevented through binding of the Ku70-Ku80 heterodimer (Lee et al., 1998; Tomita et al., 2003). The Ku proteins form a ring structure that protects the DNA end from nucleolytic degradation (Walker et al., 2001). The NHEJ reaction is stimulated by the presence of MRX (Boulton and Jackson, 1998), which may be due to the tethering capability of the complex, allowing the two ends to remain in close proximity (Chen et al., 2001). Following the recruitment
of the Ku proteins and downstream NHEJ factors including the Nej1 protein, the DNA ligase activity of the Dnl4 seals the break (reviewed in Daley et al., 2005).

In S or G2 phase, the MRX complex works with the Sae2 protein to promote resection and, concomitantly, HR (reviewed in Mimitou and Symington, 2009b). Phosphorylation by the cyclin-dependent protein kinase (CDK) Cdc28 is required for normal DNA end processing and couples the cell-cycle phase to resection (Aylon et al., 2004; Ira et al., 2004). One important target of Cdc28-dependent phosphorylation is Sae2, and a sae2-S267E allele that mimics constitutive phosphorylation complements the hypersensitivity to camptothecin, impaired end resection and other defects observed in the sae2Δ null mutant or a non-phosphorylatable sae2-S267A allele (Huertas et al., 2008). Together with MRX, Sae2 removes short oligonucleotides of approximately 50 – 100 nt from the 5’ DNA ends to initiate resection (Mimitou and Symington, 2008; Zhu et al., 2008). In G1, Sae2 is not phosphorylated and hence is not active. Thus, cleavage to trim the ends and remove MRX does not occur, and NHEJ is favored.

While the short tails produced by MRX and Sae2 are sufficient to produce gene conversion events, normal levels of HR require more extensive resection (Zhu et al., 2008). Genetic evidence suggests that two redundant pathways promote long-range resection after initial processing of the ends by MRX and Sae2. One of these pathways is dependent on Exo1, the 5’ to 3’ dsDNA exonuclease and 5’ flap endonuclease (Fiorentini et al., 1997; Tran et al., 2002). In physical analyses of end resection at a DSB, Exo1
promotes the formation of long 3’ ssDNA tails (Mimitou and Symington, 2008; Zhu et al., 2008). However, end resection still occurs at a slow rate in the mre11Δ exo1Δ double mutant, supporting the contribution of a second pathway to resection (Moreau et al., 2001). This second pathway is dependent on the DNA helicase Sgs1, the yeast ortholog of the bacterial RecQ helicase. In the absence of both Exo1 and Sgs1, very little resection occurs beyond the initial trimming by MRX and Sae2. Sgs1-dependent resection is thought to proceed by unwinding of the duplex by Sgs1 and then removal of the 5’ strand by the 5’ flap-endonuclease, Dna2 (Zhu et al., 2008). This model is supported by the reconstitution of end resection in vitro using purified MRX complex, Sgs1 with Top3 and Rmi1, Dna2 and RPA (Cejka et al., 2010a; Niu et al., 2010). Depletion of both the Sgs1 ortholog BLM and the EXO1 protein using siRNA indicates similar mechanistic redundancy for end resection in humans (Gravel et al., 2008).

1.4 Formation of the Presynaptic Filament

Additional regulatory barriers to HR follow end resection and must be overcome to successfully repair a DSB. As the 3’ ssDNA end is produced, it is quickly bound by the abundant ssDNA-binding protein, RPA (Sugawara et al., 2003; Wolner et al., 2003). However, the presence of RPA on the ssDNA prevents binding by Rad51 and inhibits HR (Figure 3). The recombination mediator protein Rad52 helps to recruit Rad51 to the DNA end, by binding Rad51 and delivering it to the RPA-coated ssDNA (New et al.,
The polymerization of additional Rad51 molecules then displaces RPA (Song and Sung, 2000; Sugiyama and Kowalczykowski, 2002).

![Diagram of presynaptic filament formation](image)

**Figure 3: Model for presynaptic filament formation.**

Following resection to create 3’ ssDNA tails, RPA binds the ssDNA and eliminates secondary structures. Rad51 is recruited to the ssDNA by Rad52 and RPA is displaced. The binding of Rad51 along with Rad55-Rad57 stabilizes the nucleoprotein filament, which is now capable of invading a duplex molecule. The resulting D loop is stabilized by the binding of Rad54. Figure adapted from Krogh and Symington, 2004.

Although biochemical data suggested that the heterodimeric complex of Rad55-Rad57 also serves as a recombination mediator helping to recruit Rad51, the formation of IR-induced Rad51 foci is not Rad55-Rad57 dependent (Lisby et al., 2004). In fact, Rad55-Rad57 focus formation requires Rad51. An alternative activity for the complex is
in stabilizing the Rad51 presynaptic filament once it is assembled (Fortin and Symington, 2002). A recent biochemical analysis indicated that Rad55-Rad57 indeed protects the Rad51 nucleoprotein filament against a salt challenge (Liu et al., 2011).

Once formed, the Rad51 filament can be dismantled. Specifically, the stability of the nucleoprotein filament is under assault by the antirecombinogenic activity of the DNA helicase Srs2 (Krejci et al., 2003; Veaute et al., 2003). However, the association of Rad55-Rad57 with Rad51 results in a nucleoprotein filament that is more resistant to dismantling by the translocase activity of Srs2 than a nucleoprotein filament containing Rad51 alone (Liu et al., 2011).

1.5 Helicase-mediated Regulation of HR Intermediates

Following the homology search, the nucleoprotein filament invades the donor duplex with the help of Rad54 and forms a D loop intermediate (Sugawara et al., 2003). Rad54 acts at multiple steps in HR but is especially important in stabilizing the D loop to allow the initiation of new DNA synthesis (reviewed in Heyer et al., 2006). The D loop can be expanded by synthesis or can migrate with the 3’ end (Ferguson and Holloman, 1996a). This intermediate is a crucial point for regulation of the HR outcome as the D loop can be dismantled to either produce NCOs or mature into an HJ, potentially yielding COs. Mechanistically, DNA helicases are presumed to affect the stability of the D loop. If adequate synthesis has occurred from the 3’ end, dismantling of the D loop will promote NCO formation via SDSA. If synthesis has not begun prior to collapse (or if
synthesis does not proceed far enough to provide a region of homology with the non-invading end), dismantling of the D loop will not repair the break, and total repair efficiency will decrease.

1.5.1 Mph1 is Proposed to Dismantle D loops

The DNA helicase presumed to dismantle D loops is Mph1, muator phenotype 1 (reviewed in Whitby, 2010)). Based on sequence homology, the Pyrococcus furiosus DNA helicase and nuclease protein, Hef, has been classified as an ortholog of Mph1 (Komori et al., 2002; Komori et al., 2004; Nishino et al., 2005). Hef contains both a superfamily 2 (SF2) helicase domain in its N-terminus and endonuclease activity in its C-terminus, and structural analysis of the N-terminal helicase domain revealed a novel structure that may promote the binding of branched and forked DNA (Nishino et al., 2005). Mutations in the gene for the human ortholog of Mph1 were identified in a patient with the rare genetic disease Fanconi anemia (FA), and it was assigned to FA complementation group M (FANCM) (Meetei et al., 2005). Work in chicken DT40 cells with the FANCM ortholog confirmed that this protein is part of the FA core complex, which is recruited to sites of DNA damage (Mosedale et al., 2005).

Mph1 is a 3’ to 5’ helicase (Prakash et al., 2005), and purified Mph1 protein can unwind D loop substrates in vitro (Prakash et al., 2009). In the absence of Mph1, the proportion of COs among the repair products of an HO-induced DSB increases, consistent with loss of pro-SDSA activity (Prakash et al., 2009). Additionally, loss of
Mph1 does not decrease the efficiency of the repair, suggesting that Mph1 dismantles the D loop after synthesis has begun. Work with a plasmid-based gap-repair system also found that loss of Mph1 increased the proportion of CO products (Tay et al., 2010). A similar increase in COs was observed with a plasmid-based assay in the absence of the *Schizosaccharomyces pombe* Mph1 ortholog, Fml1 (Sun et al., 2008).

Complicating matters is that Mph1 has another role in DNA damage repair, which may or may not be related to its role disassembling D loops. In the absence of Mph1, the rate of spontaneous mutations increases (Entian et al., 1999), as does sensitivity to DNA damaging agents (Schurer et al., 2004). This effect is presumed to be due to a role in promoting template switching over translesion synthesis (TLS) to bypass DNA lesions during DNA replication. TLS uses alternative polymerases that have less-restrictive active site pockets that can accommodate bulky lesions that block normal replicative polymerases (Yang and Woodgate, 2007). However, the TLS polymerases have lower fidelity and can cause mutations. Template switching uses the newly synthesized strand of the sister chromatid as a template to bypass a lesion, which can occur via fork reversal or strand invasion (Zhang and Lawrence, 2005). Template switching requires genes from the Rad52 epistasis group, and *rad51Δ* is epistatic to *mph1Δ* in regards to the increased mutation rate (Schurer et al., 2004). In addition, the increased mutagenesis of the *mph1Δ* strain is dependent on Rev3, the catalytic subunit of the TLS polymerase Polζ (Schurer et al., 2004). Thus, it has been proposed that Mph1
promotes lesion bypass during replication by template switching, avoiding potentially mutagenic bypass by TLS. Mechanistically, Mph1 may positively affect template switching multiple ways; options include regression of the replication fork, resetting a regressed replication fork, or dismantling a D loop formed upon invasion of a sister chromatid (Whitby, 2010).

Finally, Mph1 appears to be involved in another aspect of DNA replication: lagging strand synthesis. A multicopy suppressor of the lethality of a dna2K1080E allele, Mph1 was implicated in Okazaki fragment maturation (Kang et al., 2009). Furthermore, Mph1-stimulated cleavage of 5’ flap substrates by both Fen1 and Dna2 has been demonstrated by in vitro analysis. Although the relevance of this activity in vivo is unknown, the multiple roles of Mph1 in aspects of DNA metabolism highlight the importance of a targeted assay that assesses only a single function.

1.5.2 Srs2 Promotes NCOs during HR

Biochemical studies have determined that Srs2 removes Rad51 from ssDNA ends, inhibiting formation of the presynaptic filament and preventing recombination (Krejci et al., 2003; Veaute et al., 2003). The bacterial ortholog of Srs2, UvrD, has translocase activity as well and can remove RecA from ssDNA (Veaute et al., 2005). Fission yeast has two UvrD-like DNA helicases, SpSrs2 and SpFbh1 (Morishita et al., 2005). Fbh1 is conserved in human, and ectopic expression of FBH1 can complement srs2Δ defects in S. cerevisiae (Chiolo et al., 2007). Very recently, an additional human
homolog of Srs2, PARI, was identified by its structural similarity to Srs2 (Moldovan et al., 2012). Depletion of PARI in HeLa cells by RNA interference led to increased chromosomal rearrangements following exposure to mitomycin C, increased Rad51 foci upon IR treatment and increased HR in a direct repeat recombination assay. These results suggest that PARI, like Srs2, interacts with Rad51 and regulates HR by dismantling nucleoprotein filaments. The ability to strip Rad51 from DNA may be a mechanism for recombination “quality control” that eliminates unnecessary or suboptimal recombination substrates. This anti-recombination activity is consistent with early genetic evidence that loss of Srs2 leads to increased spontaneous recombination (Aguilera and Klein, 1988; Rong and Klein, 1993). Increased Rad51 and Rad54 foci are formed in the absence of Srs2, and many of these spontaneous recombination foci are not sites appropriate for repair via HR (Burgess et al., 2009). In addition, a truncated Srs2 protein that does not interact with Rad51 is defective in anti-recombination activity both in vitro and in vivo (Antony et al., 2009; Colavito et al., 2009).

Srs2 was originally identified as a suppressor of Rad Six sensitivity to ultraviolet light exposure, implicating the helicase in DNA damage tolerance (Lawrence and Christensen, 1979). However, suppression is not observed in the absence of genes in the Rad52 epistasis group, RAD51, RAD52, RAD54, RAD55, RAD57 (Schiestl et al., 1990), and loss of Srs2 has been suggested to promote lesion bypass through allowing additional recombination. The PCNA protein is a sliding clamp that increases
processivity during DNA replication by tethering the polymerase to the template (Moldovan et al., 2007), and post-translational modification of PCNA also coordinates the response to DNA damage at the replication fork (reviewed in Chang and Cimprich, 2009). Monoubiquitination of PCNA by Rad6 and Rad18 signals for error-prone bypass of lesions by TLS polymerases, and polyubiquitination signals for error-free template switching. SUMOylation of PCNA can also occur, and further studies have revealed that SUMOylated PCNA recruits Srs2 to the replication fork to prevent recombination during S-phase (Pfander et al., 2005; Stelter and Ulrich, 2003).

While Srs2 has anti-recombination activity during spontaneous recombination and post-replicative repair, a prorecombination role has been observed in the repair of an HO-induced DSB (Aylon et al., 2003). Additional work with the HO system revealed that loss of Srs2 led to decreased overall levels of repair and an increased proportion of COs (Ira et al., 2003). A similar increase in COs was observed in the absence of Srs2 using a spontaneous intragenic recombination assay (Robert et al., 2006) and using a gap-repair assay (Welz-Voegele and Jinks-Robertson, 2008). To investigate how deletion of Srs2 resulted in the loss of NCOs products during the repair of a DSB, Rad51 was overexpressed in an srs2Δ background, and this led to almost complete elimination of NCO products (Ira et al., 2003). Loss of Srs2 also had an effect on the kinetics of repair; the absence of Srs2 led to NCOs and COs appearing at the same time, but NCOs
appeared before COs in WT cells. Both of these results are consistent with Srs2
stimulating repair by promoting SDSA.

In investigations with a synthetic DNA structure that mimics a D loop, Srs2
binds ssDNA coated with RPA (the displaced strand) and unwinds dsDNA coated with
Rad51, supporting an active role for the protein during SDSA (Dupaigne et al., 2008).
However, the relevance of this activity in vivo has been questioned because purified Srs2
cannot dismantle a D loop in vitro at concentrations where purified Mph1 possesses this
activity (Prakash et al., 2009). Mph1 was also more efficient than Srs2 or Sgs1 in
displacing an extended primer in an in vitro assay using polymerase δ or η to extend the
single strand of an invaded D loop (Sebesta et al., 2011). An alternative explanation of
the pro-recombination role of Srs2 is that it removes residual Rad51 from the non-
invading 3′ end of the DSB, which would promote annealing and allow the completion
of SDSA. During SSA, Srs2 appears to promote the annealing of resected ends through a
similar mechanism (Carter et al., 2009). However, there is no evidence for this activity in
SDSA, and it is still unclear how Srs2 is involved in NCO formation. Studies of HO-
induced recombination revealed defects at a population level in the absence of Srs2 (Ira
et al., 2003), but using Southern blots to detect repair products does not require the
individual cell to be viable. Additionally, this method cannot differentiate between
NCOs that are formed by SDSA, by dHJ dissolution or by HJ cleavage.
1.5.3 Sgs1 Promotes NCOs during HR

A third DNA helicase that controls the NCO or CO outcome of HR in yeast is Sgs1, a member of the RecQ DNA helicase family. RecQ helicases share a similar structural organization with a core helicase domain that is dependent on both ATP and Mg$^{2+}$ to unwind dsDNA (reviewed in Bernstein et al., 2010). RecQ helicases are important for proper DNA damage repair, and homologs have been identified in Escherichia coli, fungi, plants and mammals. There are five RecQ homologs in humans (BLM, WRN, RTS/RECQ4, RECQ1/RECQL1 and RECQL5). Of these, mutations in BLM, WRN and RECQ4 are associated with a predisposition to cancer and premature aging, and the corresponding genetic disorders are referred to as Bloom’s syndrome, Werner’s syndrome and Rothmund-Thomson syndrome, respectively (reviewed in Hanada and Hickson, 2007). Patients with Bloom’s syndrome display an elevated rate of sister chromatid exchange, suggesting loss of regulation of HR (Chaganti et al., 1974).

The S. cerevisiae RecQ homolog was isolated as a suppressor of the slow growth phenotype observed in a top3Δ strain, and loss of Sgs1 increased recombination at the ribosomal DNA (rDNA) repeats (Gangloff et al., 1994). In vitro, Sgs1 exhibits 3’ to 5’ DNA helicase activity (Bennett et al., 1998; Cejka and Kowalczykowski, 2010), and spontaneous gross chromosomal rearrangements are increased in an sgs1Δ strain (Myung et al., 2001a). Consistent with a role for Sgs1 controlling excess recombination, loss of both Sgs1 and Srs2 causes synthetic lethality that is dependent on HR (Gangloff et
The interaction between Top3 and Sgs1 is necessary to prevent hyper-recombination, as a both a helicase-dead allele of Sgs1 and an allele of Sgs1 that can no longer bind Top3 display increased sister-chromatid exchange and interchromosomal heteroallelic recombination (Onoda et al., 2000; Ui et al., 2005).

While Sgs1 contributes to processive end resection (see above), this role cannot fully explain the increase in COs observed in sgs1Δ strains during the repair of mitotic or meiotic DSBs (Ira et al., 2003; Rockmill et al., 2003). Rad51-dependent joint molecules accumulate in response to damage in the absence of Sgs1, suggesting that Sgs1 promotes HJ-resolution (Liberi et al., 2005). Furthermore, these joint molecules can be removed in vivo by ectopic expression of the E. coli HJ resolvase RusA or human GEN1 (Mankouri et al., 2011). Sgs1 forms a complex with Top3 and Rmi1 (Bennett et al., 2000; Chang et al., 2005), and this complex can dissolve synthetic dHJs in vitro by decatenation of the two molecules (Cejka et al., 2010b). Initially described with purified BLM and hTOPO IIIα (the human Top3 ortholog), the dissolution of the dHJ intermediate by a RecQ helicase complex is proposed to yield exclusively NCOs (Wu and Hickson, 2003). This finding was replicated using a topologically constrained dHJ substrate with the Drosophila BLM ortholog (DmBlm) and topoisomerase IIIα (Plank et al., 2006). Dissolution activity thus provides a molecular mechanism to suppress COs during HR (reviewed in Mankouri and Hickson, 2007). However, evidence for Sgs1 performing this role in vivo is limited.
In addition to the well-documented anti-recombination activity of Sgs1, work with a plasmid-based gap repair assay has demonstrated a small but significant pro-recombination role for Sgs1 in HR (Welz-Voegele and Jinks-Robertson, 2008). A similar decrease in total repair efficiency has also been observed in the absence of Sgs1 following an HO-induced DSB (Ira et al., 2003) and in the absence of BLM in murine embryonic stem cells (LaRocque et al., 2011). The decrease in repair products in the absence of Sgs1 is not completely explained by loss of dHJ dissolution, as dHJ intermediates are rare during mitosis (Bzymek et al., 2010).

Studies by Sekelsky and colleagues have suggested that DmBlm promotes NCO formation by SDSA. Repair of DSBs formed by excision of a P element led to NCO products consistent with SDSA, but deletions between the two ends increased in the absence of DmBLM (Adams et al., 2003). These deletions were eliminated in the absence of DmRad51, suggesting that DmBlm acts downstream of invasion at the D loop intermediate (McVey et al., 2004). Further support for RecQ helicases unwinding D loop intermediates comes from biochemical studies using both static and mobile synthetic D loops (Bachrati et al., 2006; van Brabant et al., 2000).

A similarly complex role has been proposed for Sgs1 in meiosis. In S. cerevisiae, COs are modestly increased following meiosis in the absence of Sgs1 (Rockmill et al., 2003). However, in the absence of the meiotic ZMM proteins that help make up the synaptonemal complex (SC), the presence of Sgs1 dramatically decreases the proportion
of COs (Jessop et al., 2006). Finally, Sgs1 appears to dismantle aberrant recombination intermediates formed from three or more sister chromatids (Oh et al., 2007). These studies suggest that Sgs1 helps to coordinate normal CO formation in meiosis.

Despite the biochemical evidence that Sgs1 can dissolve dHJs and the increase in COs observed upon loss of Sgs1 in genetic studies, the field still lacks in vivo evidence that Sgs1 is important for the production of NCOs by dissolution. The dHJ dissolution model specifically predicts that the template molecule should remain unchanged and regions of gene conversion will be present on both sides of the break in the repaired allele (where regions of hDNA were originally formed). Regions of gene conversion formed during the repair of an HO-induced break were mapped in the absence of Sgs1, but no decrease in bidirectional tracts was observed (Lo et al., 2006). As the overall levels of HR were similar between wild-type and sgs1Δ strains, this study did not detect a role for Sgs1 catalyzing dHJ migration to yield dissolution. However, the data were difficult to interpret because only the recipient allele was examined, and the donor allele was not queried for regions of gene conversion to rule out HJ cleavage. In addition, the abundance of discontinuous gene conversion tracts highlights the importance of mapping hDNA prior to repair by the MMR machinery, which can obscure the original locations of strand transfer.


1.6 Resolution of HJ-containing Intermediates

If the D loop persists by evading helicase-mediated disassembly, an HJ can be formed. Nicking of the displaced strand will form a single HJ (sHJ), and second end capture will form a double HJ (dHJ). The dHJ intermediate is crucial to the DSBR model of (Szostak et al., 1983), as it can give rise to both NCOs and COs by symmetrical cleavage of two of the four strands. In E. coli, HJ cleavage is conducted by the HJ resolvase RuvC (Bennett et al., 1993), which acts in a complex with RuvA and RuvB (reviewed in West, 1997). RuvA forms a tetramer, and two RuvA tetramers bind the HJ, which then adopts a fourfold symmetric square-planar configuration. The DNA helicase RuvB forms a hexameric ring that is recruited by RuvA to the HJ, and two RuvB rings bind opposite arms of the junction to promote branch migration. A RuvC dimer then nicks the opposing strands of DNA at a tetranucleotide consensus sequence, and ligation of the nicks produces mature recombinant products.

The field has long been engaged in the search for the eukaryotic RuvC homolog (reviewed in Schwartz and Heyer, 2011). The RuvC paradigm made the following testable predictions about the activity of a eukaryotic endonuclease: it would preferentially cleave HJs over other forked structures, it would cleave symmetrically to generate either NCOs or COs from a common substrate and the nicked products of cleavage would be ligated directly without further processing by additional enzymes. An endonucleolytic activity capable of resolving an extruded cruciform structure was
detected and partially purified from *S. cerevisiae* as early as 1985 (Symington and Kolodner, 1985). One such protein was eventually purified from yeast cell extracts, but this protein, Cce1, was determined to be a mitochondrial endonuclease (Kleff et al., 1992). Accordingly, loss of Cce1 had no effect on mitotic or meiotic recombination. Finally, residual cruciform-cutting activity was detected in a cce1 null background, indicating that other enzymes exist to process HJs in eukaryotes.

A RuvC-like activity relevant to nuclear HJ resolution was eventually identified as Yen1 in a screen of a yeast gene fusion library (Ip et al., 2008). The same group identified the human ortholog, GEN1, by fractionation of HeLa cell-free extracts and assaying for cleavage of a synthetic HJ substrate. Both of these proteins are members of the Rad2/XPG family of structure-selective endonucleases that includes Rad2, Rad27 and Exo1. These proteins do not cleave synthetic HJs, and their endonucleolytic activity participates in other functions of DNA metabolism (reviewed in Lieber, 1997). The biochemical properties of Yen1 and GEN1 appear similar to those of RuvC, but the biological functions of these proteins remain unclear.

Another protein with an important role in HJ resolution in fungi is Mus81 (reviewed in Hollingsworth and Brill, 2004). Mus81 is related to the human XPF subunit of the ERCC1-XPF endonuclease that acts in nucleotide excision repair (NER). In *S. cerevisiae*, Mus81 forms a heterodimer with Mms4 and cleaves a variety of branched structures (Kaliraman et al., 2001). This complex is required for DNA damage tolerance.
in the absence of Sgs1 (Mullen et al., 2001). In \textit{S. pombe}, Mus81 forms a heterodimer with Eme1 that is capable of resolving a HJ into two linear duplex molecules (Boddy et al., 2001). In addition, CO formation during meiotic recombination in \textit{S. pombe} is severely disabled in the absence of this complex (Smith et al., 2003). However, the defect in sporulation in the absence of Mus81 in \textit{S. cerevisiae} is mild, indicating that meiotic recombination can occur without this complex (de los Santos et al., 2003; Interthal and Heyer, 2000; Kaliraman et al., 2001). Furthermore, Mus81 cleaves synthetic HJs with low efficiency, preferring a D loop or a nicked junction (Fricke et al., 2005; Gaillard et al., 2003; Kaliraman et al., 2001; Osman et al., 2003).

The XPF homolog in budding yeast is the NER endonuclease, Rad1, which forms a heterodimer with Rad10 (Ciccia et al., 2008). In addition to its major role in nicking DNA to remove a damage-containing oligonucleotide (Guzder et al., 1995), Rad1 can bind and cleave multiple branched and forked structures, including a synthetic HJ (Habraken et al., 1994). Rad1 specifically nicks at the junction between duplex and single stranded DNA (Bardwell et al., 1994). Genetic studies have indicated that Rad1 may be important for integration of plasmids and linear fragments into the genome (Schiestl and Prakash, 1988b) and for CO formation during mitotic gap repair (Symington et al., 2000b; Welz-Voegele and Jinks-Robertson, 2008). However, meiotic COs in yeast do not appear to have a similar requirement for Rad1 (Dowling et al., 1985; Kearney et al., 2001). In \textit{Drosophila}, the Rad1 ortholog is MEI-9, and \textit{mei-9} mutant flies are defective at
meiotic CO production but generate NCOs at levels very similar to wild-type flies (Radford et al., 2007; Sekelsky et al., 1995). Given these complicated results, additional studies are needed to clarify the role of Rad1 in generating mitotic COs.

Redundancy amongst structure-specific endonucleases has posed a major obstacle to the study of CO formation. Both Mus81 and Yen1 contribute to the repair of DSBs during mitosis (Blanco et al., 2010; Ho et al., 2010; Tay and Wu, 2010) and meiosis (Agmon et al., 2011), but Mus81 appears to be the dominant player. During meiosis, Mus81-Mms4 is phosphorylated by Cdc5, which increases the resolution of joint molecules and, as such, successful chromosome segregation in meiosis I (Matos et al., 2011). Yen1 is activated in meiosis II, following the accumulation of Clb3, to resolve persistent joint molecules. Similar post-translational regulation of the endonucleolytic activity of Mus81-Mms4 and Yen1 was also detected in synchronized mitotic cells. However, two recent studies have implicated a third major pathway for the resolution of joint molecules during meiosis: Sgs1, Exo1 and MutLγ, which consists of Mlh1-Mlh3 (De Muyt et al., 2012; Zakharyevich et al., 2012). Importantly, this newly characterized Exo1 and MutLγ CO-promoting activity is exclusively meiotic, adding to the model of how cells achieve sufficient COs to successfully complete meiosis.

1.7 Rationale for my Thesis Research

Many of the studies discussed above have used physical assays to investigate the outcome of repair of a DSB in yeast. Although these studies have been tremendously
useful, they have not provided detailed molecular information about intermediates formed during HR. Often, the CO or NCO distinction is all that can be gleaned from the detected products. In the research reported in this thesis, a plasmid-based gap repair assay has been used in an MMR-deficient haploid yeast strain background to identify regions of hDNA that remain following repair. This system allows the study of both CO and NCO products, but, importantly, it allows us to distinguish different mechanisms for the formation of COs and NCOs based on the location of hDNA in the donor and repaired alleles. Chapter 2 will present work on the molecular structures of recombination intermediates generated during gap repair. Abundant biochemical and genetic data on regulatory proteins that function in HR can be used to make predictions regarding their impact on specific classes of either NCOs or COs. The data presented in Chapter 3 demonstrate that three DNA helicases, Mph1, Sgs1 and Srs2, all promote NCOs through distinct mechanisms. Finally, the impact of the putative HJ resolvases on the structure of CO products is investigated in Chapter 4.

1.8 Notes Regarding Funding and Authorship

This work was supported by NIH grant GM038464 to SJR. KM is the primary author of all work presented in this document. In the multi-author publication that comprises Chapter 2, KM prepared Figure 6, Figure 7, and Figure 9 and contributed to the analysis and text. In the multi-author publication that comprises Chapter 3, KM was the primary author and was involved in all aspects of its production. For the
unpublished work that comprises Chapter 4, KM performed all the experiments
described, generated all figures and is the primary author of the text.
Chapter 2. Molecular Structures of Crossover and Noncrossover Intermediates during Gap Repair in Yeast: Implications for Recombination Mechanisms

Chapter two was previously published as Mitchel et al., 2010.

2.1 Summary

The molecular structures of crossover (CO) and noncrossover (NCO) intermediates were determined by sequencing the products formed when a gapped plasmid was repaired using a diverged chromosomal template. Analyses were done in the absence of mismatch repair (MMR) to allow efficient detection of strand-transfer intermediates, and the results reveal striking differences in the extents and locations of heteroduplex DNA (hDNA) in NCO versus CO products. These data indicate that most NCOs are produced by synthesis-dependent strand annealing rather than by a canonical double-strand break repair pathway, and that resolution of Holliday junctions formed as part of the latter pathway is highly constrained to generate CO products. We suggest a model in which the length of hDNA formed by the initiating strand invasion event determines susceptibility of the resulting intermediate to antirecombination and ultimately whether a CO- or a NCO-producing pathway is followed.

2.2 Introduction

In mitotically-dividing cells, homologous recombination is important for the repair of double-strand breaks (DSBs) generated directly by agents such as ionizing radiation, as well those that arise spontaneously through encounters of the replication
machinery with DNA damage. In meiosis, the repair of programmed DSBs via recombination between homologous chromosomes is essential for proper chromosome segregation. The basic mechanisms of recombination are highly conserved between mitosis and meiosis, and the ability to study both has made the yeast *Saccharomyces cerevisiae* a particularly attractive model system. Here, we use the repair of a gapped plasmid in mitosis to model DSB repair in yeast.

Multiple mechanisms of homologous recombination are used to repair DSBs in yeast (for reviews see Paques and Haber, 1999b; San Filippo et al., 2008a; Symington, 2002b), and all begin with resection of the 5′ ends to yield 3′ single-stranded tails. In the classic DSB repair (DSBR) model of recombination (Szostak et al., 1983), one of the 3′ tails thus exposed invades a homologous duplex DNA molecule, creating a region of heteroduplex DNA (hDNA) and a displaced, single-stranded D-loop (Figure 4, step A). Extension of the invading 3′ end by DNA polymerase enlarges the D-loop, thereby exposing sequences complementary to the other side of the original DSB. Annealing of the D-loop to the other (noninvading) 3′ end yields an intermediate with two Holliday junctions (HJs; Figure 4, step B), the resolution of which either maintains the original linkages of sequences that flank the junctions (a noncrossover or NCO event) or switches the flanking sequences (a crossover or CO event). Resolution of HJs can occur by sequential nicking and ligation reactions, or can be mediated by the combined action of a helicase and topoisomerase (Figure 4, steps C and D, respectively). Whereas the
former mechanism is generally assumed to generate both CO and NCO products, the latter “dissolution” mechanism generates only NCOs. In the synthesis-dependent strand annealing (SDSA) model, the D-loop collapses and the extended 3’ end is displaced from the invaded duplex, allowing it to anneal to the single-stranded tail on the other side of the DSB (Figure 4, step E). Because HJs are not formed, SDSA results only in NCO events. A distinguishing feature of DSBR is the formation of hDNA on both sides of the repaired break; in SDSA only a single tract of hDNA persists in the repaired products (gray boxes in Figure 4).

Recombination is a homology-driven process that can involve sister chromatids, homologous chromosomes or ectopic sequences dispersed throughout the genome. While interactions between identical sister chromatids are typically of no genetic consequence, recombination between homologs can uncover recessive markers and ectopic interactions can generate a wide variety of genome rearrangements. When mitotic recombination occurs, it clearly is important that it involve the “correct” sequences in order to avoid undesirable genetic outcomes. The rate of ectopic recombination in yeast is directly proportional both to the length of total homology (Jinks-Robertson et al., 1993) and to the degree of sequence identity (Datta et al., 1997) between the substrates.
Figure 4: SDSA and DSBR models of gap repair.

Solid blue and orange lines correspond to single strands of the gapped and intact alleles, respectively; arrowheads represent the 3' ends of DNA strands. Dotted lines correspond to new DNA, which is colored according to the template directing its synthesis. The solid triangle at the distal end of the D-loop formed in step A represents the position of Rad1-Rad10 cleavage to facilitate second-end capture and generate an intermediate with a single HJ. With the double HJ, a CO results when the exchanged strands are nicked at one junction and the unexchanged strands are nicked at the other junction; the CO position occurs at the junction where the unexchanged strands are nicked. Gray boxes indicate the positions of hDNA in intermediates and products.

When interacting sequences are not identical, an hDNA intermediate can contain mismatches that are processed by the mismatch repair (MMR) machinery. The repair of such mismatches results in the classic genetic phenomenon of gene conversion, which is defined as the unidirectional transfer of information from one DNA duplex to another. If mismatches fail to be repaired, the mismatched strands will segregate at the next
round of replication, resulting in a sectored colony. In addition to initiating a simple repair process, mismatches in hDNA intermediates can block the production of mature recombinants (reviewed by Surtees et al., 2004). The enforcement of stringent identity requirements during recombination derives primarily from antirecombination activity of the MMR system, a system best characterized with respect to its role in correcting DNA synthesis errors. In yeast, the major players in nuclear mismatch recognition/repair are Msh2, Msh3 and Msh6, which are MutS homologs, and the MutL homologs, Pms1 and Mlh1 (for reviews see Harfe and Jinks-Robertson, 2000; Kunkel and Erie, 2005). In addition to these core MMR proteins, the repair of replication errors involves a 5′ > 3′ exonuclease (Exo1) and the PCNA sliding clamp. Whereas the core MMR proteins and Exo1 are also important for regulating mitotic recombination fidelity in yeast (e.g., see Nicholson et al., 2000b), PCNA appears to play little, if any, role in this process (Stone et al., 2008). In addition, the Sgs1 helicase is important in antirecombination (Myung et al., 2001b; Spell and Jinks-Robertson, 2004b; Sugawara et al., 2004), but has no known function during the general repair of mismatches. These genetic studies suggest a basic mechanistic difference between the replication- and recombination-related activities of the MMR system.

In the current study, gapped plasmids were transformed into a haploid, MMR-defective strain containing a diverged chromosomal repair template. Following the selection of recombinants, the extent of hDNA in CO and NCO intermediates was
inferred by sequencing the products derived from individual repair events. Results
demonstrate that the structures of CO and NCO intermediates are distinctly different, an
observation most easily explained if COs and NCOs are generated by DSBR and SDSA,
respectively. Additional examination of gap-repair products generated in the presence
of MMR provides molecular confirmation that the MMR-directed correction of
mismatches in hDNA is distinct from MMR-associated antirecombination.

2.3 Results

The transformation-based gap-repair system used previously to examine the
effect of sequence divergence on mitotic CO and NCO events is shown in Figure 5
(Welz-Voegele and Jinks-Robertson, 2008). In this system, a plasmid-encoded HIS3 gene
contains a gap that must be repaired using a 2%-diverged chromosomal template (a
his3Δ3′ allele integrated at the CAN1 locus on chromosome V; see Appendix A Figure 23
for sequence polymorphisms) in order to generate a His⁺ transformant. Following the
selection of His⁺ colonies, CO and NCO events are distinguished based on the relative
stability and instability, respectively, of the plasmid-encoded URA3 marker. To
preserve hDNA in NCO versus CO intermediates, recombination products were initially
derived in an MMR-deficient (mlh1Δ) background. In the absence of mismatch
correction, hDNA is resolved by DNA replication to yield a sectored colony in which
Figure 5: Gap-repair system.

The plasmid contains an ARS, the *URA3* gene (gray) and a gapped *his3* allele (orange). The *his3Δ3’* allele is (blue) located at the *CAN1* locus on chromosome V. NCO and CO events generate His⁺ transformants with an unstable and stable Ura⁺ phenotype, respectively. The positions of primers used to selectively amplify recombination products are indicated.

Half of the cells contain one allele and half contain the other allele. To allow the detection of both alleles, DNA was isolated from His⁺ transformants without prior colony purification. In the case of NCO recombinants, both the reconstituted, plasmid-encoded *HIS3* allele and the chromosomal repair template (recipient and donor alleles, respectively) were sequenced; in CO recombinants, both chromosomal alleles produced by plasmid integration were sequenced. Finally, to examine the antirecombination-related function of the MMR system, recombinants also were derived in an MMR-competent host strain.
2.3.1 NCO Intermediates in the BglII Gap-Repair System

In the BglIII system used previously, removal of BglIII fragment from the HIS3 coding sequence generates an approximately 60-bp gap relative to the chromosomal repair template (see Appendix A, Figure 23). This gap is asymmetrically positioned within the region of donor homology, with 600 and 160 bp of homology upstream and downstream of the gap, respectively. In addition, the first upstream polymorphism is farther away from the gap than the first downstream polymorphism (approximately 80 and 30 bp, respectively). In the 91 NCO products analyzed, all sequence transfer was confined to the repaired allele; we did not detect any acquisition of plasmid sequences by the chromosomal donor allele. The structures of the repaired alleles are presented in Figure 6A. The presence of two complementary donor strands in the recipient allele corresponds to a gene conversion event, the extent of which is indicated by a blue donor segment in an otherwise yellow recipient allele. Sequence flanking the gap should be exchanged in the form of single strands to yield hDNA intermediates, and tracts are indicated as green segments. There was transfer of donor sequences flanking the gap in 75% (68/91) of the repaired plasmids and hDNA was detected >95% of the time (66/68). The few examples of gene conversion of markers flanking the gap could reflect nucleolytic expansion of the original gap, a failure to detect both products following replicative hDNA resolution, or a repair system that acts in the absence of the canonical MMR system.
Figure 6: NCO products generated in the absence of MMR.

The chromosomal his3∆3′ and gapped alleles are indicated at the top of each panel in blue and yellow, respectively. Gap positions are indicated by the long, vertical blue lines; the positions of silent sequence polymorphisms are indicated by the short, vertical black lines in the gapped alleles (see Appendix A, Figure 23 for sequence changes). Each horizontal line corresponds to an independent gap-repair event, and the extent of DNA transferred from the chromosome is indicated. Green lines correspond to hDNA and blue lines to gene conversion events. Tract endpoints were placed between the most gap-distal polymorphism transferred and the next, unchanged polymorphism.

Of the 66 plasmids with hDNA, ~90% acquired donor sequence on only one side of the gap. The strong bias for unidirectional hDNA suggests that most NCO events were derived from SDSA rather than from DSBR, and hence did not involve an HJ-containing intermediate. Although an alternative interpretation is that the accompanying hDNA on the other side of the gap is usually too short to be detected,
additional results with the BssHII system indicate that bidirectional hDNA is indeed rare (see below). In the SDSA model, the hDNA formed by invasion of the donor is dismantled and the newly-extended 3’ end anneals to the single-stranded tail on the other side of the DSB. The single tract of hDNA is thus on the side of the break opposite the side that initiates strand invasion (Figure 4), and the extent of hDNA in the product reflects the amount of DNA synthesis primed from the invading end. All but one of the unidirectional hDNA tracts was downstream of the gap, indicating that most of the initiating strand invasion occurred upstream of the gap. We note that the upstream side has the most total homology as well as the greatest length of gap-adjacent uninterrupted sequence identity, either of which could potentially bias the distribution of unidirectional hDNA.

When SDSA is initiated by strand invasion upstream of the gap, the invading 3’ end must be extended 60 nt to traverse the gap, plus an additional distance to allow annealing to the single-stranded tail on the other side. In any given NCO event, DNA synthesis extends past the most gap-distal polymorphism transferred, but presumably not as far as the next polymorphism. The average length of DNA synthesis associated with strand invasion was calculated by assuming that synthesis began precisely at the upstream edge of the gap and ended halfway between the two relevant polymorphisms. We estimate that the invading end was used to prime ~170 nt of DNA synthesis from the donor template. Although we assume here for simplicity that hDNA length directly
reflects the extent of DNA synthesis from the invading end, is also possible that it instead reflects the extent of end resection on the annealing side of the gap. In the latter case, the invading end could be over-replicated relative to the end to which it anneals, which would require removal of the over-replicated end before ligation.

Although most NCOs in the BglIII system had an hDNA tract, there were 23 events in which no hDNA was detected. If these reflect a failure to detect segregated hDNA, then there should be an equivalent number of NCOs with only donor polymorphisms (i.e., gene conversions). Only two gene conversions were detected, however, suggesting that most of the “no hDNA” class reflects short tracts of DNA synthesis that did not extend to the first polymorphism. In these cases, it is impossible to discern whether the initiating strand invasion occurred upstream or downstream of the gap. Finally, there were four NCOs with hDNA on both sides of the repaired gap, characteristic of a DSBR intermediate resolved by dissolution rather than by cleavage (Figure 4D).

2.3.2 NCO Intermediates in the BssHII System

As noted above, the asymmetry of the gap in the BglIII system could limit the length and/or direction of hDNA detected among NCOs. The presence of a 60-bp gap rather than a simple DSB could also affect recombination pathway choice and/or outcome. To address these issues, we used the same diverged his3 sequences to develop a second gap-repair system. This was accomplished by introducing a BssHII
site near the center of the 820-bp donor-recipient homology (see Appendix A, Figure 23). Digestion of the resulting plasmid with BssHII prior to transformation generates an 8-bp gap relative to the chromosomal donor allele. In addition to the smaller gap, the first flanking polymorphisms are closer to the gap and are more symmetrically positioned than in the BglII system; both are ~20 bp from the BssHII-generated gap. As with the BglII system, no transfer of DNA from the broken plasmid to the chromosomal donor was detected among the 88 NCOs analyzed. The structures of the repaired plasmid alleles are shown in Figure 6B.

The locations of the hDNA tracts with respect to the gap were similar to those in the BglII system: 92% of the tracts detected were unidirectional (66/72), consistent with the SDSA mechanism, and there was a strong bias for the unidirectional tracts to be downstream of the gap (54/66). The small number of bidirectional tracts detected in the BssHII system (6/72), which are consistent with HJ dissolution rather than cleavage, was also similar to that observed with the BglII system (4/66). Eight of the unidirectional hDNA tracts were associated with contiguous, gap-proximal gene conversion tracts. Because of their hDNA association, these gene conversion tracts most likely reflect gap expansions. Using the downstream, unidirectional hDNA tracts to calculate DNA synthesis, we estimate an average length of ~235 nt of DNA synthesis in the BssHII system, which is ~40% longer than that in the BglII system. Finally, there were seven unidirectional gene conversion tracts that were not associated with contiguous
hDNA. The occurrence of an equivalent number of NCOs with no detectable hDNA suggests that these gene conversions likely reflect a failure to detect both of the sectors derived from hDNA segregation.

In the BssHII system, there were more unidirectional hDNA tracts that extended upstream of the gap than in the BglII system (12/72 versus 1/60, respectively). Although this could reflect a true shift to more upstream tracts in the BssHII system, a consideration of the average length of upstream tracts suggests an inability to detect comparable tracts in the BglII system. Only ~150 nt of DNA synthesis were primed when the invading end was downstream of the BssHII-generated gap; approximately 140 nt of DNA synthesis would be required to reach the first upstream polymorphism in the BglII system. We speculate that the abundant “no hDNA” class in the BglII system includes events initiated by downstream invasion, followed by limited DNA synthesis that failed to extend past the first polymorphism upstream of the gap.

**2.3.3 CO Intermediates in the BglII System**

The extents of DNA transfer associated with CO events were determined by sequencing the full-length and truncated alleles generated by plasmid integration in the *mlh1Δ* background (Figure 7A). Strand transfer was detected in all 60 CO recombinants analyzed, and these can be divided into four classes. The most abundant class (34/60) was that in which hDNA was continuous on one side of the gap in one allele, and continuous on the other side of the gap in the other allele.
Figure 7: CO products generated in the absence of MMR.

The cartoon above the sequenced events illustrates the products expected if the gap is repaired but no additional flanking sequence is transferred. Plasmid sequence is in yellow, chromosomal sequence in blue and hDNA in green. Classes 1-4 are described in the text.
This is precisely the pattern of hDNA predicted by the DSBR model (Figure 4, step C). Strikingly, hDNA was always present downstream of the gap in the full-length allele and always upstream of the gap in the truncated allele. As illustrated in Figure 8A, this pattern is expected only if the initiating strand invasion and the site of crossing over occur on the same side of the gap. The second class of CO events (9/60) contained continuous hDNA on only one side of the gap, and this single tract of hDNA adhered to exactly the same pattern as in the class 1 events: always upstream or downstream of the gap if present in the truncated or full-length allele, respectively.

In the DSBR model, one tract of hDNA reflects the initiating strand invasion event and the other is formed by second-end capture. Although it is not possible to distinguish which hDNA tract corresponds to which event the first class of CO events can nevertheless be used to calculate the average length of hDNA upstream and downstream of the gap in intermediates. There are ~600 bp of total homology upstream of the gap, and the average upstream hDNA length, including DNA synthesis across the 60 bp gap, was ~520 bp. Downstream of the gap, where homology was limited to 160 bp, the average length of DNA synthesis was only ~180 bp.

A third class of CO events (9/60) had a gene conversion tract on only one side of the gap. We suggest that these events reflect only one-half of the sectored colony expected to form when a hDNA intermediate is resolved by DNA replication (see Appendix A, Figure 24).
Figure 8: Relationship between the end that invades the donor, the CO position relative to the gap, and the location of hDNA in CO products.

Dotted lines represent DNA synthesized during gap repair and are colored according to the template. Arrowheads represent the 3’ ends of DNA strands and gray boxes highlight the positions of hDNA. In Panel A, the donor allele is invaded by the 3’ end upstream of the gap, and intermediates with one or two HJs are shown. The single HJ postulated to result from D-loop nicking should always be upstream of the gap, as shown. Panel B illustrates nick-directed cleavage of single or double HJs. Filled circles correspond to 5’ ends at nicked HJs and the dashed black arrows indicate the positions where a nicked HJ is predicted to be cleaved by Mus81-Mms4. Such cleavage generates
exclusively CO products. See Appendix A, Figure 24 for gene conversion tracts predicted if segregated hDNA fails to be detected.

We again note that when a single conversion tract was present, it was always upstream or downstream of the gap in the truncated or full-length allele, respectively. Finally, there was a fourth class of CO events (8/60) that contained more complex events. Some of these had hDNA at the same positions in both alleles ("symmetric" hDNA), which can be explained by branch migration of an HJ. Others had a mix of hDNA and gene conversion tracts, and could result from MMR-independent repair. With regard to this possibility, an MMR-independent, short-patch repair pathway that corrects mismatches in recombination intermediates has been reported in *S. cerevisiae* (Coic et al., 2000) as well as in *Schizosaccharomyces pombe* (Kunz and Fleck, 2001).

### 2.3.4 CO Intermediates in the BssHII System

Forty full-length and truncated alleles created by integration of the BssHII plasmid in an MMR-defective background are shown in Figure 4B. Most had either a tract of hDNA adjacent to the gap in each allele (class 1) or a single gene conversion tract (class 3). No COs with hDNA in only one allele (class 2 in the BglII system) were observed, perhaps because fewer events were examined and/or strand transfer was more efficiently detected with polymorphisms closer to the gap. Finally, there was a small number of class 4 events that had a complex pattern of hDNA and/or gene conversion tracts. Strikingly, the same pattern of strand transfer seen in the BglII system
was present in the class 1 and 3 products: hDNA/gene conversion upstream and downstream of the gap in the truncated and full-length alleles, respectively. Using the class 1 events to calculate the average amount of DNA synthesis associated with CO intermediates, we estimate ~280 nt upstream and ~350 nt downstream of the BssHII-generated gap. The length of upstream DNA synthesis was less and the length of downstream synthesis greater in the BssHII system than in the BglII system (~520 nt and 180 nt, respectively), which likely reflects the relative positions of the gaps in the two systems. Despite the differences in gap positions and sizes, however, the overall patterns of strand transfer among COs in the BglII and BssHII system were remarkably similar.

2.3.5 Effect of MMR on Gap Repair in the BglII System

In the BglII system, most MMR-associated antirecombination activity is directed toward CO intermediates (Welz-Voegele and Jinks-Robertson, 2008; see also Tay et al., 2010). To provide insight into why CO intermediates are preferentially targeted and how antirecombination is effected, 59 CO and 90 NCO products derived from the WT background were sequenced (Figure 9). As in the mlh1Δ background, all sequence transfer in NCOs was from the chromosomal repair template to the gapped plasmid. In contrast to the persistent hDNA in the mlh1Δ background, however, only four examples of unrepaired hDNA were found among the 74 NCO recombinants with detectable strand transfer. Based on these data, we estimate that the efficiency of mismatch
correction in NCO intermediates is greater than 90%. Although the persistence of hDNA was rare, it was present on both sides of the gap in three of the four bidirectional tracts, which are diagnostic of HJ dissolution. Because MMR was efficient among CO products derived from HJ cleavage (see below), an interesting possibility is that the repair of mismatches in DSBR intermediates is linked to junction cleavage.

**Figure 9: NCO and CO products of the BglII system in the presence of MMR.**
See legends to Figures 6 and 7 for NCO and CO details, respectively.

The distributions of gene conversion tracts in NCOs isolated in the presence of MMR (mostly unidirectional tracts extending downstream of the repaired gap) were very similar to those of hDNA tracts in the mlh1Δ background. This suggests that, as in the mlh1Δ background, SDSA is the primary mechanism for generating NCOs and that
most of the initiating strand invasion occurs upstream of the gap in WT. Using the unidirectional gene conversion tracts downstream of the gap, we calculate that ~160 nt of DNA synthesis occurs in the WT background, an amount very similar to that estimated in the \textit{mlh1\Delta} background. Finally, the similar proportion of the no-transfer class in the WT and \textit{mlh1\Delta} backgrounds (29\% and 25\%, respectively) suggests that hDNA repair in NCO intermediates is strongly biased to favor the donated (chromosomal) allele. Altogether, the molecular comparison of NCO products derived in the presence versus absence of MMR indicates that mismatches affect neither the stability of the corresponding intermediates nor the mechanism that generates them.

The general patterns of strand transfer relative to the recombination-initiating gap also appeared similar among CO products isolated in the presence versus absence of MMR (compare Figures 7A and 9B). As in the \textit{mlh1\Delta} background, detectable strand transfer in the full-length and truncated CO products always occurred downstream and upstream of the gap, respectively, in the WT background. The average length of DNA synthesized upstream and downstream of the gap in the WT CO products was ~470 nt and ~180 nt, respectively, which is similar to that calculated in the \textit{mlh1\Delta} background (520 nt and 180 nt, respectively). Although hDNA was repaired in most NCOs isolated in the WT background, pure hDNA tracts persisted in ~22\% (13/59) of the CO products. There were 12 tracts of hDNA upstream and 4 tracts downstream of the gap; unrepaired hDNA was present in both alleles in only three CO events. The repair efficiency of
downstream hDNA tracts appears similar among CO and NCO products; the rarity of strand transfer upstream of the gap among NCO products precluded an estimation of repair efficiency. These data indicate that the MMR-dependent repair of hDNA occurs efficiently in those CO intermediates that escape MMR-dependent antirecombination activity, suggesting that the two processes are temporally distinct.

2.4 Discussion

An implicit prediction of the DSBR model is the formation of hDNA on both sides of the initiating break, with each tract of hDNA being bordered by an HJ (Figure 1). The resulting double HJs can be cleaved to produce a NCO or CO outcome (step C), generating products which each contain hDNA (trans configuration). Since its inception, there have been several modifications to the DSBR model. First, it has been proposed that HJs can be resolved by helicase-driven dissolution, a process that generates only NCO products. In contrast to the trans hDNA associated with HJ cleavage, dissolution specifically predicts the presence of both hDNA tracts on the molecule with the initiating break (the cis configuration; step D in Figure 4). Second, the idea that NCO and CO events correspond to alternative modes of HJ cleavage has been challenged by the discovery that NCO can precede CO formation in both mitosis (Ira et al., 2003) and meiosis (Allers and Lichten, 2001). Consistent with the observed temporal separation, the SDSA mechanism of DSB repair provides a way to generate NCO events without going through an obligatory HJ intermediate (step E in Figure 4; Ferguson and
Holloman, 1996b; Nassif et al., 1994a; Paques et al., 1998b). Of particular relevance to the results reported here, the NCO-specific SDSA model predicts that hDNA will be present in only the repaired allele and that it will be limited to only one side of the gap.

In the present study, a diverged chromosomal allele was used as a template to repair a plasmid with a BglII- or BssHII-generated gap. Transformation experiments were done in the absence of MMR, thereby allowing the positions and extents of strand transfer in NCO and CO intermediates to be directly inferred by sequencing both products of individual recombination events. As discussed below, results suggest that NCOs result primarily from SDSA and that HJ cleavage is highly constrained to generate only CO products. In addition, repair products isolated in a WT background were examined for the BglII plasmid, for which the antirecombination activity of the MMR machinery has been shown to inhibit primarily CO events (Welz-Voegele and Jinks-Robertson, 2008). Results provide additional support for a model in which MMR-directed antirecombination is distinct from mismatch correction.

2.4.1 NCO Events Are Produced Primarily by SDSA

Despite differences in the initiating-gap size and position, the overall patterns of strand transfer in the BglII and BssHII systems were very similar. In the absence of MMR, hDNA was detected in the majority of the recipient, plasmid alleles, but no examples of hDNA were found in the corresponding chromosomal, donor alleles. A similar donor-to-recipient pattern of transfer has been observed during the repair of HO-
induced breaks introduced into tandem repeats (Paques et al., 1998a) and during mating
type switching (Ira et al., 2006). The confinement of hDNA to the recipient allele in
earlier studies was consistent with either the dismantling of D-loops or with HJ
dissolution (steps D and E, respectively, in Figure 4), but these alternatives could not be
distinguished. While the association of unidirectional gene conversion tracts with the
mitotic repair of an HO-induced DSB (Palmer et al., 2003) and with meiotic
recombination (Gilbertson and Stahl, 1996; Jessop et al., 2005; Merker et al., 2003) are
consistent with D-loop collapse, studies done in the presence of MMR make it
impossible to tell whether such conversion tracts reflect unidirectional hDNA or the
manner in which bidirectional hDNA is repaired. A unique feature of our system is that
it allows the location of hDNA relative to the initiating DSB in individual recombinants
to be accurately assessed. More than 90% of the hDNA detected in NCO recombinants
derived using either the BglII or BssHII gap-repair system was unidirectional, providing
direct evidence that most NCOs are generated by SDSA rather than by HJ dissolution.
Finally, it should be noted that the few NCO intermediates that did contain bidirectional
tracts had the cis rather than the trans configuration of hDNA. The exclusive cis
configuration suggests that HJ cleavage rarely generates NCOs in this system, which is
consistent with the biased resolution of HJ-containing intermediates inferred in meiotic
analyses (Allers and Lichten, 2001).
In the BglII system, there was a very striking asymmetry in the location of hDNA among NCO products, with >95% of the tracts extending downstream of the gap in the \textit{mlh1}\textDelta strain (Figure 6). Because hDNA reflects second-end annealing during SDSA, invasion of the donor thus appeared to be almost exclusively initiated by the end located upstream of the gap (see step E in Figure 4). Additional data obtained with the BssHII system suggest that much of the perceived bias in the BglII system likely resulted from an inability to efficiently detect short, unidirectional upstream hDNA tracts. We note, however, that there was still a strong bias for unidirectional hDNA to extend downstream of the BssHII-generated gap and that a similar downstream bias has been reported during the repair of a chromosomal HO break (Palmer et al., 2003). Finally, the presence of shorter downstream hDNA tracts in the BglIII than in the BssHII system indicate that asymmetry of the BglIII-generated gap may have limited the extent of DNA synthesis following strand invasion.

\textbf{2.4.2 CO Events Are Produced by Constrained Cleavage of Holliday Junctions}

Random cleavage of double HJs is predicted to yield equivalent numbers of CO and NCO products, with the locations of the associated \textit{trans} hDNA being random. Not only did we fail to detect a \textit{trans} pattern of hDNA among ~200 NCO products sequenced, the hDNA in the full-length CO products was always located downstream of the gap and that in the truncated products was always upstream of the gap. This
particular pattern of hDNA is predicted only if the CO site is on the same side of the gap as the single-stranded tail that initiates donor invasion (Figure 8A).

Both an exclusive production of CO products by HJ cleavage and the biased location of hDNA within these products can be explained if the resolution of HJ intermediates is nick-directed. Nicked HJs, which are precursors to mature, fully ligated HJs, are the preferred substrate for Mus81-Mms4 (Mus81-Eme1 in S. pombe and vertebrates) \textit{in vitro}, and this complex is required for meiotic CO formation in \textit{S. pombe} (Boddy et al., 2001). Importantly, Mus81-Mms4 uses the 5' end of a nick near an HJ intermediate to direct cleavage of the opposing strand (Osman et al., 2003). As shown in Figure 5B, the net result of such directed cleavage is an obligatory CO that occurs on the side of the break that initiates strand invasion – exactly the pattern observed in our system. We thus speculate that Mus81-Mms4 may be responsible for resolving nicked HJs in this system, and predict that the positions of \textit{trans} hDNA in CO products will be randomized upon its loss. Given the redundancy of HJ-resolving activities in budding yeast (Klein and Symington, 2009), however, it seems unlikely that Mus81-Mms4 will be absolutely required for CO production in our system.

CO events in the BgIII system, as well as in similar gap-repair systems, are strongly dependent on the Rad1-Rad10 endonuclease (Schiestl and Prakash, 1988a; Symington et al., 2000a; Welz-Voegele and Jinks-Robertson, 2008) Welz-Voegele, 2008 #3], but this complex is not required for CO formation in other types of assays in
budding yeast (Schiestl and Prakash, 1988b). Rad1-Rad10 cleaves at junctions between single-stranded and duplex DNA \textit{in vitro} (Bardwell et al., 1994), and it has been suggested that it might nick D-loops to facilitate second-end capture (Symington et al., 2000b). Such an activity would generate an intermediate with a single HJ adjacent to the invading rather than the captured end (Figure 8), and hence at the position where the CO event presumably occurs in our system. An additional activity (e.g., Mus81-Mms4), however, would still be required to produce an exclusive CO outcome. An alternative possibility to D-loop processing is that Rad1-Rad10 directly processes HJs or that it removes the 3′ tails produced when the invading end is extended past the region of donor-recipient homology. With regard to a possible role of Rad1-Rad10 in processing recombination intermediates, it should be noted that single HJs are characteristic of meiotic recombination in \textit{S. pombe} (Cromie et al., 2006) and that the Rad1 homolog (MEI-9) is required for most meiotic COs in Drosophila (Sekelsky et al., 1995).

### 2.4.3 MMR-Directed Antirecombination in the BglII System

In the BglIII gap-repair system, antirecombination activity of the MMR system has a profound effect on the production of CO products, but little effect on NCOs (Welz-Voegele and Jinks-Robertson, 2008). The sequencing data reported here not only suggest that CO and NCO products are produced by distinct mechanisms, they also indicate that hDNA is more extensive in CO than in NCO intermediates. Although the associated mismatches could interfere with a late step that is unique to the DSBR
pathway (e.g., HJ resolution or second-end capture by the displaced D-loop), we favor a model in which the initiating strand invasion event, which is common to the DSBR and SDSA pathways, is targeted by antirecombination. We suggest that the initiation of DSBR generally requires more extensive hDNA formation, and hence that early CO intermediates will be more efficiently detected and removed by the MMR machinery than early NCO intermediates. In terms of antirecombination mechanism, it is important to note that the rare CO events that escaped antirecombination in a WT background contained gene conversion rather than persistent hDNA tracts. This suggests that the mechanism of antirecombination is distinct from a canonical mismatch correction process. Interestingly, the MMR-PCNA interactions that are important in the removal of both replication- and recombination-generated mismatches appear to play little if any role during antirecombination (Stone et al., 2008). An attractive hypothesis is that the differing PCNA requirements reflect a temporal separation between antirecombination and recombination-associated mismatch correction, with PCNA-independent antirecombination occurring prior to the initiation of 3’-end extension, which depends on PCNA \textit{in vitro} (Li et al., 2009).

2.4.4 A Unifying Model for Mitotic Gap Repair

The data presented here bring together in a single assay observations made using a variety of systems, and these data form the basis of the model presented in Figure 10.
The central feature of this model is that the length of hDNA formed upon invasion of the donor molecule is related to whether the DSBR or SDSA pathway will be followed, and hence whether a CO or NCO event will ultimately be produced. Long tracts of initiating hDNA would generally lead to DSBR and CO formation, while short hDNA would
likely result in SDSA and a NCO outcome. We note that one version of SDSA invokes D-loop migration towards the extending end, which can collapse the D-loop and free the end to pair with a complementary 3' tail on the other side of the DSB (Ferguson and Holloman, 1996a). The initial D-loop size is expected to reflect the length of initiating hDNA, and we suggest that this size determines D-loop stability. Short hDNA tracts would be associated with a small D-loop that has a high probability of being dismantled by D-loop migration, thus favoring NCO production via SDSA. Longer hDNA, on the other hand, would generate a correspondingly larger D-loop that would persist longer and ultimately favor HJ formation and CO production. A specific model presented in Figure 10 is that length of hDNA formed upon invasion of the donor duplex directly reflects the length of the invading, single-stranded tail, which in turn will be determined by the extent of broken-end resection. The exonucleases responsible for end processing in yeast have recently been identified (Mimitou and Symington, 2008; Zhu et al., 2008), and it will be interesting to see if and how their presence/absence affects the NCO-CO outcome. We have shown that both Sgs1 and Exo1 are important in MMR-associated antirecombination and have assumed that this reflects a role in processing recombination intermediates (Nicholson et al., 2000a; Spell and Jinks-Robertson, 2004b). An alternative possibility is that the antirecombination activity derives from an end-resection role that promotes more extensive hDNA formation and increases the probability of mismatch detection.
The model in Figure 10 also incorporates the differential effects of the MMR machinery on CO and NCO events (Welz-Voegele and Jinks-Robertson, 2008). Because the DSBR pathway is associated with the formation of more extensive hDNA at the initial strand-invasion step, the accompanying presence of more mismatches would be more likely trigger MMR-associated antirecombination, reversing the intermediate and thereby precluding CO formation. An intermediate with short hDNA, and relatively few mismatches, would be more likely to evade the MMR machinery, but less likely to mature into an HJ-containing intermediate. The final feature of the model in Figure 10 is that it incorporates the observation that mismatch correction has a strong PCNA requirement, but mitotic antirecombination does not (Stone et al., 2008). We suggest that antirecombination occurs early, before PCNA is loaded to initiate end extension, and involves reversal of intermediate. Mismatch detection that occurs after PCNA engagement could be coupled to DNA synthesis, as it presumably is during DNA replication, or it could occur later during the recombination process, after the interacting duplexes have been resolved. It has been suggested that the interaction of the MMR machinery with PCNA provides strand discrimination information during replication (Umar et al., 1996), and a similar situation might exist during recombination. As shown in Figure 10, if PCNA targets the nascent strand for removal, the genetic result would be gene conversion rather than restoration, as observed here. Not only is the proposed
model consistent with observations made using diverse recombination assays in yeast, it
makes specific predictions that can be further tested in the gap-repair system.

2.5 Conclusions

NCO products of gap repair contain hDNA only in the repaired plasmid and
most often on only one side of the gap, consistent with predictions of the SDSA model.
In contrast, a bidirectional, trans pattern of hDNA is most often observed in CO
products, as predicted by the DSBR model. Data additionally suggest that when an HJ-
containing intermediate forms, cleavage of the junctions is highly constrained to
generate the CO outcome. Finally, sequence analysis of the gap-repair products
demonstrates that CO intermediates contain more extensive hDNA and hence more
potential mismatches than do NCO intermediates, providing an explanation for why
COs are more efficient targets of MMR-associated antirecombination. Together, these
observations suggest a model in which the length of the invading 3’ end determines
whether the DSBR or SDSA pathway will be followed, and hence whether a CO or NCO
outcome is produced.

2.6 Experimental Procedures

2.6.1 Media and Growth Conditions

Yeast strains were grown nonselectively in YEPD (1% Bacto-yeast extract, 2%
Bacto peptone, 2% dextrose) supplemented with 500 µg/ml adenine hemisulfate.
Selective growth was on synthetic complete (SC) medium lacking the appropriate
nutrient. Ura− segregants were selected on SC plates containing 0.1% 5-flourourotic acid. All growth was at 30°C.

2.6.2 Gap-repair Experiments

Haploid strain SJR1501 and its isogenic mlh1Δ derivative (SJR2157) contain the diverged gap-repair template and have been described previously (Welz-Voegele and Jinks-Robertson, 2008). The fragment for the BglIII gap-repair assay was generated by linearizing plasmid pSR840 (Welz-Voegele and Jinks-Robertson, 2008) with BglIII, followed by treatment with Mung bean nuclease to remove overhangs. pSR897 was used in BssHII gap-repair experiments and was derived in two steps: the BglIII fragment of pSR840 was restored and then a unique BssHII site was introduced by site-directed mutagenesis, replacing 5′-TTTCTGGA with 5′-CGCG (see Appendix A, Figure 23). pSR987 was digested with BssHII prior to transformation of SJR2157.

To distinguish NCO and CO recombinants, His+ colonies were transferred directly to SC-His liquid medium and grown to saturation in 96-well microtiter plates. Cells were diluted 100-fold into nonselective YPD medium, grown again to saturation, and 5 μl of a 1:100 dilution were spotted onto 5-FOA medium. Spots with full growth after two days were scored as NCOs and those with no growth or only a few colonies were scored as COs.
2.6.3 DNA Sequence Analysis of Recombinants

Transformants from the original microtiter plates were diluted into fresh SC-His and grown for 2 days in microtiter plates; all subsequent DNA manipulations were in a 96-well format (http://jinks-robertsonlab.duhs.duke.edu/protocols/yeast_prep.html). DNA was extracted following cell lysis with zymolyase, and appropriate PCR fragments were amplified from total DNA using primers M43, M45, M46 and B32 as appropriate. PCR products were sequenced by the High Throughput Genomic Unit at the University of Washington (Seattle, WA) or by the Duke Comprehensive Cancer Center DNA Analysis Facility using reverse primers M42, M46, R60 and/or R61. Primers sequences are provided in Appendix A Supplemental Table 1.

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Chapter 3. Heteroduplex DNA Position Defines the Roles of the Sgs1, Srs2 and Mph1 Helicases in Promoting Distinct Recombination Outcomes

3.1 Summary

The contributions of the Sgs1, Mph1 and Srs2 DNA helicases to mitotic double-strand break (DSB) repair were investigated by transforming a linearized plasmid into yeast strains containing a diverged chromosomal repair template. In addition to monitoring overall DSB repair efficiencies and the proportions of crossovers (COs) versus noncrossovers (NCOs) produced, both products of individual NCO events were sequenced to determine the location of heteroduplex DNA (hDNA) formed during recombinational repair. As hDNA positions are expected to differ depending on whether a NCO is produced by synthesis-dependent strand-annealing (SDSA) or through a Holliday junction (HJ)-containing intermediate, the underlying molecular mechanism can be inferred. Data demonstrate that all three helicases promote the formation of NCOs via the SDSA pathway and confirm that Sgs1 generates the hDNA pattern predicted by HJ dissolution.

3.2 Introduction

As the major DSB repair pathway in the yeast *Saccharomyces cerevisiae*, error-free homologous recombination (HR) promotes high-fidelity repair through the use of an intact template DNA sequence. However, HR can also lead to loss of heterozygosity and gross chromosomal rearrangements and thus requires tight regulation. To initiate
HR, the 5’ ends of the DSB are resected to yield 3’ single-stranded regions of DNA (for reviews, see Paques and Haber, 1999a; San Filippo et al., 2008b; Symington, 2002a). These 3’ ends are coated with Rad51 to form nucleoprotein filaments that are competent to conduct a homology search and invade a donor duplex DNA molecule, promoting pairing with the complementary strand. Successful strand invasion of a homologous duplex results in the formation of a D-loop structure consisting of a region of heteroduplex DNA (hDNA) and a displaced single strand of DNA (Figure 11). New DNA synthesis occurs using the 3’ invading end as a primer, and this reaction enlarges the D-loop. Expansion of the D-loop, or its movement with the extending 3’ end (Ferguson and Holloman, 1996a), eventually exposes sequences complementary to the other side of the break (Figure 11A). In the canonical DSB repair (DSBR) model of recombination (Szostak et al., 1983), annealing between the D-loop and the non-invading end of the DSB (“2nd end capture”) results in the formation of a double Holliday junction (dHJ) intermediate (Figure 11B). Alternatively, if the D-loop is nicked, annealing of the nicked strand to the non-invading end of the DSB will create an intermediate with a single HJ (Symington et al., 2000b). HJ-containing intermediates can be resolved by cleavage (Figure 11C), and this process is generally assumed to yield either noncrossover (NCO) products that maintain the original linkages of DNA flanking the break, or crossover (CO) products in which the linkages of flanking DNA are switched. As an alternative to cleavage, a dHJ-containing intermediate can be “dissolved” to yield
exclusively NCO products (Figure 11D) (reviewed in Mankouri and Hickson, 2007)). In lieu of engaging the second end of the DSB and subsequent HJ formation, the D-loop can be dismantled (Figure 11E). Annealing of the newly synthesized DNA to the non-invading 3’ end of the break then provides a template for the synthesis of the other strand of the damaged molecule. As this Synthesis-Dependent Strand-Annealing (SDSA) pathway does not go through an HJ-containing intermediate, it yields exclusively NCO products (Nassif et al., 1994b).

**Figure 11: Gap-repair pathways.**

Single strands of DNA are represented by orange and black lines, and arrowheads indicate 3’ ends. Regions of hDNA are boxed, and newly synthesized DNA is depicted as dashed lines in the same color as the template allele. Additional detail is provided in the text.
In *S. cerevisiae*, three 3’ to 5’ DNA helicases - Srs2, Sgs1 and Mph1 - have been implicated in regulating the outcome of mitotic DSB repair (Heyer et al., 2010), and each increases the frequency of NCO events relative to CO events (Ira et al., 2003; Prakash et al., 2009). Srs2 (suppressor of rad6 sensitivity) was the first of the three helicases to be identified, and its gene was discovered in a screen for mutations that suppress the UV sensitivity of *rad6* strains (Lawrence and Christensen, 1979). Because suppression depends on the HR machinery, it was suggested that this helicase normally inhibits recombinational bypass of DNA lesions (Schiestl et al., 1990). In spontaneous recombination assays, loss of Srs2 increases the rate of recombination, confirming that Srs2 can inhibit recombination (Aguilera and Klein, 1988; Rong and Klein, 1993). The anti-recombination activity of Srs2 has been attributed to its translocase activity, which removes the Rad51 protein from single-stranded DNA ends and thereby prevents strand invasion (Krejci et al., 2003; Veaute et al., 2003). However, when Srs2 function was examined in the context of an HO endonuclease-induced DSB, it was paradoxically found to play a pro-recombination role (Aylon et al., 2003). The loss of Srs2 not only decreased the overall level of DSB repair, it led to a proportional increase of COs among the recovered products (Ira et al., 2003), suggesting a specific role in NCO formation. Although it has been suggested that Srs2 directly dismantles D-loops to promote NCOs via SDSA (Dupaigne et al., 2008), an alternative possibility is that its pro-recombination role reflects the removal of Rad51 from single-stranded DNA (ssDNA) ends, which
would promote annealing between the 2nd end of the DSB and the newly extended strand upon D-loop collapse. Consistent with this possibility, biochemical studies have shown that Rad51 complexed with ssDNA is a potent inhibitor of Rad52-mediated annealing reactions (Wu et al., 2008).

Sgs1 (slow growth suppressor) was identified based on genetic interactions with Top3, with sgs1 mutations suppressing the genetic instability and slow growth of top3 strains (Gangloff et al., 1994). Sgs1 is a member of the RecQ family of 3’ to 5’ DNA helicases and is the ortholog of the human helicase BLM (Cejka and Kowalczykowski, 2010; Gangloff et al., 1994). Mutations in the BLM gene lead to the autosomal recessive disorder Bloom’s syndrome, which is characterized by genetic instability and increased sister chromatid exchange (Bernstein et al., 2010). Like Srs2, the Sgs1 helicase has multiple roles in recombination. First, Sgs1 acts with the endonuclease Dna2 to promote extensive 5’ to 3’ resection of the DSB ends (for a review, see Mimitou and Symington, 2009a). Second, biochemical and in vivo studies suggest that Sgs1, together with Top3 and Rmi1, promotes NCO formation by dissolving dHJ-containing intermediates that could alternatively be cleaved to yield COs (Cejka et al., 2010b; Mankouri et al., 2011). Dissolution involves migration of the two HJs towards each other, followed by decatenation of the two linked strands. Consistent with a role in dHJ dissolution, loss of Sgs1 results in increased CO formation during repair of an HO-induced DSB (Ira et al., 2003).
MPH1 was identified in a screen for mutants exhibiting a mutator phenotype (Entian et al., 1999), and the encoded protein is the ortholog of the human Fanconi Anemia protein FANCM (Meetei et al., 2005; Mosedale et al., 2005). The participation of Mph1 in HR was initially inferred from epistasis analysis (Schurer et al., 2004). In its absence, the frequency of HO-induced COs was found to be elevated, but overall levels of repair were not affected (Prakash et al., 2009). The increase in COs was suggested to specifically reflect a loss of SDSA events, and consistent with this, Mph1 can efficiently dismantle D-loops in vitro (Zheng et al., 2011). In a plasmid-based gap-repair assay, COs were similarly found to be elevated in the absence of Mph1 (Tay et al., 2010).

Although biochemical studies have suggested specific roles for Srs2, Sgs1 and Mph1 in promoting either SDSA or dHJ dissolution, corresponding in vivo evidence has been lacking. In particular, prior studies have not been able to distinguish whether a given NCO product was produced by HJ cleavage, HJ dissolution or SDSA. To more rigorously assess the specific functions of the Mph1, Srs2 and Sgs1 helicases in NCO formation, gapped plasmids were transformed into wild-type and mutant strains that were MMR-defective and contained a diverged chromosomal template for repair. We measured gap-repair efficiency, determined the CO-NCO distribution among repair events and sequenced both products of individual NCO repair events to detect regions of hDNA. As the location of hDNA can be used to infer the underlying molecular mechanism of NCO formation, the data provide novel insight into how recombination
intermediates are processed by these helicases. Results both confirm the presumed roles of these helicases and uncover novel functions.

### 3.3 Results

To analyze the roles of helicases in mitotic DSB repair, we used a transformation-based gap-repair system described previously (Mitchel et al., 2010). Briefly, the introduced plasmid contained an 800 bp *HIS3* gene within which a centrally located, 8-bp gap was created by restriction digest. As a repair template, a *his3* allele missing the C-terminal 11 amino acids and containing 19 single nucleotide polymorphisms (SNPs) was inserted on chromosome V. Because the donor sequence and plasmid are not identical, regions of hDNA formed during HR will contain mismatches. In the absence of mismatch repair (MMR), mismatches persist and segregate at the next round of replication. To allow detection of hDNA, all experiments were conducted in an MMR-defective background (*mlh1*Δ), and this parental strain served as the reference “wild-type” (WT). All helicase-defective strains will be referred to hereafter only by their relevant genotype: *mph1*Δ, *sgs1*Δ or *srs2*Δ.

The unique aspect of analyses done here is the tracking of hDNA in NCO products, as distinct patterns are predicted by SDSA, HJ dissolution and HJ cleavage. As illustrated in Figure 11, each of the NCO products generated by HJ cleavage is expected to contain a single region of hDNA. One region should be present on one side of the break in the repaired plasmid allele, and the other on the opposite side of the
break in the chromosomal, donor allele. In contrast to NCO products generated by HJ cleavage, no change is expected to the donor duplex following HJ dissolution. Both regions of hDNA are predicted to be in the repaired allele, one on either side of the break. Finally, NCOs produced by SDSA are expected to contain a single region of hDNA in the repaired allele located specifically on the annealing side of the gap. As with HJ dissolution, no change to the donor allele is expected.

3.3.1 Helicases and their Effects on Gap-repair Efficiency

To control for variations in transformation efficiency, a linearized plasmid containing a LEU2 marker was co-transformed with the gapped HIS3 plasmid. His\(^+\) and Leu\(^+\) colonies were selected separately during each transformation, with the His\(^-\)::Leu\(^+\) ratio being a measure of gap-repair efficiency. In the WT and the mph1\(\Delta\) strain, the median His\(^-\)::Leu\(^+\) ratio was 1.66 (Figure 12), indicating that Mph1 does not affect the overall efficiency of gap repair. The median His\(^-\)::Leu\(^+\) ratio following transformation of the sgs1\(\Delta\) strain was reduced to 1.00, and this decrease was significant when the His\(^-\)::Leu\(^+\) ratios obtained in independent transformations of the WT and sgs1\(\Delta\) strains were compared by a Mann-Whitney U-test (p<0.01). Finally, the median His\(^-\)::Leu\(^+\) ratio obtained in the srs2\(\Delta\) strain was reduced 3-fold relative to that obtained in the WT (p<0.001). These data demonstrate that loss of either Sgs1 or Srs2, but not of Mph1, leads to decreased gap repair in this system.
Figure 12: Efficiency of gap repair in WT and helicase-deficient strains. Ratios of His\(^+\) to Leu\(^+\) transformants in individual transformations are indicated by filled circles; the median is indicated with a black bar.

3.3.2 Effects of Helicase Deficiencies on CO and NCO Production

The gapped plasmid used in the transformation experiments contained an autonomously replicating sequence (ARS) but no centromere (CEN) sequence, allowing the repaired plasmid either to integrate into the chromosome with the repair template (a CO event) or to remain autonomous (a NCO event). These two outcomes were distinguished by examining the stability of the plasmid-encoded URA3 marker, allowing His\(^+\) products to be partitioned into NCO and CO events. Simply comparing
the proportions of COs versus NCOs in different genetic backgrounds (see Appendix B, Figure 25) can be misleading, however, as it does not take into account changes in overall gap-repair efficiency. For example, an elevation in the proportion of COs could reflect either a specific gain in CO events with no effect on NCOs, a channeling of potential NCO products into the CO pathway or a specific loss of NCO products with no effect on COs. The efficiency of CO (or NCO) repair was thus calculated by multiplying the median repair efficiency by the proportion of CO (or NCO) events (Table 1).

Table 1: Gap-repair efficiency and CO production in WT and helicase-deficient strains.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Repair efficiency (median His*:Leu*)</th>
<th>NCO Outcome</th>
<th>CO Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Proportion</td>
<td>Efficiency</td>
</tr>
<tr>
<td>WT</td>
<td>1.66</td>
<td>243/271</td>
<td>1.49</td>
</tr>
<tr>
<td>mph1Δ</td>
<td>1.66</td>
<td>218/276</td>
<td>1.31</td>
</tr>
<tr>
<td>sgs1Δ</td>
<td>1.00*</td>
<td>150/176</td>
<td>0.84*</td>
</tr>
<tr>
<td>mph1Δ sgs1Δ</td>
<td>1.41*</td>
<td>145/186</td>
<td>1.10*</td>
</tr>
<tr>
<td>srs2Δ</td>
<td>0.54*</td>
<td>144/189</td>
<td>0.41*</td>
</tr>
</tbody>
</table>

Asterisks indicate an efficiency distribution significantly different from WT (p<0.05).

To allow a statistical comparison of the efficiency of CO (or NCO) production, the His*:Leu* ratios measured in individual transformation experiments were multiplied by the proportion of COs among gap-repair products, yielding a distribution of CO-type (or
NCO-type) His⁺:Leu⁺ ratios. The distributions in different strains were then compared using a Mann-Whitney U-test.

In the WT yeast strain, only ~10% of repair events were COs. With an overall gap-repair efficiency of 1.66, the median CO and NCO efficiencies were thus 0.17 and 1.49, respectively (Table 1). Although the overall efficiency of gap repair in the mph1Δ strain was indistinguishable from that in WT, the proportion of COs increased to 21%. The median CO efficiency in the mph1Δ strain was thus 0.35, an increase that was highly significant when the WT and mph1Δ distributions were compared (p<0.0001). The median NCO efficiency in the mph1Δ strain decreased only slightly (from 1.49 to 1.31), a change that was not statistically different from that in the WT strain (p=0.98). It should noted that the gain in COs matched the loss in NCOs (each ~0.18), suggesting that upon loss of Mph1, events that would normally become NCOs are instead channeled into a CO pathway.

In the sgs1Δ strain, the proportion of COs among the repaired products increased to ~15%. Because the overall efficiency of gap repair decreased, however, there was no significant change in the efficiency of CO production in the sgs1Δ background relative to WT (0.15 and 0.17, respectively; p=0.44). By contrast, the median efficiency of NCOs decreased from 1.49 in the WT to 0.85 in the sgs1Δ strain, a change that was highly significant (p<0.01). Finally, in the srs2Δ strain, a proportional increase in COs (to ~24%) accompanied the 3-fold decrease in overall gap-repair efficiency. The efficiency of CO
formation was thus 0.13 in the srs2Δ strain, only slightly less than the value of 0.17 in the WT strain. Although the distribution of CO efficiencies in the srs2Δ strain was not significantly different from the WT distribution (p=0.13), the median NCO efficiency decreased from 1.49 in WT to only 0.41 in the srs2Δ mutant (p<0.0001). Thus, with either an Sgs1 or Srs2 deficiency, the reduction in overall gap-repair efficiency reflects a specific reduction in NCO formation that is not accompanied by a compensatory gain in COs.

3.3.3 Differentiating NCO Products Generated by SDSA and HJ-processing

Alterations in CO or NCO production could reflect an effect on the NCO-specific SDSA pathway, a change in the efficiency of forming HJ-containing intermediates and/or a change in how HJ-containing intermediates are resolved. To differentiate between these possibilities, a HIS3-containing CEN plasmid, which generates only viable NCO products, was used in transformation experiments, and both alleles involved in individual gap-repair events were sequenced. Of 249 NCO products sequenced from the WT strain, regions of hDNA were detected on the plasmid allele in 159 (Table 2). In 18 of these, hDNA was present on both sides of the gap (bidirectional hDNA), consistent with dHJ dissolution; the remaining 141 had hDNA on only one side of the gap (unidirectional hDNA), diagnostic of SDSA (Figure 13). This distribution of hDNA among NCOs isolated using the NCO-only plasmid was similar to that previously reported using an ARS-containing plasmid (Mitchel et al., 2010).
**Table 2: Efficiency of repair outcomes based on hDNA position in the repaired plasmid allele.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total repair efficiency (median His⁺Leu⁺)</th>
<th>CO efficiency</th>
<th>NCO Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Unidirectional hDNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Proportion (%)</td>
</tr>
<tr>
<td>Wild-type</td>
<td>1.66</td>
<td>0.17</td>
<td>141/159 (88.7%)</td>
</tr>
<tr>
<td>mph1Δ</td>
<td>1.66</td>
<td>0.35*</td>
<td>150/176 (85.2%)</td>
</tr>
<tr>
<td>sgs1Δ</td>
<td>1.00*</td>
<td>0.15</td>
<td>136/149 (91.3%)</td>
</tr>
<tr>
<td>mph1Δ sgs1Δ</td>
<td>1.41*</td>
<td>0.31*</td>
<td>36/47 (76.6%)</td>
</tr>
<tr>
<td>srs2Δ</td>
<td>0.54*</td>
<td>0.13</td>
<td>102/12 (79.1%)</td>
</tr>
</tbody>
</table>

1 Total of 47 NCOs includes 1 NCO with pattern of hDNA consistent with HJ cleavage
NCO outcome was determined using only transformants where hDNA was detected. Asterisks indicate p<0.05
Figure 13: Position of hDNA in NCO products of WT and helicase-deficient strains.

Each line represents the plasmid allele of a single NCO isolate, where plasmid sequence is depicted in yellow, chromosomal sequence in blue and hDNA in green. Only those NCOs with hDNA detected on the plasmid allele are plotted, as they were the samples used for statistical analysis. Samples are arranged into the relevant classes: bidirectional hDNA and unidirectional hDNA.

Although the corresponding chromosomal alleles were sequenced for each NCO product isolated, none had the hDNA pattern predicted by HJ cleavage, confirming that HJ cleavage does not contribute significantly to NCO formation in this system (Appendix B, Table 7). To determine the efficiency with which SDSA and HJ dissolution occurred, the proportion of unidirectional and bidirectional hDNA among NCOs was
multiplied by the median efficiency of NCO production. In WT, the median NCO efficiency of 1.49 was thus broken down into a bidirectional hDNA value of 0.17 and a unidirectional hDNA value of 1.32 (Table 2).

3.3.4 Mph1 Promotes NCOs through the SDSA Pathway

The sequences of 242 NCO products from the \textit{mph1\Delta} strain were analyzed. Of the 176 NCOs with detectable hDNA, ~85\% had unidirectional hDNA, and ~15\% had bidirectional hDNA (Figure 13; Table 2). Compared to WT, there was a small but significant reduction in unidirectional hDNA in the absence of Mph1 (from 1.32 to 1.12, respectively; p<0.02). Although NCOs with bidirectional hDNA increased from 0.17 in the presence of Mph1 to 0.19 in its absence, this increase was not significant (p=0.06). Thus, in the absence of Mph1, the 2-fold increase in COs was compensated for by a specific decrease in SDSA, with little if any change in NCO products generated through an HJ-containing intermediate.

3.3.5 Sgs1 Promotes NCOs both through SDSA and HJ Dissolution

In the absence of Sgs1, the overall gap-repair efficiency dropped to approximately 60\% of the WT level, and this reflected a selective loss of NCO events. The products of 285 NCOs isolated from the \textit{sgs1\Delta} strain were sequenced, 149 of which had detectable hDNA on the plasmid. Thirteen hDNA tracts were bidirectional, and 136 were unidirectional (Figure 13; Table 2). Taking into account the reduction in overall repair efficiency, the NCOs with bidirectional hDNA decreased from 0.17 in the
presence of Sgs1 to 0.07 in its absence (p<0.002). Additionally, NCOs with unidirectional hDNA dropped significantly from 1.32 in WT to 0.78 in the sgs1Δ strain (p<0.01). These data are consistent with a role for Sgs1 in promoting NCO formation via dHJ dissolution and additionally indicate that Sgs1 promotes SDSA.

### 3.3.6 Mph1 and Sgs1 Act Separately to Promote NCOs through SDSA

To determine the relationship between Sgs1 and Mph1 during gap repair, we constructed a double-mutant strain. The overall repair efficiency decreased from 1.66 in WT to 1.41 in the double mutant (p<0.04) (Figure 12). The repair efficiency was significantly higher than that obtained in the sgs1Δ single mutant (1.41 and 1.00, respectively; p<0.03), indicating that the pro-recombination function of Sgs1 is largely bypassed in the absence of Mph1. The CO production increased from 0.17 in WT to 0.31 in the mph1Δ sgs1Δ strain (p<0.0002), a value similar to that in the mph1Δ strain (0.35; p=0.26), but 2-fold greater than that in the sgs1Δ strain (0.15; p<0.002). By contrast, NCO production decreased from 1.49 in WT to 1.10 in the mph1Δ sgs1Δ strain (p<0.003) and was more similar to that obtained in the sgs1Δ than in the mph1Δ background (p=0.10 and p<0.04, respectively).

To examine the molecular basis of the decreased NCO efficiency in the double mutant, 85 NCO products were sequenced. hDNA was detected in 47 of these; 10 had bidirectional and 35 had unidirectional hDNA (Figure 13; Table 2). A single example of an NCO event with the pattern of hDNA consistent with HJ cleavage was detected in the
mph1Δ sgs1Δ background (Appendix B, Table 7). Thus, although extremely rare, cleavage of an HJ to yield a NCO is nevertheless possible in this system. The efficiency of NCOs with unidirectional hDNA decreased from 1.32 in WT to 0.84 in the mph1Δ sgs1Δ strain (p<0.0007), a value significantly less than that in the mph1Δ strain (1.12; p<0.002) but similar to that the sgs1Δ strain (0.78; p=0.75). By contrast, the efficiency of NCOs with bidirectional hDNA increased from 0.17 in WT to 0.23 in the double mutant (p<0.003). When compared to the single mutants, the efficiency of NCOs with bidirectional hDNA in the mph1Δ sgs1Δ strain was significantly greater than in either the mph1Δ strain (0.19; p<0.01) or the sgs1Δ strain (0.07; p<0.001).

3.3.7 Srs2 Promotes NCOs both through SDSA and HJ-containing Intermediates

In the absence of Srs2, the overall gap-repair efficiency decreased 3-fold, and this reflected a specific reduction in NCO events (Table 11). Among 254 NCOs produced in the srs2Δ strain, hDNA was detected in 129. Unidirectional hDNA was present in 102 of the hDNA-containing products and bidirectional hDNA in the remaining 27 (Figure 13; Table 2). In the srs2Δ strain, the efficiency of NCOs with unidirectional hDNA decreased 4-fold, from 1.32 in WT to 0.33 (p<0.0001). Unexpectedly, the NCOs with bidirectional hDNA also decreased significantly, from 0.17 in WT to 0.09 in the srs2Δ mutant (p<0.001). These data indicate that the unidirectional and bidirectional hDNA predicted by SDSA and HJ dissolution, respectively, are promoted by Srs2.
The pro-recombination role of Srs2 in the gap-repair assay could reflect its helicase and/or its translocase activity. To examine the relevance of each to NCO production, we used the translocase-deficient srs2-860 allele, which truncates the protein and eliminates the Rad51-interaction domain (Colavito et al., 2009). If only the helicase activity of Srs2 is important, then the efficiency of NCOs in the srs2-860 strain is expected to be the same as in the WT background. If the translocase activity of Srs2 is relevant, however, then the efficiency of NCOs should be reduced in the srs2-860 strain. The WT, srs2Δ, and srs2-860 strains were transformed in parallel with an ARS-containing gapped-plasmid mix (note: the plasmid mix used in previous experiments had been exhausted, and this analysis was performed with a new mix), and the efficiencies of CO and NCO formation were determined (Table 3).

Table 3: The Srs2 translocase activity is relevant after HR is initiated

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Repair efficiency (median His⁺:Leu⁺)</th>
<th>NCO Outcome</th>
<th>CO Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Proportion</td>
<td>Efficiency</td>
</tr>
<tr>
<td>WT</td>
<td>0.94</td>
<td>169/182</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(92.9%)</td>
<td></td>
</tr>
<tr>
<td>srs2Δ</td>
<td>0.30*</td>
<td>113/154</td>
<td>0.22*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(73.4%)</td>
<td></td>
</tr>
<tr>
<td>srs2-860</td>
<td>0.73</td>
<td>152/178</td>
<td>0.62*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(85.4%)</td>
<td></td>
</tr>
</tbody>
</table>

Asterisks indicate p<0.05

Consistent with the results shown in Table 1, there was a highly significant, 3-fold decrease in total gap-repair efficiency in the srs2Δ relative to the WT strain (from 0.94 to 0.30*.
0.30; p<0.0001). In addition to the similar relationship between total repair efficiencies, it should be noted that the proportions of COs and NCOs obtained in a given strain background were indistinguishable when data obtained using the old and new plasmid mixes were compared (p=0.32 and p=0.62 for WT and srs2Δ, respectively, by Fisher’s exact test). As before, the reduction in overall gap-repair efficiency in the srs2Δ background was due to the specific loss of NCO events, with no compensatory gain in COs.

The median His\(^+\):Leu\(^+\) ratio decreased from 0.94 in WT to 0.73 in the srs2-860 strain, but this reduction was not significant (p=0.07). Even though the srs2-860 allele had little effect on the overall gap-repair efficiency, it significantly altered NCO and CO production. Compared to the WT strain, there was a reduction in NCOs (from 0.87 to 0.62; p<0.05) and an increase COs (from 0.07 to 0.11; p<0.001). These data suggest that the translocase activity of Srs2 is important both for promoting the NCO outcome and for limiting COs.

### 3.4 Discussion

Previously, DSB repair studies have measured relative levels of CO and NCO products through physical analysis of large populations of repaired molecules (Ira et al., 2003; Prakash et al., 2009). Here, sequencing both products of individual NCO events produced in a gap-repair assay has allowed us to discern underlying molecular mechanisms, thereby providing a unique tool for assessing the specific structures being
dismantled by candidate helicases. Specifically, the position of hDNA relative to the initiating gap allows us to infer whether a given NCO event was generated by SDSA ("unidirectional" hDNA on only one side of the repaired gap), HJ cleavage (hDNA on one side of the gap in the repaired plasmid and on the other side of the gap in the chromosomal donor allele) or HJ dissolution ("bidirectional" hDNA on both sides of the gap in the repaired plasmid). As we reported previously, NCO production via HJ cleavage is extremely rare in our gap-repair assay; ~90% of NCO events in a WT background had an hDNA pattern consistent with SDSA and the remainder were consistent with HJ dissolution (Mitchel et al., 2010). The data obtained in our analyses are summarized in Figure 14, and the major conclusions are as follows. First, all three helicases are important for promoting the SDSA pathway. Second, the SDSA intermediates normally promoted by Mph1 are diverted into an alternative pathway that yields primarily CO events. By contrast, loss of SDSA-derived NCOs in an sgs1Δ or srs2Δ background is not accompanied by a compensatory gain in HJ-derived products, resulting in a decrease in overall gap-repair efficiency. Third, loss of Sgs1 is associated with a reduction in bidirectional as well as SDSA-type unidirectional hDNA tracts, providing direct evidence for the presumptive role of this helicase in the dissolution of dHJs. Unexpectedly, there also was a decrease in efficiency of bidirectional hDNA products in an srs2Δ background.
Figure 14: Distribution of gap-repair products in WT and helicase-deficient strains.

A. The height of each bar indicates the efficiency with which each type of product is formed. COs are in red, and NCOs are in blue. As described in the text, the srs2-860 data were generated using a different plasmid mix. To plot these data on the same scale, values were normalized using the median His⁺:Leu⁺ ratios obtained from the WT strain with both mixes (i.e. srs2-860 values x 1.66/0.94). Asterisks indicate p<0.05. B. NCOs with bidirectional hDNA are in dark blue, and NCOs with unidirectional hDNA are in light blue. Asterisks indicate p<0.05.
Below, we discuss the roles of Sgs1, Mph1 and Srs2 deduced here and place these in the context of those previously inferred in vivo and demonstrated biochemically.

3.4.1 Sgs1 Dissolves dHJs and Promotes SDSA

As reported using a similar gap-repair assay (Welz-Voegele and Jinks-Robertson, 2008), we observed a clear reduction in total gap-repair efficiency upon loss of Sgs1. Studies of DSB-induced recombination in mammalian cells have similarly found a pro-recombination role for BLM (LaRocque et al., 2011), and a slight, 20-30% reduction in repair of HO-induced breaks was previously noted in an sgs1Δ background (Ira et al., 2003). The 2-fold increase in the proportion of COs that we observed upon loss of Sgs1 reflected a specific loss of NCOs, with no corresponding gain in COs. The proportional gain in COs among recombination products is consistent with earlier studies of yeast spontaneous and DSB-induced recombination (Gangloff et al., 2000; Ira et al., 2003; Tay et al., 2010; Welz-Voegele and Jinks-Robertson, 2008), as well as with studies in mammalian cells (LaRocque et al., 2011) and Drosophila (McVey et al., 2004).

In a time-course analysis of HO-induced DSB repair in yeast, the appearance of early NCOs, which were assumed to reflect SDSA, was not affected by loss of Sgs1, leading to the suggestion that Sgs1 specifically promotes the dHJ dissolution pathway (Ira et al., 2003). There have been numerous in vitro and in vivo studies that support a role for Sgs1, together with Top3 and Rmi1, in dHJ dissocation. In vitro, for example, human and Drosophila BLM/TopIIIa and yeast Sgs1/Top3 can dissolve dHJs (Cejka et
In vivo, a return-to-growth protocol was used to generate unresolved meiotic joint molecules whose resolution could then be followed in mitosis. In an sgs1-ΔC795 mutant, which lacks the endogenous helicase and HJ-binding activities of the protein, joint molecules persisted longer and their eventual resolution led to proportionally more COs, consistent with dHJ dissolution by Sgs1 (Dayani et al., 2011). In a different in vivo approach, exposure of sgs1 cells to DNA damage was associated with an accumulation of recombination-dependent X-shaped molecules (Liberi et al., 2005; Mankouri et al., 2011). Importantly, these molecules disappeared when DNA was treated with bacterial HJ resolvases, suggesting that they correspond to fully-ligated HJs that are normally dissolved by the Sgs1/Top3/Rmi1 complex (Mankouri et al., 2011). Although biochemical studies have demonstrated that Sgs1 can promote the dissolution of dHJs and there are in vivo data consistent with this activity, prior in vivo experiments have not been able to distinguish NCO products produced by HJ dissolution from those produced by SDSA. Because our assay allows this critical distinction to be made, we have been able to confirm that loss of Sgs1 is associated with a decrease in the specific class of NCOs predicted as the product of Sgs1-driven dHJ dissolution: NCOs with bidirectional hDNA on the repaired molecule (Figure 15).
Figure 15: Models for helicase-mediated regulation of gap repair.
The helicases Sgs1, Srs2 and Mph1 are represented by green, yellow and pink symbols, respectively. See text for details.

A reduction in the bidirectional hDNA pattern predicted by dHJ dissolution was expected upon loss of Sgs1, but a similar reduction in the unidirectional hDNA diagnostic of SDSA was not (Figure 15). Previous studies in Drosophila, however, have proposed a role for BLM in dismantling D-loops to promote SDSA following P element excision (Adams et al., 2003; McVey et al., 2007). There is also supporting biochemical evidence that human BLM binds and dismantles D-loops (Bachrati et al., 2006; van
Brabant et al., 2000). Recent data suggest that Sgs1/Top3-driven D-loop disruption could occur via a novel mechanism involving migration of back end of the D-loop, rather than the more canonical helicase-mediated removal of the invading strand (Bachrati et al., 2006; Chen and Brill, 2010). Such a basic difference in mechanism could at least partially explain why Srs2, Mph1 and Sgs1 cannot fully substitute for each other to promote SDSA. With regard to the mitotic D-loop dismantling activity inferred here based on hDNA patterns, recent studies indicate that it is Sgs1 that promotes early SDSA-type NCOs in meiotic time-course analyses (De Muyt et al., 2012; Zakharyevich et al., 2012).

3.4.2 Mph1 Promotes SDSA at the Expense of CO Formation

Although mph1Δ mutants were reported to accumulate proportionally more COs in HO studies, an important distinction was that NCOs were converted into COs in an mph1Δ background and were not simply “lost” as in an srs2Δ or sgs1Δ mutant (Ira et al., 2003). Our mph1Δ results are entirely consistent with these earlier data; the 2-fold increase in CO events was accompanied by a coordinate decrease in SDSA-type NCOs so that the overall efficiency of gap repair was maintained. A curious aspect of our data is that the increase in COs was not accompanied by an increase in dissolution-type NCOs. Why this might be the case is unclear, but one possibility is that the D-loops that fail to be dismantled by Mph1 are nicked and give rise to single (ligated) HJs, which would not be substrates for Sgs1/Top3-driven dissolution (Figure 15). While there are no data from higher eukaryotes that address the CO-NCO distribution in the absence of
Mph1, *Schizosaccharomyces pombe* mutants defective in the ortholog Fml1 have normal efficiencies of gap repair accompanied by a strong proportional increase in COs (Sun et al., 2008). In terms of how Mph1 might dismantle D-loops, it has been argued that it is the only one of the three helicases examined here that can unwind a mobile D-loop created by Rad51 (Prakash et al., 2009; Sebesta et al., 2011).

In our strain background, we were only able to examine gap repair in an mph1Δ sgs1Δ double mutant; combining srs2Δ and sgs1Δ results in synthetic lethality (Gangloff et al., 2000), and the srs2Δ mph1Δ double mutant was too sick to generate meaningful data. One very intriguing result was suppression of the sgs1Δ gap-repair deficiency upon additional loss of Mph1, indicating that Sgs1 is more important for the completion of gap repair when Mph1 is present. This was not evident in previous studies of HO-induced recombination (Prakash et al., 2009), perhaps because the effect of Sgs1 loss on overall repair efficiency was much more subtle than seen in our gap-repair assay. The reduction in the production of NCOs with unidirectional hDNA was no greater in the mph1Δ sgs1Δ double mutant than in the sgs1Δ single mutant, suggesting that Sgs1 and Mph1 work in the same pathway to promote SDSA. An interesting possibility is that as the Mph1 helicase unwinds the invading strand, the catenating activity of Sgs1/Top3 is required to “rewind” the duplex that was part of the D-loop (Chen and Brill, 2010).

While the double mutant was similar to sgs1Δ in terms of SDSA efficiency, it was more similar to the mph1Δ single mutant in terms of CO efficiency. Finally, with regard to the
efficiency of HJ-dissolution (bidirectional hDNA) events, there was a significant increase in double mutant relative to the WT strain, an increase that was not evident in either single mutant. As Srs2 is the remaining helicase, we can only surmise that its cognate substrate (see below) was elevated.

3.4.3 Roles of Srs2 in Promoting NCO Pathways during DSB Repair

A pro-recombination role for Srs2 has been demonstrated in physical studies of HO-induced mitotic recombination (Aylon et al., 2003; Ira et al., 2003) and in a plasmid-based gap-repair assay similar to the one used here (Welz-Voegele and Jinks-Robertson, 2008). Importantly, loss of Srs2 has been associated with an increase in the proportion of COs produced during HO-induced DSB repair (Ira et al., 2003), during gap repair (Welz-Voegele and Jinks-Robertson, 2008) and during spontaneous recombination (Robert et al., 2006). Because only the early-appearing NCOs were lost following HO induction in an srs2Δ background, it was suggested that Srs2 promotes the NCO-specific SDSA pathway (Ira et al., 2003). As expected, the efficiency of gap repair in the current analyses decreased 3-fold upon loss of Srs2, and there was an increase in the proportion of CO events. Significantly, by analyzing the position of hDNA, we were able to demonstrate a corresponding 3-fold reduction in SDSA-type NCO products in an srs2Δ background; no increase in CO events or bidirectional hDNA was detected. These data confirm that SDSA is the “early” NCO-specific pathway identified in HO studies, and that the elevation in proportion of COs in an srs2Δ background reflects a specific loss of
SDSA events rather than a maturation of intermediates into HJs. As observed in HO assays (Ira et al., 2003), the pro-recombination role of Sgs1 was not as strong as that of Srs2 during gap repair, and our data are consistent with independent functions with respect to promoting gap repair.

The most straightforward way for a helicase to promote SDSA is through the dismantling of an extended D-loop (Figure 15), but whether this is the most relevant function of Srs2 in vivo has been the subject of debate (Dupaigne et al., 2008; Prakash et al., 2009). The Srs2-860 protein retains helicase activity but does not interact with Rad51, and hence is defective specifically in the translocase activity that removes Rad51 from DNA (Colavito et al., 2009). A comparison of the overall gap-repair efficiencies in WT, \textit{srs2}\textDelta and \textit{srs2-860} strains indicates that the pro-recombination role of Srs2 is due primarily to its helicase activity. Because most gap repair (~80%) in WT reflects SDSA, our results are consistent with helicase-driven dismantling of D-loops by Srs2. Although NCOs were produced much more efficiently in the \textit{srs2-860} than in the \textit{srs2}\textDelta background, there was nevertheless a significant decrease in NCOs in the translocase-defective mutant. This suggests that the disruption of Rad51 nucleoprotein filaments by Srs2 (Krejci et al., 2003; Veaute et al., 2003) also helps promote SDSA. The translocase activity of Srs2 could be important for removing Rad51 from the 2\textsuperscript{nd} end of the break to promote the requisite Rad52-dependent annealing reaction, or it could be necessary for D-loop disruption when Rad51 remains bound to duplex DNA within the D-loop.
Either role would be consistent with the observation that overexpression of Rad51 in an srs2Δ background almost completely eliminates NCOs (Ira et al., 2003). It should be noted that the 2nd-end engagement required to generate a dHJ could occur either through an annealing reaction or through a second, Rad51-catalyzed strand invasion event. The latter should be more efficient in an srs2-860 background, which might account for the small increase in COs observed in this strain.

The loss of the SDSA pattern of hDNA among NCO events in the srs2Δ background was expected based on prior studies, but the accompanying 2-fold reduction in the bidirectional hDNA pattern assumed to be diagnostic of dHJ dissolution was not. The unwinding of a 4-way structure that mimics an HJ by yeast Srs2 has been examined, and it was concluded that it is a no better substrate for yeast Srs2 than a blunt-ended duplex (Dupaigne et al., 2008). A recent analysis of the putative homolog of Srs2 from Arabidopsis thaliana, however, reported that the helicase has significant activity against a nicked HJ (Blanck et al., 2009). We thus speculate that, in addition to a D-loop, a nicked dHJ is a cognate substrate for Srs2 in vivo (Figure 15). A nicked dHJ can be formed by a mechanism analogous to that assumed to occur when SDSA-mediated repair of a gapped plasmid requires that each end invade a template on a different chromosome (Miura et al., 2012; Paques et al., 1998a). In the case of the assay used here, independent invasion of the same repair template by each end, followed by extension and unwinding - basically two SDSA reactions - would produce a repaired
plasmid with bidirectional hDNA. The contributions of Srs2 and Sgs1 to bidirectional hDNA among NCO products appear to be independent (Table 2), which would be consistent with these helicases working on different structures.

3.4.4 Concluding Remarks

Given the numerous roles that have been identified for the Mph1, Sgs1 and Srs2 helicases, determining their specific regulatory activities once HR has initiated has been problematic. Although a gapped plasmid was used here to model DSB repair, it is important to note that both the efficiencies of repair and the distributions of repair products are completely consistent with those reported in HO-initiated chromosomal assays. Through monitoring of hDNA among NCO products, the results presented here provide molecular confirmation that both Srs2 and Mph1 promote SDSA and that Sgs1 participates in the dissolution of dHJs. Importantly, additional roles for Sgs1 in promoting SDSA and for Srs2 in dismantling HJs have been inferred, broadening the potential range of activities of these helicases in vivo.

3.5 Experimental Procedures

3.5.1 Media and Growth Conditions

Cells were grown nonselectively in YEPD (1% Bacto-yeast extract, 2% Bacto peptone, 2% dextrose) supplemented with 500 µg/mL adenine hemisulfate. Selective growth was on synthetic complete (SC) medium lacking the appropriate nutrient. Ura
segregants were identified on SC plates containing 0.1% 5-fluoroorotic acid (5-FOA). All growth was at 30°C.

**3.5.2 Gap-Repair Experiments**

A complete strain list is provided in Appendix B, Table 8. Helicase-defective derivatives of the haploid *mlh1Δ* strain SJR2157, which contains the diverged gap-repair template (Welz-Voegele and Jinks-Robertson, 2008), were constructed by targeted gene deletion. The substrate for gap repair was generated by *Bss*HII linearization of either the *ARS*-containing plasmid pSR987 (Mitchel et al., 2010) or the *CEN/ARS*-containing plasmid pSR1015. pSR1015 was constructed by inserting an *XhoI/XbaI* *HIS3* fragment from pSR987 into *XhoI/XbaI*-digested pRS316 (Sikorski and Hieter, 1989).

The OD$_{600}$ of the exponentially growing cultures, each of which was derived from an independent colony, was measured to determine cell density. Six cultures with OD$_{600}$ values between 0.7 and 1.0 were selected for parallel transformation using the protocol described previously (Welz-Voegele and Jinks-Robertson, 2008). Each experiment was repeated with at least six more cultures derived from independent colonies. His$^+$ and Leu$^+$ colonies were counted 5 days after selective plating. To avoid bias when partitioning recombinants into CO and NCO events, plates were divided into sections and every His$^+$ transformant within a given section was picked. His$^+$ transformants were frozen in 20% glycerol without prior purification, and an aliquot was grown nonselectively in YEPD prior to spotting an appropriate dilution on 5-FOA. Spots with
full growth on 5-FOA after 3 days were scored as NCO events; those with no growth or only a few papillae were scored as CO events.

3.5.3 DNA Sequence Analysis of Recombinants

Transformations with pSR1015 and selection of His\(^+\) transformants was as described above. An aliquot of the frozen stock of each His\(^+\) colony was transferred to SC-his liquid medium and grown to saturation in 96-well microtiter plates. Following DNA extraction (http://jinks-robertsonlab.duhs.duke.edu/protocols/yeast_prep.html) the plasmid and chromosomal alleles were separately amplified with the appropriate primers (Appendix A, Table 6) and products were sequenced by the Duke Comprehensive Cancer Center DNA Analysis Facility. Sequence chromatograms were examined visually to detect the double peaks indicative of hDNA at a given SNP. Samples with only gene conversion or with no detectable sequence transfer were not included in further analysis because it was not possible to infer a recombination intermediate.

3.5.4 Statistical Analysis

The His\(^+\):Leu\(^+\) ratio distributions for the various events in different strain backgrounds were compared using the Mann-Whitney U-test (http://vassarstats.net/), and two-tailed p-values are reported.
3.6 Acknowledgements

We thank Hannah Klein for providing a construct to introduce the srs2-860 allele, and members of the SJR lab for helpful discussions throughout the course of this work. We especially appreciate comments provided by Nayun Kim during the preparation of the manuscript.
Chapter 4. Candidate Resolvases Impact CO Formation during Gap Repair

4.1 Introduction

The chromosome repair process homologous recombination (HR) is critical for cell survival during normal vegetative growth, as exposure to DNA damaging agents is ubiquitous and can result in double-strand breaks (DSBs). Repair of a DSB by HR can yield either a non-crossover (NCO) that maintains the original linkage of the sequences flanking the break or a crossover (CO) that switches the linkages (Paques and Haber, 1999a). The least disruptive outcome of HR is recovery of the lost sequence without change to the nearby genes, and numerous mechanisms exist to generate NCOs. To begin HR, the single-stranded 3’ end of a broken sequence invades a donor duplex, and a D-loop intermediate is formed. New DNA synthesis is primed off this invading end to recover the damaged sequence, and the release of the invading strand allows annealing to other end of the broken molecule. DNA synthesis to regenerate the other strand and ligation to seal the nicks results in a NCO product. This synthesis-dependent strand-annealing (SDSA) pathway yields exclusively NCO products and is the predominant NCO generating mechanism (Mitchel et al., 2010).

If the D-loop persists, a Holliday Junction (HJ) can be formed. Nicking of the displaced top strand of the D loop and its annealing to the other (non-invading) end of the break will form a single Holliday Junction (sHJ). A double Holliday Junction (dHJ) can be formed through second end capture (Figure 16). Helicase-mediated dissolution of
the dHJ intermediate comprises an alternate pathway for the production of NCOs, while resolution of an HJ by cleavage can yield either a NCO or CO.

![Diagram of DSB, HJ formation, and HJ processing](image)

**Figure 16: Repair of a double-strand break (DSB), formation of a Holliday-Junction (HJ) intermediate and HJ processing**

Cleavage of both HJs in the same orientation (both sets of exchanged strands or both sets of non-exchanged strands) will result in NCOs, and COs are generated when the two HJs are cleaved in different orientations, where one set of exchanged strands and one set of non-exchanged strands are cut (Figure 17a). In the case of an sHJ, cleavage of the exchanged strands will create a NCO, and cleavage of the non-exchanged strands will yield a CO (Figure 17b).
Figure 17: HJ cleavage results in either CO or NCO products. Individual strands of DNA are represented by lines, and 3’ ends are indicated with arrows. Red lines depict the DNA duplex with the initiating break, and blue lines represent the template. Closed carets represent cleavage of non-exchanged strands at a HJ, and open carets represent cleavage of exchanged strands. A. Cleavage of a dHJ intermediate. B. Cleavage of an sHJ intermediate.
The HJ is a key component of the canonical model of DSB repair, and this 4-stranded structure physically links two DNA duplexes. Resolution of HJ-containing intermediates is crucial because the covalent linkage of DNA duplexes will prevent successful chromosome segregation at the next cell division. In bacteriophage, bacteria and archaea, specialized enzymes that resolve HJs by endonucleolytic cleavage have been isolated (Lilley and White, 2001). The best characterized of these “resolvase” enzymes is the *E. coli* protein RuvC (West, 1997). RuvC symmetrically nicks two strands of the HJ, and these strands can be ligated (without any additional processing) to result in either a NCO or CO. In eukaryotes, identifying the HJ resolvase has been more difficult. One candidate identified from lysed cells, Cce1, can nick cruciform structures to yield ligatable DNA ends (Kleff et al., 1992). However, Cce1 is located exclusively in the mitochondria, and its loss does not effect nuclear recombination. The search for the *S. cerevisiae* resolvase has yielded several additional candidates that nick branched DNA structures and/or synthetic HJs: Yen1, Mus81-Mms4, Slx1-Slx4 and Rad1-Rad10 (reviewed in Schwartz and Heyer, 2011). Given the scarcity of COs produced in mitosis and the complex regulation of HR, determining the *in vivo* activities of these proteins is an ongoing topic of study.

Yen1 (*yeast endonuclease 1*) was identified in 2008 as a nuclease capable of resolving a synthetic HJ *in vitro* (Ip et al., 2008). Yen1 and its human ortholog GEN1 contain domains with homology to the Rad2/XPG family of nucleases. Conforming to
the RuvC paradigm, analysis of the cleavage products indicated that Yen1 cleaves symmetrically and that the nicks can be ligated without further processing. However, further study has revealed that deletion of Yen1 alone does not impact survival following exposure to genotoxic agents nor does deletion appear to impact CO or NCO outcome during meiotic DSB repair (Blanco et al., 2010; Ho et al., 2010; Tay and Wu, 2010). Thus, the relevance of Yen1 to \textit{in vivo} HJ resolution remains uncertain.

The Mus81-Mms4 complex (Mus81-Eme1 in \textit{Schizosaccharomyces pombe}) has been implicated in CO formation. Although survival is not decreased in the absence of Mus81 following exposure to ionizing radiation or endonuclease-induced DSBs (Boddy et al., 2000; Interthal and Heyer, 2000), decreased mitotic CO formation during the repair of an I-SceI generated DSB has been observed (Ho et al., 2010). Added support for a role of Mus81-Mms4 in generating COs comes from its importance in meiotic recombination. In \textit{S. cerevisiae}, approximately 35\% of COs require Mus81 (de los Santos et al., 2003; de los Santos et al., 2001). The effect is more dramatic in \textit{S. pombe}, where both meiotic CO production and spore viability are severely affected in the absence of Mus81 (Boddy et al., 2001; Smith et al., 2003). \textit{In vitro} Mus81-Mms4 cleaves HJs asymmetrically, and the products of cleavage require further processing before ligation can occur (Boddy et al., 2001). Furthermore, \textit{in vitro} analysis suggests that the preferred substrate of Mus81-Mms4/EME1 contains a 5' end near the joint to be cleaved, which results in exclusively CO formation (Ehmsen and Heyer, 2008; Fricke et al., 2005; Osman et al., 2003). The
DSBR model assumes that HJs are fully ligated, although the presence of nicks could direct cleavage of HJs by Mus81-Mms4.

Previous work with a plasmid-based gap-repair assay in haploid yeast has demonstrated a requirement for the heterodimeric Rad1-Rad10 complex in mitotic crossing over (Symington et al., 2000b; Welz-Voegele and Jinks-Robertson, 2008). Rad1-Rad10 primarily acts in the nucleotide excision repair (NER) pathway, which removes damage from the genome by excising a small oligonucleotide containing the lesion (Guzder et al., 1995). During NER, Rad1-Rad10 incises 5’ of the lesion, but it can also bind and cleave other substrates, including synthetic HJs (Habraken et al., 1994). Rad1-Rad10 also has a role in single-strand annealing (SSA), in which annealing between directly repeated sequences flanking a break site repairs the DSB (Paques and Haber, 1997). Following removal of heterologous tails and ligation of the remaining nicks, SSA generates NCOs with only one copy of the repeated sequence and containing a deletion of the region between the repeats. In this process, nucleases are presumed to cleave the tails following annealing between the repeats. During SSA, as well as during microhomology-mediated end joining (Ahmad et al., 2008; Lee and Lee, 2007), Rad1-Rad10 specifically removes the overhanging 3’ tails that remain after annealing. In *Drosophila*, loss of the Rad1 homolog MEI-9 leads to decreased formation of COs (but normal NCO production) during meiotic recombination (Radford et al., 2007; Sekelsky et al., 1995). Rad1-Rad10 appears to have little effect on meiotic CO formation in
budding yeast (Dowling et al., 1985), and it remains unclear why mitotic COs resulting from gap repair exhibit a dependence on this complex.

Slx1 and Slx4 form a complex and were identified on the basis of their synthetic lethality in an sgs1Δ background, suggesting a role in HR (Mullen et al., 2001). The complex can cleave a variety of DNA junctions in vitro and generates ligatable ends (Fricke and Brill, 2003). The Drosophila Slx4 homolog (MUS312) does facilitate meiotic crossing over (Yildiz et al., 2002), however, this activity also depends on the Rad1-Rad10 homologs, MEI-9-ERCC1 (Andersen et al., 2009) and not SLX1. Although Slx4 has not been implicated in HJ resolution in yeast, it is required for SSA, and its role appears to be dependent on an interaction with Rad1-Rad10 (Flott et al., 2007; Toh et al., 2010). Spore viability is not affected in yeast strains deficient in Slx1 or Slx4, and this complex is not presumed to participate in HJ resolution in S. cerevisiae (Fricke and Brill, 2003; Mullen et al., 2001; Schwartz and Heyer, 2011).

A previous investigation of the molecular structures of COs isolated using a gap-repair system revealed that most events shared a common pattern of strand transfer (Mitchel et al., 2010). In this assay, a small gap in HIS3 located on a plasmid is repaired via HR from a chromosomal template, and CO products integrate the plasmid into the genome. Following plasmid integration, the chromosome contains one full length and one truncated allele of HIS3. Intriguingly, in the majority of COs analyzed, the repaired full-length allele had heteroduplex DNA (hDNA) downstream of the gap, while the
truncated allele had hDNA upstream of the gap. The reverse pattern of hDNA (hDNA upstream in the full-length and downstream in the truncated alleles) was not detected in any of the 40 COs analyzed in the initial report, as would be predicted if HJ cleavage were random. Furthermore, no NCO products were observed with patterns of hDNA expected as a result of HJ cleavage. Thus, we suspect that the activity of a specific endonuclease may be responsible for constraining the cleavage pattern.

To investigate the contributions of Mus81-Mms4, Yen1 and Rad1-Rad10 to constrained cleavage during mitotic crossing over, haploid strains containing deletions of Mus81, Yen1 or Rad1 were transformed with gapped plasmids. To select CO products exclusively, we used a vector that did not contain an autonomously replicating sequence (ARS). Regions of hDNA in the CO products were detected by sequencing, allowing the location of cleavage to be inferred. This approach allows an *in vivo* examination of the CO-generating roles of these nucleases at a finer scale than previously possible.

### 4.2 Results

We used a transformation-based gap-repair system described previously (Mitchel et al., 2010 and Chapter 3) to analyze constrained cleavage during crossing over. This assay enables us to determine the locations of strand transfer during HR by sequencing the products of gap-repair. A full-length copy of *HIS3* located on a plasmid was digested with a restriction enzyme to create an 8-bp gap. This digested plasmid was transformed into haploid strains containing a truncated copy of *HIS3* (*his3Δ3′*) inserted
into chromosome V to serve as a repair template. The chromosomal \textit{his3}\Delta 3' allele also contains 19 single nucleotide polymorphisms (SNPs) that distinguish the two alleles. Repair by HR using this diverged allele yields a His\textsuperscript{+} colony. An MMR-defective background (\textit{mlh1}\Delta) was used in all experiments so that the regions of heteroduplex DNA (hDNA) formed during HR would not be repaired and could be detected later. This \textit{mlh1}\Delta parental strain acted as the reference “wild-type” (WT), and this deletion is shared by all the resolvase mutant strains. The resolvase mutant strains will be referred to solely by their unique genotype: \textit{mus81}\Delta, \textit{yen1}\Delta or \textit{rad1}\Delta.

The original gapped plasmid contained an ARS but no centromere (CEN) sequence, which allowed the repaired plasmid either to integrate into the chromosome with the repair template (a CO event) or to remain autonomous (a NCO event). However, as COs occur at a low frequency in this system (approximately 10% of events), a “CO-only” derivative of the gapped plasmid was used to examine the role of the resolvase proteins. This CO-only plasmid does not contain an ARS or a CEN, and repair as a NCO will yield a plasmid that will not persist as the yeast cells divide. Thus, transformation with the CO-only plasmid produces exclusively COs and allows us to study these relatively rare events.

\textbf{4.2.1 The Majority of CO Products are Generated by Constrained Cleavage}

The initial phenomenon of constrained cleavage was observed in COs generated with the ARS-containing plasmid, and only a small number of events were analyzed. As
discussed above, the majority of these COs had hDNA downstream of the gap in the repaired full-length allele and hDNA upstream of the gap in the truncated allele; this will be referred to as the “conventional” cleavage pattern. To confirm that the conventional cleavage pattern is reproduced in the CO-only plasmid, additional COs were isolated from the WT strain. Both the repaired and truncated alleles were sequenced from 89 COs, and hDNA was detected in 73 of these events (Figure 18). The COs were divided into four classes based on the sequencing results. The largest class (40/89) had hDNA present in both alleles (referred to as class A), and the hDNA pattern was indicative of conventional cleavage (Table 1). In seventeen COs, hDNA was detected in only one allele, and we define these as class B events. In each of the class B events, the hDNA was located as predicted by the conventional cleavage pattern, and the other allele had either a gene-conversion tract or no detectable sequence transfer. This pattern can be explained if hDNA was originally present in both alleles and an aberrant gene conversion event occurred in only one allele. We observed 16 events with a more complex pattern of hDNA (class C events). In many of these COs, regions of hDNA were present on both sides of the gap within one allele (bidirectional hDNA), or regions of hDNA were present at the same location in both alleles (symmetrical hDNA). Both of these patterns are consistent with HJ migration.
Figure 18: Location of hDNA in the full-length and truncated alleles of CO events isolated in the WT strain.

Both alleles from individual events are aligned, and events are grouped by class. Chromosomal sequence is depicted in blue, plasmid sequence in yellow and hDNA in green.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total number of COs sequenced</th>
<th>Class D COs without detectable hDNA</th>
<th>COs with hDNA</th>
<th>Conventional Cleavage</th>
<th>Reversed Cleavage</th>
<th>Class C complex tracts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Class A (%)</td>
<td>Class B (%)</td>
<td>Class A (%)</td>
<td>Class B (%)</td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>89</td>
<td>40/73 (54.8%)</td>
<td>17/73 (23.3%)</td>
<td>0</td>
<td>0</td>
<td>16/73 (21.9%)</td>
</tr>
<tr>
<td>mus81Δ</td>
<td>88</td>
<td>41/70 (58.6%)</td>
<td>18/70 (25.7%)</td>
<td>1/70 (1.4%)</td>
<td>2/70 (2.9%)</td>
<td>8/70 (11.4%)</td>
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<tr>
<td>yen1Δ</td>
<td>90</td>
<td>34/78 (43.6%)</td>
<td>32/78 (41.0%)</td>
<td>0</td>
<td>0</td>
<td>12/78 (15.4%)</td>
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<tr>
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<td>90</td>
<td>26/46 (56.5%)</td>
<td>11/46 (23.9%)</td>
<td>0</td>
<td>0</td>
<td>9/46 (19.6%)</td>
</tr>
<tr>
<td>rad1Δ</td>
<td>86</td>
<td>5/57 (8.8%)</td>
<td>23/57 (40.4%)</td>
<td>1/57 (1.8%)</td>
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</tbody>
</table>
In the remaining 16 events, no hDNA was detected (class D events), and the original cleavage pattern cannot be determined. It is important to note that no CO products were observed with the reversed cleavage pattern. Based on these results, we can conclude that the pattern of cleavage observed with the ARS-containing plasmid is shared by COs generated from the CO-only plasmid.

4.2.2 Mus81 is Not Essential for the Conventional Cleavage Pattern

Based on previous studies of the endonucleolytic activity of Mus81 (Kaliraman et al., 2001; Osman et al., 2003), we predicted that Mus81 is responsible for the conventional cleavage pattern observed in the CO products of gap-repair. If Mus81 is indeed the major HJ resolvase in this system, we would expect to see a decrease in Class A events in the absence of Mus81. We analyzed the sequences of 88 CO products isolated from the mus81Δ strain; 70 of which had detectable hDNA (Figure 19; Table 1). The remaining 18 COs were class D events with no hDNA. Eight class C events with bidirectional or symmetric hDNA were detected in the mus81Δ strain, which is 2-fold decrease compared to WT (21.9% versus 11.4%). Similar to WT, the majority of the COs with hDNA (41/70) were class A events. An additional 18 COs were class B events, consistent with the conventional pattern of cleavage. Together, these products of conventional cleavage made up more than 80% of the COs with detectable hDNA. To determine if the class A events resulting from conventional cleavage were effected by the loss of Mus81,
Figure 19: Location of hDNA in the full-length and truncated alleles of CO events isolated in the *mus81Δ* strain.
the length of the hDNA tracts downstream of the gap in the full-length allele were compared between WT and the \textit{mus81Δ} samples (Table 5).

Table 5: Tract length of hDNA in the full-length allele of class A CO products

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of class A COs</th>
<th>Median length</th>
<th>Average length</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>40/73 (54.8%)</td>
<td>407.0</td>
<td>373.7</td>
<td>46.3</td>
</tr>
<tr>
<td>\textit{mus81Δ}</td>
<td>41/70 (58.6%)</td>
<td>407.0</td>
<td>365.8</td>
<td>57.3</td>
</tr>
<tr>
<td>\textit{yen1Δ}</td>
<td>34/78 (43.6%)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>\textit{mus81Δ yen1Δ}</td>
<td>26/46 (56.5%)</td>
<td>407.0</td>
<td>357.1</td>
<td>62.5</td>
</tr>
<tr>
<td>\textit{rad1Δ}</td>
<td>5/57 (8.8%)</td>
<td>354.5</td>
<td>306.3</td>
<td>77.7</td>
</tr>
</tbody>
</table>

The average tract length differed by less than 10 basepairs and the standard deviation of the two distributions overlapped, which does not support a significant change in tract length. Based on this analysis, the class A conventional cleavage COs do not appear to be affected by loss of Mus81. It should be noted that one class A CO was observed with a reversed pattern of cleavage in the \textit{mus81Δ} strain, indicating that the reversed cleavage pattern is possible in this system. Two additional COs were detected with hDNA in only one allele (class B), and the hDNA location was consistent with the reversed cleavage pattern. These data indicate either that Mus81 is only a minor contributor to CO formation or that it is redundant with an activity that has a similar cleavage bias.
4.2.3 Conventional Cleavage Remains Unchanged in the Absence of Yen1

In the absence of Mus81, the class A events remained dominant, but there was evidence of reversed cleavage to yield COs. These results suggest that a second resolvase is involved in the cleavage of HJs. Yen1 has recently been identified as a HJ resolvase (Ip et al., 2008), and we examined its contribution to the hDNA patterns among CO events (Figure 20; Table 4). Twelve of the 90 COs sequenced were class D events without hDNA. Of the 78 events with hDNA, 12 (15.4%) were more complex class C events consistent with HJ migration. Class C events decreased slightly in the absence of Yen1 compared to WT (21.9% class C events), but not as dramatically as in the absence of Mus81 (11.4% class C events). The remaining COs with hDNA were consistent with the conventional cleavage pattern, and they were composed of 34 class A events and 32 class B events. The similarity of these results with the COs isolated from the WT strain suggests that Yen1 is not the major HJ resolvase in this system. In the yen1Δ strain, no COs were detected that had hDNA patterns diagnostic of reversed HJ cleavage.

4.2.4 Conventional Cleavage Persists in the Absence of Both Mus81 and Yen1

Although the loss of either Mus81 or Yen1 did not reduce the proportion of class A events dramatically, it is possible that these two proteins are acting redundantly.
Figure 20: Location of hDNA in the full-length and truncated alleles of CO events isolated in the \textit{yen1Δ} strain.
If this is the case, then the presence of the other protein in either of the single mutant strains may be masking the effect of the eliminated protein. To explore this possibility, a double mutant mus81Δ yen1Δ strain was constructed, and COs were collected (Figure 21; Table 4). Strikingly, the majority of the 90 COs sequenced were class D events without hDNA (44/90), and this increase compared to WT is highly significant (p<0.0001 as determined by a Fisher exact probability test). A small proportion of class C events with complex hDNA patterns was detected (9/46). The remaining 37 COs all had patterns of hDNA consistent with the conventional cleavage pattern; 26 COs were class A events, and 11 COs were class B events. Despite the abundance of class D events in this double mutant, the lack of reversed cleavage COs does not support the model that either of these proteins are responsible for the phenomenon of constrained cleavage. The lack of hDNA in individual COs can be explained by aberrant (non-MMR) repair or by segregation (either missing one half of the sectored colony when collecting the sample or the other cell died at an early stage).

4.2.5 Rad1 is Important for Constrained Cleavage Yielding COs

Previous studies have found that Rad1 promotes CO formation (Symington et al., 2000b; Welz-Voegele and Jinks-Robertson, 2008). To determine if Rad1 is generating COs by constrained cleavage in this system, we isolated and analyzed 86 COs in a rad1Δ strain (Figure 22; Table 4). Similar to the double mutant mus81Δ yen1Δ strain, class D events without hDNA were abundant within the sequencing spectrum (29/86).
Figure 21: Location of hDNA in the full-length and truncated alleles of CO events isolated in the *mus81Δ yen1Δ* strain.
Figure 22: Location of hDNA in the full-length and truncated alleles of CO events isolated in the \textit{rad1}\Delta strain.
The proportion of class D events was significantly greater that that of the WT strain (p<0.02 as determined by a Fisher exact probability test). Of the events with hDNA, class C events with complex patterns made up a substantial proportion of the COs in the absence of Rad1 (21/57). Twenty-eight COs demonstrating the conventional cleavage pattern were observed; 5 of these COs were class A events, and 23 were class B events. Importantly, 8 COs were detected with the reversed cleavage pattern. One of these was a class A event with hDNA in both alleles, and the remaining 7 COs had hDNA in only one allele (class B). These differences in the distribution of events (conventional cleavage, reversed cleavage and class C events) between WT and the rad1Δ strain were highly significantly (p<0.0001 as determined by Fisher exact probability test). However, class A events still outnumbered reversed cleavage events at more than 3 to 1. Additionally, in the rad1Δ strain, the complex class C events and no hDNA class D events made up more than 50% of the COs. Based on these data, we conclude that Rad1 is not the only protein responsible for the constrained cleavage pattern in this system.

4.3 Discussion

Constrained cleavage leading to crossing over during mitotic HR was examined in WT and in strains defective in candidate resolvases. Our plasmid-based gap-repair assay in an MMR-defective background (mlh1Δ) allowed us to track regions of hDNA in COs produced by HR. After confirming the phenomenon of constrained cleavage, the contributions of three candidate HJ resolvases, Mus81, Yen1 and Rad1, were quantified
by classifying CO events based on the location of hDNA in both the repaired and donor alleles. Our data demonstrate that none of the endonucleases tested here are exclusively responsible for the conventional cleavage pattern observed in this system. However, significant differences were observed between the spectrum of events in the rad1Δ strain and WT, supporting a role for Rad1 in CO formation in this system. Reversed cleavage was detected in the mus81Δ strain, suggesting that Mus81 participates in constrained cleavage. Additionally, a significant loss of events with hDNA was detected in the mus81Δ yen1Δ strain, which was not expected. Below, we examine the roles of Rad1, Mus81 and Yen1 revealed by our sequencing data and discuss relevant in vivo and biochemical studies.

4.3.1 Rad1 Promotes Normal CO Formation

Rad1-Rad10 participates in flap removal during DSB repair (Ivanov and Haber, 1995; Liefshitz et al., 1995) and has been implicated in HJ resolution (Habraken et al., 1994). However, a direct role in HJ cleavage has been debated (West, 1995). Our data confirm that Rad1 is important for the normal formation of COs during gap repair. Indeed, in the absence of Rad1, the distribution of hDNA in CO events changed significantly compared to the WT strain. Notably, class A events with the conventional hDNA pattern were almost eliminated, and an increase in reversed cleavage events was detected. These findings are consistent with Rad1 participating in the processing of HJs, leading to the conventional cleavage pattern.
Determining the role of Rad1 in HJ processing during gap-repair is complicated by its potential activity in both the formation and resolution of HJs. Previous studies have implicated Rad1 in D-loop nicking, presumably leading to sHJ formation (Symington et al., 2000b), and one may expect that loss of a major player in HJ formation could result in changes to the types or amount of COs produced. Without frequency data, we cannot confirm that the efficiency of CO formation is decreasing in the absence of Rad1, as was seen previously in a plasmid-based system with a larger gap (Welz-Voegele and Jinks-Robertson, 2008). Thus, one of the following explanations may be responsible for the changes to the spectrum of COs observed in this strain: only very rare events are being detected (because overall COs are severely decreased), one class of products is disappearing (and other classes are not impacted) or one class of products is converted into another type of product.

A significant increase in class D products was detected in the rad1∆ strain. If the absence of Rad1 indeed leads to loss of class A and B events but does not impact class D events, the class D events may appear to be increasing as they make up a larger proportion of the remaining products. Alternatively, class A and B events may be converted to class D events when Rad1 is absent. One mechanism for the production of class D events is the resolution of an sHJ intermediate by replication, which is predicted to result in a region of gene conversion in one allele and integration of the plasmid into the chromosome as a CO. If sHJ intermediates are not resolved in the absence of Rad1,
resolution by replication may increase and lead to additional class D events (Lorraine Symington, personal communication).

4.3.2 Loss of Mus81 and/or Yen1 Does Not Eliminate Conventional Cleavage

Both the \textit{mus81\textDelta} and the \textit{yen1\textDelta} single mutants yielded COs with patterns of hDNA very similar to the distribution obtained in the WT strain. No evidence of reversed cleavage was detected in the \textit{yen1\textDelta} strain. Although we did detect reversed cleavage in the \textit{mus81\textDelta} strain, this change was not statistically significant (p=0.05 by Fisher exact probability test). These results suggest that neither is solely responsible for the constrained pattern of HJ resolution in this system.

Several recent publications have investigated the impact of loss of both Mus81-Mms4 and Yen1 on HR. Agmon et al., 2011 showed that though loss of either led to only small defects in repair of an HO-induced DSB, loss of both genes specifically reduced CO formation. Work with a synthetic HJ also found that loss of both Mus81 and Yen1 decreased resolution (Tay and Wu, 2010). Given these results and the previously reported growth defect of the \textit{mus81\textDelta yen1\textDelta} double mutant (Blanco et al., 2010), we anticipated seeing an effect on the molecular structures of COs formed in this background. However, we found that conventional cleavage was not significantly reduced, and we did not detect an elevation in the reversed cleavage pattern. A highly significant increase in class D events was detected in this background, even more dramatic than that of the \textit{rad1\textDelta} CO spectrum. Similar to the explanation given above,
this increase may be the result of an overall decrease in the efficiency of crossing over and the resolution of joint molecules by replication. Our finding is supported by the observation that the number of sectored colonies resulting from repair of DSB in the absence of both Mus81 and Yen1 decreased significantly, which was presumably due to death of one of the recombinant cells (Ho et al., 2010). More importantly, both COs and NCOs are lost in favor of break-induced replication (BIR) in the \textit{mus81Δ yen1Δ} double mutant (Ho et al., 2010). As our assay does not detect BIR events, we have not observed this effect. Based on the data available at this time, we can conclude that neither Mus81 nor Yen1 are required alone or in combination for the constrained cleavage observed during gap repair.

\textbf{4.3.3 A Model for Constrained Cleavage Giving Rise to COs During Mitotic Gap Repair}

The strong effect of Rad1 on the CO products of gap-repair and the evidence that Rad1-Rad10 is not a traditional HJ resolvase require a new model to explain the genesis of COs in this system (West, 1995). If the canonical dHJ intermediate were cleaved by a resolvase, the prediction is that half of the products will be NCOs and half will be COs. Of the COs resulting from dHJ cleavage, equal proportions of two classes of products are expected, and these two classes can be distinguished by the location of hDNA in the donor and repaired alleles. As COs are created when the two HJs are cleaved asymmetrically, the conventional cleavage pattern is expected when the non-exchanged strands are cleaved in the first HJ (upstream of the gap) but the exchanged strands are
cleaved in the second HJ (downstream of the gap) (Figure 17a). When the exchanged strands are cleaved in the first HJ (upstream of the gap) but the non-exchanged strands are cleaved in the second HJ (downstream of the gap), the reversed cleavage pattern is expected. One possibility that explains the absence of the reversed cleavage pattern is that the two HJs are distinguished by which was formed by invasion (as opposed to 2nd end capture). The results of the sequencing of NCOs generated by synthesis-dependent strand annealing suggest that invasion happens preferentially upstream of the gap. If this upstream HJ is cleaved to generate the CO, the conventional pattern of hDNA would be generated. However, the bias towards invasion on the upstream side of the gap (determined from the NCO products of SDSA presented in Chapter 3) is only observed in ~75% of NCOs with unidirectional hDNA (108 out of 141), which is not sufficient to explain the complete absence of reversed cleavage in COs isolated from the WT strain.

Another possibility is that the dHJ is not fully ligated, and nicks are available to direct cleavage of this intermediate. However, Mus81-Mms4 is predicted to be the relevant endonuclease in regards to this activity (Osman et al., 2003), and the data presented here do not support a role for nick directed cleavage by Mus81 yielding the conventional pattern of hDNA. During recombination between diverged substrates, such as the plasmid-based system used here, homology may be limited. In this case, annealing of the D loop to the non-invading other end of the DSB will produce a single-
stranded gap if resection has been proceeded past the extent of homology, and this gap is a substrate for cleavage by Rad1-Rad10 (Lorraine Symington, personal communication). In this model, Rad1-Rad10 cleaves the annealed D loop to produce an sHJ, which could then be cleaved by Mus81-Mms4 (or Yen1). The gap versus nick model for sHJ processing is supported by the *in vitro* evidence that Mus81-Mms4 cleaves branched structures with gaps <4 nt, but Rad1-Rad10 will cleave structures with larger gaps (Bastin-Shanower et al., 2003; Ehmsen and Heyer, 2009).

Our data are compatible with an sHJ being a relevant intermediate during plasmid integration. Although dHJs have been detected in mitotic recombination, their abundance was very low relative to the number of DSBs formed (Bzymek et al., 2010). An sHJ intermediate is consistent with the Rad1 dependence of COs in a similar system (Welz-Voegele and Jinks-Robertson, 2008), as well as the previously characterized biochemical activity of Rad1-Rad10. There is only one way to cleave an sHJ that results in a CO, and this type of cleavage also results in the conventional cleavage pattern (if the HJ forms upstream of the gap). An sHJ intermediate could also explain why the conventional cleavage pattern remained even in the absence of the candidate resolvase proteins. Loss of these proteins may have reduced overall CO formation, but the conventional cleavage pattern was detected in the COs that remained (because an sHJ can only generate one type of CO). However, cleavage of an sHJ formed downstream of the gap would result in reversed cleavage, which was never detected in the WT strain,
and one remaining issue with the sHJ model is why it would be preferentially formed on the upstream side.

Although the majority of our results are consistent with cleavage of an sHJ intermediate, reversed cleavage was detected at significant levels in rad1Δ COs and at low levels in mus81Δ COs. If Rad1 is nicking the D-loop to promote sHJ formation, the absence of Rad1 may lead to second end capture of persistent D-loops, forming higher levels of dHJs in this background. Indeed, the reversed cleavage events among the mus81Δ COs could be the result of the processing of dHJs and support a low level of dHJs even when Rad1 is present. Alternatively, Mus81 may be nicking the D-loop and the non-exchanged strand to form COs (reviewed in Hollingsworth and Brill, 2004), independent of Rad1. In both the rad1Δ and the mus81Δ strains where we observed reversed cleavage, conventional cleavage was still more abundant than reversed cleavage. Analysis of COs in a rad1Δ mus81Δ double mutant may be a way to test for the existence other mechanisms that promote conventional cleavage and/or the sHJ intermediate.

One reason for the absence of the cleavage products of dHJs may be that dHJ are processed by mechanisms other than cleavage. The Sgs1 helicase has been proposed to specifically target dHJs for resolution by dissolution that yields exclusively NCO products. Data presented in the previous chapter demonstrate that the dHJ dissolution activity of Sgs1 contributes to NCO production during gap repair. In addition, recent
work has implicated Sgs1 as a major regulator of CO production and joint molecule resolution during meiosis (De Muyt et al., 2012; Zakharyevich et al., 2012). In the absence of both Mus81 and Yen1, successful JM processing and crossing over can occur (De Muyt et al., 2012). This activity is dependent on Sgs1, as well as the MMR components Exo1 and Mlh1-Mlh3 (Zakharyevich et al., 2012). Further study is required to confirm if, similar to the meiotic regulation, Sgs1 acts in a mitotic HJ resolution pathway that produces COs.

4.3.4 Concluding Remarks

Determining the relevance of the Mus81, Yen1 and Rad1 endonucleases to HJ cleavage has been problematic, as there are numerous regulatory mechanisms that control HR prior to this step. By monitoring the position of hDNA among individual CO products, the results presented here provide molecular confirmation that both Rad1 and Mus81 participate in HJ cleavage in this system. An additional role for Rad1 in promoting COs has been inferred, and we conclude that both dHJ and sHJ intermediates contribute to the production of COs during gap repair.

4.4 Experimental Procedures

4.4.1 Media and Growth Conditions

Yeast strains were grown nonselectively in YEPD (1% Bacto-yeast extract, 2% Bacto peptone, 2% dextrose) supplemented with 500 µg/mL adenine hemisulfate.
Selective growth was on synthetic complete (SC) medium lacking the appropriate nutrient, and all growth was at 30°C.

### 4.4.2 Gap-Repair Experiments

All strains were derived from the haploid strain SJR1501, which contains the diverged gap-repair template that has been described previously (Welz-Voegele and Jinks-Robertson, 2008). Resolvase-defective, \textit{mlh1}\textDelta derivatives of SJR1501 were constructed by targeted gene deletion, and a complete strain list is provided in Appendix C, Table 1. All deletions were confirmed by PCR. Linearizing plasmid pSR1008, a CO-only derivative of pSR987 (Mitchel et al., 2010) generated the substrate for gap repair. Plasmid pSR1008 was constructed by inserting a \textit{NotI}/\textit{SalI} fragment containing the \textit{his3}\Delta3' allele from pSR987 into \textit{NotI}/\textit{SalI} -digested pRS306 (Sikorski and Hieter, 1989), which does not contain an \textit{ARS}. Prior to transformation, plasmids were digested with \textit{BssHII}, and complete digestion was confirmed by gel electrophoresis.

The transformation protocol described previously (Welz-Voegele and Jinks-Robertson, 2008) was used, with the following modifications. After overnight growth of individual colonies in 5 ml of liquid YEPD, varying amounts of the saturated culture were added to a fresh 5 ml of liquid YEPD. These cultures were then incubated for a second night. The \textit{OD}_{600} of the cultures was measured to determine cell density, and two cultures (each derived from a different colony) with \textit{OD}_{600} values between 0.7 and 1.0 were selected for transformation. Cells were harvested by centrifugation at room
temperature, washed twice, and resuspended in 15 µl of H₂O. Cells were then combined with 165 µl of transformation mix consisting of 120 µl of PEG 3350 (50%, w/v), 18 µl of 1 M LiAc, 5 µl of 10 mg/ml boiled salmon sperm carrier DNA, 2 µl H₂O and 20 µl [20 ng] of gapped p1008. The tubes were vortexed for 1 min and incubated at 42°C for 1 h. The cells were then pelleted and resuspended in 300 µl of sterile water. Two 150-µl aliquots were plated on SC-his plates to select recombinants. After 4 days of incubation at 30°C, individual His⁺ transformants were frozen down in 20% glycerol without prior purification. To avoid bias, the selection plates were divided into sections, and all His⁺ transformants within a particular section were analyzed.

4.4.3 DNA Sequence Analysis of Recombinants

Using 96-well microtiter plates, 95 individual His⁺ recombinants were grown to saturation in SC-his liquid medium after inoculation with a 10 µl aliquot of the frozen stock described above. Cells were collected by centrifugation at room temperature and, following cell lysis with zymolyase, the genomic DNA was extracted (http://jinks-robertsonlab.duhs.duke.edu/protocols/yeast_prep.html). The full length and truncated alleles were amplified by polymerase chain reaction (PCR) with the appropriate primers (Appendix C, Table 2). The Duke Comprehensive Cancer Center DNA Analysis Facility performed the PCR purification and sequencing.

Following manual examination of the sequence chromatograms, the regions of hDNA were mapped using the presence of double peaks at the 19 SNPs, which
differentiate the two alleles. The full length and truncated alleles of each CO were examined for hDNA, and the location of hDNA in each allele was used to classify the individual repair events. Samples without detectable sequence transfer or with gene conversion only were not included in further analysis as one unique recombination intermediate cannot be inferred from their structure.

Individual tract lengths were calculated based on the presence of hDNA and gene conversion at the 19 SNPs, and the SNP with hDNA or gene conversion furthest from the gap was used to calculate the endpoint for an individual tract. The midpoint between the last SNP with hDNA/converted and the first SNP that was unchanged was used for the tract length, as in theory the hDNA tract could be only one nucleotide past the furthest SNP with hDNA or one nucleotide before the closest unchanged SNP. The median length and the average length in different backgrounds were calculated using the full-length allele of conventional cleavage class A events.
Chapter 5. Concluding Remarks

Recombination is vital to genome integrity in its role as the primary pathway for high-fidelity repair of DNA damage. Homologous recombination (HR) also contributes to the repair of stalled or broken replication forks and telomere maintenance (for reviews see Jain and Cooper, 2010; McGlynn and Lloyd, 2002). However, recombination can also be intentionally initiated and have beneficial consequences. Crossovers (COs) formed during programmed meiotic recombination allow for the proper segregation of homologous chromosomes and generate diversity amongst the haploid products. Gene conversion of the mating-type locus in haploid yeast is the result of mitotic recombination, which leads to mating-type switching and allows mating with the existing population to create diploid cells.

Defects in HR are associated with aging and human diseases, including cancer, highlighting its importance in DNA repair. During mitosis, COs between homologous chromosomes or repetitive sequences can uncover recessive alleles through loss of heterozygosity or lead to large-scale genome rearrangements, respectively. Chromosomal instability is a widely documented feature of human cancers (Berger et al., 2011; Chapman et al., 2011; Lengauer et al., 1998; Stratton et al., 2009). In the repeat-laden human genome, HR between diverged sequences can be particularly problematic, and control of mitotic COs is essential. DNA helicases contribute to the prevention of mitotic COs in yeast, and mutations in the human homologs of these genes lead to
devastating genetic diseases. Defects in three of the five human RecQ homologs, BLM, WRN and RecQ4, are linked to syndromes that are characterized by premature aging and a predisposition to cancer (Bernstein et al., 2010). FANCM, the human homolog of the yeast DNA helicase Mph1, is involved in the repair of interstrand crosslinks, and loss of FANCM activity has been observed in patients with Fanconi anemia, a disease of progressive bone marrow failure and increased risk of cancer, particularly of leukemia (Deans and West, 2011). Given the potential consequences of engaging in HR, regulation of recombination is particularly important.

At the time that this thesis work was initiated, the following model was in place to explain the mechanism of HR. The ends of the chromosome containing the initiating lesion, a double-strand break (DSB), are processed to yield 3’ single-strand DNA (ssDNA) tails by resection of the 5’ end. The 3’ ends are coated with Rad51 to form a nucleoprotein filament capable of invading a homologous duplex. Following invasion of the 3’ end to form a D loop, new synthesis is primed by the invading 3’ end using the complementary strand of the invaded molecule as a template. Multiple regulatory steps were proposed at this point to determine the outcome of this process as either a CO or noncrossover (NCO). Capture of the non-invading end of the DSB would form an intermediate with two Holliday junctions (HJ), and cleavage of an HJ can produce either a CO or a NCO. Collapse of the D loop would prevent CO formation by avoiding an HJ intermediate. Although multiple pathways for the production of COs versus NCOs had
been described, the specific regulatory steps leading to the CO or NCO outcome remained unclear.

In the experiments described here, a small gap on a plasmid was used to model a DSB, which allowed us to examine the regulation of mitotic HR in haploid yeast. The benefits of the transformation-based plasmid assay used in this work include the following features: a specific initiating lesion, the ability to recover both products of recombination and the presence of SNPs differentiating the donor and recipient alleles. Following transformation of this gapped plasmid into mismatch-repair (MMR) defective haploid yeast with a diverged chromosomal template, repair events were identified by selecting for restoration of a prototrophic marker, and the products of HR were categorized as COs or NCOs. In the absence of MMR, regions of heteroduplex DNA (hDNA) formed during HR are not repaired to give rise to gene conversion and can be detected by the presence of SNPs engineered to differentiate the two alleles. Both alleles of NCO and CO products were examined for hDNA by sequencing, and, as the location of the HR-initiating gap was known, these regions of hDNA could be used to infer the mechanism of HR. This system allows us to examine the molecular structures of recombination intermediates at a level of detail currently unmatched among in vivo assays.

In this system, a small gap on the plasmid introduced by restriction enzyme digestion served as the initiating lesion for recombination. In many other assays used to
study HR, the precise location and nature of the lesion is unknown, adding an element of uncertainty to inferred mechanisms. However, one drawback to our system is that our events are derived from plasmids containing a small gap, which may not be representative of the physiologically relevant lesions that induce spontaneous mitotic recombination. While a small gap is very similar to a DSB and DSBs are generally agreed to initiate HR, single stranded nicks may also initiate recombination (Strathern et al., 1991), and we cannot examine the products of nick-initiated recombination. However, the ability to directly detect hDNA prior to processing by MMR added a unique level of clarity and precision to our studies. While regions of gene conversion can be observed following HR in the presence of MMR, MMR can either lead to gene conversion or restoration, and restoration cannot be distinguished from a molecule that never possessed hDNA. The $\textit{mlh1}\Delta$ strain background used in these studies provides clarity regarding the location of strand transfer during HR. A plasmid-based system allowed the selection of both NCO and CO events from the same assay and the recovery of both products of recombination from individual HR events. The presence/absence and location of hDNA in both alleles of an individual recombination event allows the mechanism to be inferred. While some systems can detect both products of recombination in either CO or NCO events, few are designed to allow the comparison of both alleles in both types of products produced within the same experiment. This aspect of our assay enables the direct comparison of CO and NCO production.
The work presented in Chapter 2 demonstrated that the location and extent of hDNA differed significantly in NCO versus CO products. NCOs were often found to have one region of unidirectional hDNA, consistent with synthesis-dependent strand annealing (SDSA). Few NCOs were observed with two regions of hDNA, one on either side of the gap, and no NCOs were detected with one region of hDNA in the plasmid allele and one on the chromosomal allele. This distribution of events suggests that NCOs are primarily formed by SDSA in this system and that NCOs resulting from an HJ are the products of dissolution as opposed to cleavage. COs often had two regions of hDNA, one in each allele, and this pattern is diagnostic of HJ cleavage. In general, COs had longer hDNA tracts than NCOs, which suggested a model where the amount of resection (and, thus, the length of the invading end) determines the CO or NCO outcome.

This model poses the following fundamental and as yet unanswered question regarding the regulation of HR: what determines the nature of a recombination intermediate? To begin answering this question, we investigated how recombination intermediates were affected by proteins known to influence the CO or NCO outcome. Informed by previous in vitro and in vivo work, the impact of DNA helicases on NCO production was investigated in Chapter 3. We observed that the DNA helicases Mph1, Sgs1 and Srs2 all contribute to the formation of NCOs by SDSA, and Sgs1 and Srs2 promote NCOs with bidirectional hDNA. In the absence of Sgs1 or Srs2, COs were
unaffected, but the loss of Mph1 increased the proportion of COs. This result suggests that some recombination intermediates can either become NCOs or COs, depending on their processing, while other intermediates (those acted upon by Sgs1 and Srs2) cannot be redirected into a CO-producing pathway.

The role of structure-specific endonucleases on the formation of COs was investigated in Chapter 4. Using a CO-only plasmid in order to isolate these fairly rare events (~10% of His⁺ repair products in the wild-type strain), the constrained cleavage pattern initially observed and characterized in (Mitchel et al., 2010) was confirmed. The pattern of hDNA in COs produced in the absence of Mus81, Yen1 or Rad1 was examined, and substantial reversed cleavage was observed in the absence of Rad1. However, the major effect of loss of Rad1 was the absence of hDNA, and the bias towards the conventional pattern remained in the residual products where hDNA was detected. The activity of the candidate resolvases thus cannot explain why the HJ is preferentially formed and cleaved to yield a CO upstream of the gap.

In addition to the absence of COs products formed by cleavage of the non-exchanged strands of a HJ located downstream of the gap, the NCO product of HJ cleavage was never detected in the wild-type strain. One NCO with hDNA in both alleles consistent with HJ cleavage was detected in the mph1Δ sgs1Δ double mutant, indicating that this mechanism exists in this system but is very rarely used. The lack of these NCOs does not fit into the canonical model of DSB repair, where a HJ can be
cleaved to yield either a CO or NCO. This model predicts that random cleavage should yield equal proportions of COs and NCOs as the HJ is four fold symmetric when in a square-planar configuration. Even accounting for helicase-mediated dissolution of HJs prior to cleavage, the complete absence of these NCOs suggests a mechanism that prevents their formation. One such mechanism would be the nick-directed cleavage of Mus81-Mms4, which is predicted to yield exclusively COs (Hollingsworth and Brill, 2004). To test this model, the NCO products of gap repair in the absence of Mus81-Mms4 should be examined.

On a more fundamental level, it is still unknown why some D loop intermediates are unstable and collapse leading to SDSA, while others persist and mature into HJs. SDSA was not eliminated in the mph1Δ strain, the sgs1Δ strain, or the srs2Δ strain (nor in the mph1Δsgs1Δ or mph1Δsrs2Δ double mutants), which suggests that D loop collapse can occur spontaneously or that Sgs1 and Srs2 play a redundant role in this function. The sgs1Δsrs2Δ double mutant is lethal, so we cannot eliminate this second possibility. However, experiments could be designed to test the first option. If most D loop collapse occurs stochastically, the stability of the D loop may be determined by the length of the invading end. This model could be tested by artificially resecting the ends of the plasmid substrate. Commercially available nicking endonucleases could be used to generate 3’ single-stranded ends of a known length and transformation into an sgs1Δexo1Δ double mutant strain would eliminate the pathways for extensive resection. If D loop stability is
determined by the length of the invading end, smaller amounts of resection will produce a higher proportion of SDSA products and plasmids with longer resection will yield a lower proportion of SDSA products. The system could also be modified to extend the amount of homology between the two alleles by using an alternative gene, such as LYS2, as the selectable marker for repair. Greater homology would allow for more heterogeneity in hDNA tract lengths among recovered HR products. In particular, mitotic CO products have been detected with long gene conversion tracts in other assays (Lee et al., 2009).

Studies of recombination are crucial to our understanding of how damaged DNA is repaired, of how crossing over is prevented during mitotic HR and of the maintenance of genome stability as a whole. While these studies were performed in the budding yeast, *Saccharomyces cerevisiae*, most features of DNA metabolism are conserved among eukaryotes. In addition, the HR repair pathway has been detected in eukaryotes, prokaryotes and archaea, indicating that recombination is an evolutionarily ancient adaptation. Finally, the relevance of these studies to our knowledge of human diseases is underscored by genetic diseases that are due to defects in specific HR regulatory proteins, such as DNA helicases, as mentioned above. The experiments presented here provide further insight into how COs and NCOs are generated in response to a DSB and thus constitute a substantial contribution to the field of recombination and DNA repair.
Appendix A

Figure 23: Silent polymorphisms and gaps in the HIS3 gene.
Changes introduced by site-directed mutagenesis are indicated in red. The start and stop codons for the ORF are highlighted in yellow, as is the KpnI site that marks the end of the chromosomal \textit{his3}Δ3′ allele. The extent of the BglIII-generated gap is highlighted in gray. The 8 nt replaced with 4 nt to generate the BssHII site are boxed and highlighted gray, respectively. Digestion with BssHII generates 4-nt 5′ overhangs, which will be removed by the resection required to produce 3′ tails. It should be noted that the tails thus generated contain no terminal nonhomology relative to the chromosomal repair template.
Figure 24: Gene conversion tracts expected from hDNA segregation in CO products.

Orange and blue lines correspond to plasmid and chromosomal DNA, respectively; 
HIS3 and HIS3-flanking sequences are solid and dashed lines, respectively. Dotted lines 
represent DNA synthesized during gap repair and are colored according to the 
template. Arrowheads represent the 3’ ends of DNA strands, dashed boxes indicate the 
positions of gaps, and gray boxes highlight the positions of hDNA. The hDNA in the 
CO products has the trans pattern observed experimentally: upstream and downstream 
of the gap in the truncated and full-length products, respectively. Segregation of the 
hDNA-containing strands produces a single gene conversion tract upstream or 
downstream of the repaired gap in the truncated or full-length allele, respectively.
### Table 6: Primer sequences for PCR amplification and sequencing of gap-repair products

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M42</td>
<td>5′-CAATCACTTTTGGCCTTGGAAAC</td>
<td>Reverse sequencing primer; anneals to flanking CAN1 sequence</td>
</tr>
<tr>
<td>M43</td>
<td>5′-TAATCCATGCGCCAGTGGAAAC</td>
<td>Amplification of his3Δ3; anneals to flanking CAN1 sequence</td>
</tr>
<tr>
<td>M45</td>
<td>5′-CGCCAAGCTCGGAATTAAC</td>
<td>Amplification of repaired HIS3; anneals to flanking vector sequence</td>
</tr>
<tr>
<td>M46</td>
<td>5′-GTAATACGACTCACTATAGGGCG</td>
<td>Amplification of repaired HIS3; anneals to flanking vector sequence</td>
</tr>
<tr>
<td>B32</td>
<td>5′-CGCCGACATAGAGGAGAAG</td>
<td>Amplification of his3Δ3; anneals to flanking CAN1 sequence</td>
</tr>
<tr>
<td>R60</td>
<td>5′-TGCAAAACCAAGTGCACAAC</td>
<td>Reverse sequencing primer; anneals to HIS3 sequence</td>
</tr>
<tr>
<td>R61</td>
<td>5′-GCCTGTTGTCTGACTGCTTC</td>
<td>Reverse sequencing primer; anneals to HIS3 sequence</td>
</tr>
</tbody>
</table>
Appendix B

Figure 25: Proportion of COs produced during gap repair in WT and helicase-deficient strains.

The percentage of COs out of the total number of transformants is plotted by strain. The number of CO and NCO products were compared between WT and the helicase mutant strains, and a Fisher exact 2x2 probability test was used to calculate p-values.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total number of events</th>
<th>Chromosomal sequence change</th>
<th>Corresponding plasmid sequence</th>
<th>Number of events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>5</td>
<td>Gene conversion</td>
<td>No plasmid sequence detected</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hDNA</td>
<td>No plasmid sequence detected</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hDNA</td>
<td>No hDNA detected (gap-only)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hDNA</td>
<td>Gene conversion</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hDNA</td>
<td>Bidirectional hDNA</td>
<td>1</td>
</tr>
<tr>
<td>mph1Δ</td>
<td>5</td>
<td>Gene conversion</td>
<td>Gene conversion</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hDNA</td>
<td>No plasmid sequence detected</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hDNA</td>
<td>Gene conversion</td>
<td>2</td>
</tr>
<tr>
<td>srs2Δ</td>
<td>9</td>
<td>hDNA</td>
<td>No plasmid sequence detected</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hDNA</td>
<td>No hDNA detected (gap-only)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hDNA</td>
<td>Gene conversion</td>
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<td></td>
<td></td>
<td>Gene conversion</td>
<td>Gene conversion</td>
<td>2</td>
</tr>
<tr>
<td>sgs1Δ</td>
<td>7</td>
<td>hDNA</td>
<td>No plasmid sequence detected</td>
<td>3</td>
</tr>
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<td></td>
<td></td>
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<td>hDNA at same location</td>
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<td></td>
<td>Gene conversion</td>
<td>hDNA</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hDNA</td>
<td>Bidirectional hDNA</td>
<td>1</td>
</tr>
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<td>mph1Δ sgs1Δ</td>
<td>8</td>
<td>hDNA</td>
<td>Bidirectional hDNA</td>
<td>1</td>
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<td></td>
<td></td>
<td>hDNA</td>
<td>No plasmid sequence detected</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gene conversion</td>
<td>Bidirectional hDNA</td>
<td>1</td>
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<td></td>
<td></td>
<td>hDNA</td>
<td>hDNA at same location</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>hDNA</td>
<td>hDNA (consistent w HJ cleavage)</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 8: Length of hDNA tracts in the plasmid allele of NCO events detected in WT and helicase defective strains

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Event type</th>
<th>Median length</th>
<th>Average length</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>SDSA- downstream</td>
<td>292.0</td>
<td>265.8</td>
<td>127.0</td>
</tr>
<tr>
<td>mph1Δ</td>
<td>SDSA- downstream</td>
<td>321.5</td>
<td>289.6</td>
<td>120.0</td>
</tr>
<tr>
<td>sgs1Δ</td>
<td>SDSA- downstream</td>
<td>292.0</td>
<td>274.9</td>
<td>122.7</td>
</tr>
<tr>
<td>srs2Δ</td>
<td>SDSA- downstream</td>
<td>256.0</td>
<td>253.7</td>
<td>130.5</td>
</tr>
<tr>
<td>Wild-type</td>
<td>SDSA- upstream</td>
<td>217.5</td>
<td>235.5</td>
<td>99.7</td>
</tr>
<tr>
<td>mph1Δ</td>
<td>SDSA- upstream</td>
<td>217.5</td>
<td>225.5</td>
<td>96.4</td>
</tr>
<tr>
<td>sgs1Δ</td>
<td>SDSA- upstream</td>
<td>217.5</td>
<td>203.3</td>
<td>104.1</td>
</tr>
<tr>
<td>srs2Δ</td>
<td>SDSA- upstream</td>
<td>272.5</td>
<td>246.6</td>
<td>110.1</td>
</tr>
<tr>
<td>Wild-type</td>
<td>bidirectional</td>
<td>491.0</td>
<td>462.8</td>
<td>135.5</td>
</tr>
<tr>
<td>mph1Δ</td>
<td>bidirectional</td>
<td>479.5</td>
<td>491.6</td>
<td>149.1</td>
</tr>
<tr>
<td>sgs1Δ</td>
<td>bidirectional</td>
<td>572.0</td>
<td>564.9</td>
<td>148.3</td>
</tr>
<tr>
<td>srs2Δ</td>
<td>bidirectional</td>
<td>509.5</td>
<td>504.3</td>
<td>126.4</td>
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<tr>
<td>Strain</td>
<td>Genotype</td>
<td>Plasmid used</td>
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<td></td>
</tr>
<tr>
<td>--------</td>
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</tr>
<tr>
<td>SJR2157</td>
<td>MATα ade2-101oc his3Δ200 ura3-Nhe lys2ΔRV::hisG leu2-R can1::his3ΔKpn, 19 mut mlh1Δ::kan Gal⁺</td>
<td>pSR987/pSR1015</td>
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<td></td>
</tr>
<tr>
<td>SJR2862</td>
<td>MATα ade2-101oc his3Δ200 ura3-Nhe lys2ΔRV::hisG leu2-R can1::his3ΔKpn, 19 mut mlh1Δ::kan sgs1Δ::hyg Gal⁺</td>
<td>pSR987/pSR1015</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJR3137</td>
<td>MATα ade2-101oc his3Δ200 ura3-Nhe lys2ΔRV::hisG leu2-R can1::his3ΔKpn, 19 mut mlh1Δ::kan mph1Δ::hyg Gal⁺</td>
<td>pSR987/pSR1015</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJR3208</td>
<td>MATα ade2-101oc his3Δ200 ura3-Nhe lys2ΔRV::hisG leu2-R can1::his3ΔKpn, 19 mut mlh1Δ::kan mph1Δ::hyg sgs1Δ::nat Gal⁺</td>
<td>pSR1015</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJR3297</td>
<td>MATα ade2-101oc his3Δ200 ura3-Nhe lys2ΔRV::hisG leu2-R can1::his3ΔKpn, 19 mut mlh1Δ::kan srs2Δ::hyg Gal⁺</td>
<td>pSR1015</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJR3511</td>
<td>MATα ade2-101oc his3Δ200 ura3-Nhe lys2ΔRV::hisG leu2-R can1::his3ΔKpn, 19 mut mlh1Δ::kan srs2-860 Gal⁺</td>
<td>pSR1015</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## Appendix C

Table 10: *Saccharomyces cerevisiae* strains employed in Chapter 4

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Plasmid used</th>
</tr>
</thead>
<tbody>
<tr>
<td>SJR2157</td>
<td>MATα ade2-101oe his3Δ200 ura3-Nhe lys2ΔRV::hisG leu2-R can1::his3ΔKpn, 19 mut mlh1Δ::kan Gal+</td>
<td>pSR987/pSR1008</td>
</tr>
<tr>
<td>SJR2924</td>
<td>MATα ade2-101oe his3Δ200 ura3-Nhe lys2ΔRV::hisG leu2-R can1::his3ΔKpn, 19 mut mlh1Δ::URA3 mus81Δ::kan Gal+</td>
<td>pSR1008</td>
</tr>
<tr>
<td>SJR2925</td>
<td>MATα ade2-101oe his3Δ200 ura3-Nhe lys2ΔRV::hisG leu2-R can1::his3ΔKpn, 19 mut mlh1Δ::URA3 yen1Δ::kan Gal+</td>
<td>pSR1008</td>
</tr>
<tr>
<td>SJR2937</td>
<td>MATα ade2-101oe his3Δ200 ura3-Nhe lys2ΔRV::hisG leu2-R can1::his3ΔKpn, 19 mut mlh1Δ::URA3 mus81Δ::kan yen1Δ::hyg Gal+</td>
<td>pSR1008</td>
</tr>
<tr>
<td>SJR3183</td>
<td>MATα ade2-101oe his3Δ200 ura3-Nhe lys2ΔRV::hisG leu2-R can1::his3ΔKpn, 19 mut mlh1Δ::hyg rad1Δ::hisG Gal+</td>
<td>pSR1008</td>
</tr>
</tbody>
</table>
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**Biography**

Katrina Mitchel was born in Santa Cruz, CA on September 30\textsuperscript{th}, 1982. She was raised in Carmel Valley, CA and attended Reed College in Portland, OR. Completing her undergraduate thesis in the Chemistry department, Katrina received her Bachelor of Arts degree from Reed College in 2004 from the Biochemistry and Molecular Biology program. Katrina began graduate school in 2006 in the Molecular Genetics and Microbiology department at Duke University, and joined the lab of Sue Jinks-Robertson in 2008, studying homologous recombination and double-strand break repair in yeast. Some of her thesis work has been published in the article “Molecular structures of crossover and noncrossover intermediates during gap repair in yeast: implications for recombination” in the journal Molecular Cell, in 2010. She received the DeLill Nasser award for professional development from the Genetics Society of America in 2011.