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# Mechanisms and Regulation of Mitotic Recombination in *Saccharomyces cerevisiae*

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**ABSTRACT** Homology-dependent exchange of genetic information between DNA molecules has a profound impact on the maintenance of genome integrity by facilitating error-free DNA repair, replication, and chromosome segregation during cell division as well as programmed cell developmental events. This chapter will focus on homologous mitotic recombination in budding yeast *Saccharomyces cerevisiae*. However, there is an important link between mitotic and meiotic recombination (covered in the forthcoming chapter by Hunter *et al.* 2015) and many of the functions are evolutionarily conserved. Here we will discuss several models that have been proposed to explain the mechanism of mitotic recombination, the genes and proteins involved in various pathways, the genetic and physical assays used to discover and study these genes, and the roles of many of these proteins inside the cell.

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IN the course of this review, we will touch on many of the genes and processes conserved between mitosis and meiosis. Indeed, early studies in yeast and other fungi showed that mitotic recombination exhibited many of the same properties of meiotic recombination. For example, gene conversion, the nonreciprocal transfer of genetic information (see below), is sometimes associated with exchange (*i.e.*, crossover). Heteroduplex DNA, which is detected by the failure to repair mismatches between genetically distinct DNA molecules, is indicative of strand exchange and is often found at or near sites of crossovers. Importantly, the unrepaired mismatched sequences segregate after the next round of DNA replication and can be seen as sectored colonies, similar to postmeiotic segregation observed by tetrad analysis.

For simplicity, homologous recombination (HR) is minimally defined as the repair of DNA lesions using homologous sequences. During S phase and G2, in both haploid and diploid cells, repair of the damage uses the unbroken sister chromosome as the homologous sequence (Figure 1A). Such repair is the main role of mitotic recombination and it can lead to genetic consequences. When sister chromatid repair is accompanied by a crossover, it results in sister-chromatid exchange (SCE). If the repair event occurs between misaligned repetitive sequences in a tandem array, it results in an unequal SCE (USCE) (Figure 1B). In diploids, repair can also be templated from the unbroken homologous chromosome and if associated with a crossover, the exchange can lead to loss of heterozygosity (LOH) (Figure 1C). Mitotic gene conversion results when there is a nonreciprocal transfer of genetic information from one chromosome to the other during the repair event (Figure 1D). DNA repair from homologous sequences at nonallelic positions, called ectopic recombination, can lead to deletions, inversions, translocations, and acentric or dicentric chromosomes if repair is associated with a crossover (Figure 1, E and F).

Most of our attention focuses on the repair of double-strand breaks (DSBs); however, the exact nature of the initiating spontaneous lesion is unknown. Indeed nicks can be processed into single-stranded DNA (ssDNA) gaps or DSBs as the result of ligation failure from the previous round of DNA replication or during the repair of damaged or misincorporated nucleotides via processes such as nucleotide excision repair (NER), mismatch repair (MMR), base excision repair (BER), or transcription-coupled repair (TCR). Nicks can also be formed after the failed catalysis of Top1 covalently attached to DNA. Upon subsequent replication, these protein-bound nicks can also become DSBs. Reactive oxygen

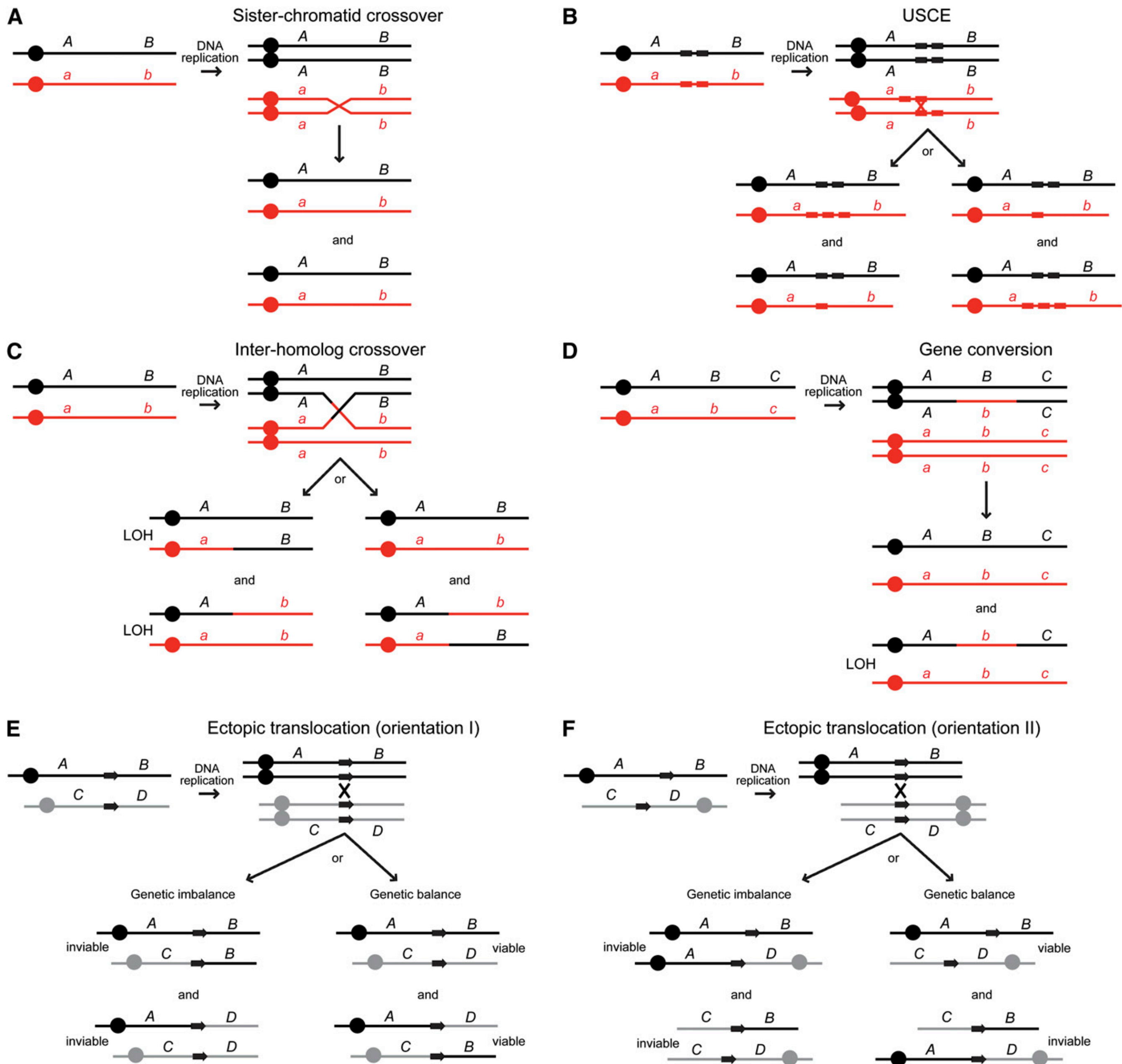


**Figure 1** Genetic outcomes of homologous recombination. The letters A/a and B/b indicate heteroalleles. Circles indicate centromeres. Colors red and black indicate homologous chromosomes in diploid cells. (A) Sister-chromatid crossover. A crossover between sister chromatids results in two genetically identical cells. (B) Unequal sister-chromatid exchange (USCE). Within repetitive sequence elements (boxes), a crossover between misaligned repeats results in repeat copy number expansion and contraction. (C) Interhomolog crossover. A crossover between homologs leads to loss of heterozygosity (LOH), if the recombinant molecules segregate to different cells in the ensuing cell division. (D) Gene conversion. A nonreciprocal genetic exchange between homologs leads to LOH in one of the resulting cells. (E) Productive ectopic translocation. A crossover between homologous sequences (boxes with arrows) with the same orientation relative to the centromere (circles) on different chromosomes in gray and black results in a productive ectopic translocation. Cosegregation of the recombinant molecules results in genetically balanced cells, shown on the right. Segregation of the recombinant molecules to different cells, shown on the left, leads to lethality if the regions represented by B and D are essential. (F) Nonproductive ectopic translocation. If the recombining sequences have opposite orientation with respect to the centromere, the reciprocal translocation results in inviable dicentric and acentric chromosomes.

species (ROS), cellular metabolism, and exogenous damage from ultraviolet light or gamma-irradiation can also produce nicks. Similarly, collapsed and stalled replication forks can lead to structures that can be processed into DSBs. Finally, DNA ends are produced when gamma-irradiation breaks both strands or when telomeres are uncapped due to problems in assembling the shelterin complex. In some cases, simple ligation of the ends, so-called nonhomologous end joining (NHEJ), results in repair that may or may not be error-free. For example, in a G1 cell, NHEJ is likely the preferred repair choice. However, given the many different sources of DNA lesions, it is clear that one of the central questions in the

regulation of recombination is how the cell "determines" how to repair a DSB—NHEJ or HR. Among some of the issues the cell must confront are the necessity to interpret whether it is haploid or diploid (controlled by the *MAT* locus), where it is in the cell cycle (controlled by CDK), what kind of processing the DNA ends need [controlled in part by the Mre11–Rad50–Xrs2 (MRX) complex] and the chromatin environment of the DNA lesion. All of this information must be integrated for the appropriate repair decision to be made. Understanding this integration will clarify how all of the pathways that impact recombination are intertwined to lead to repair of a DNA lesion and a viable cell.

Many of the genes described in this chapter that affect genetic recombination were originally identified by their requirement to repair radiation-induced DNA damage. For example, most of the genes of the *RAD52* epistasis group are ionizing radiation (IR) sensitive, while *RAD1* and *RAD10* are ultraviolet light sensitive (Game and Cox 1971; Game and Mortimer 1974). In addition, genes have been identified that are sensitive to other types of DNA damaging agents, such as methyl methanesulfonate (MMS), camptothecin (CPT), 4-nitroquinoline 1-oxide (4-NQO), etc. Over the years, genes involved in many pathways have been shown to affect genetic recombination. Some of these genes were discovered in mutation analyses that looked for effects on recombination and repair assays, while others were identified when the yeast gene disruption library was systematically tested for gross chromosomal rearrangements (Huang and Koshland 2003; Smith *et al.* 2004), mitotic crossing over (Andersen *et al.* 2008), sensitivity of polyploidy (Storchova *et al.* 2006), Rad52 foci (Alvaro *et al.* 2007), doxorubicin sensitivity (Westmoreland *et al.* 2009), sensitivity to R-loops (Gomez-Gonzalez *et al.* 2011), fragility of triplex structure-forming GAA/TTC (Zhang *et al.* 2012), stability of quasipalindromes (Zhang *et al.* 2013), and many more. Bioinformatic analyses have also revealed many genes involved in genome stability (Putnam *et al.* 2012). Furthermore, screens for hyperrecombination (hyper-rec) uncovered genes involved in a multitude of pathways (Aguilera and Klein 1988; Keil and McWilliams 1993; Scholes *et al.* 2001).

Finally, it is worth mentioning that most assays that have been constructed to identify genes that affect recombination were applied without knowing the precise recombination pathway being assayed. By isolating mutations that affect that pathway, we can start to understand the mechanism of the assay. At the same time, the precise function of the gene is refined by understanding its role in the assay. This "yin–yang" situation makes the study of homologous recombination so challenging.

## II. Mechanisms of Recombination

### A. Models for DSB-initiated homologous recombination

***DSB repair and synthesis-dependent strand annealing models:*** The DSB repair (DSBR) model was first proposed to explain the mechanism of plasmid gap repair and is currently the most accepted model to rationalize the association of crossing over with gene conversion during homologous recombination (Orr-Weaver *et al.* 1981; Szostak *et al.* 1983). In this model, the 5′ ends at the DSB are degraded to yield 3′ ssDNA tails, one of which invades a homologous double-stranded DNA (dsDNA) to form a displacement loop (D-loop) and is used to prime DNA synthesis, templated by the donor duplex (Figure 2). The 3′-terminated strand at the other side of the break anneals to the displaced strand from the donor duplex and primes a second round of leading strand synthesis. After ligation of the newly synthesized DNA to the resected 5′ strands, a double Holliday junction intermediate (dHJ) is generated. To segregate the recombinant duplexes, the HJs must be removed, which can occur by the activity of a helicase and topoisomerase to produce only noncrossover (NCO) products (dissolution), or by endonucleolytic cleavage (resolution). Cutting the inner strands of both HJs yields NCO products, whereas cleavage of the inner strands of one HJ and the outer strands of the other generates crossovers (COs). Heteroduplex DNA (hDNA), which is formed by pairing one strand from one duplex with a complementary strand from the other duplex, is a hallmark of homologous recombination and can be formed during the initial strand invasion and/or by second end capture. Repair of mismatches present in hDNA can give rise to gene conversion or restoration to the original sequence (Boiteux and Jinks-Robertson 2013).

While the DSBR model explains many properties of meiotic recombination, mitotic recombination generally shows a lower association of crossing over with gene conversion than observed during meiotic recombination. This observation led to two variations of the DSBR model, the synthesis-dependent strand annealing (SDSA) and migrating D-loop models, to explain the lower incidence of associated COs during mitotic DSB repair (Nassif *et al.* 1994; Ferguson and Holloman 1996; Paques *et al.* 1998). The SDSA model proposes that both 3′ ssDNA tails invade the homologous duplex(es) and after limited DNA synthesis are displaced by DNA helicases; the nascent complementary strands anneal and after fill-in synthesis and ligation generate exclusively NCO products (Nassif *et al.* 1994). The migrating D-loop model proposes that only one of the two 3′ ssDNA tails invades the homologous DNA duplex and after limited DNA synthesis is dissociated and anneals to the 3′ ssDNA tail at the other side of the DSB (Ferguson and Holloman 1996). Gap filling and ligation yields only NCO products (Figure 2). SDSA is the acronym generally used to refer to both models.

***Break-induced replication:*** Break-induced replication (BIR) is a recombination-dependent replication process that results in the nonreciprocal transfer of DNA from the donor to recipient chromosome. For repair by BIR, a single end of a DSB invades a homologous duplex DNA and initiates replication to the chromosome end (Kraus *et al.* 2001; Llorente *et al.* 2008) (Figure 2). As BIR from one of the two ends of a DSB would result in extensive loss of heterozygosity (LOH), it suggests BIR is suppressed when DSBs have two homologous ends in order for repair to occur by a more conservative HR mechanism.

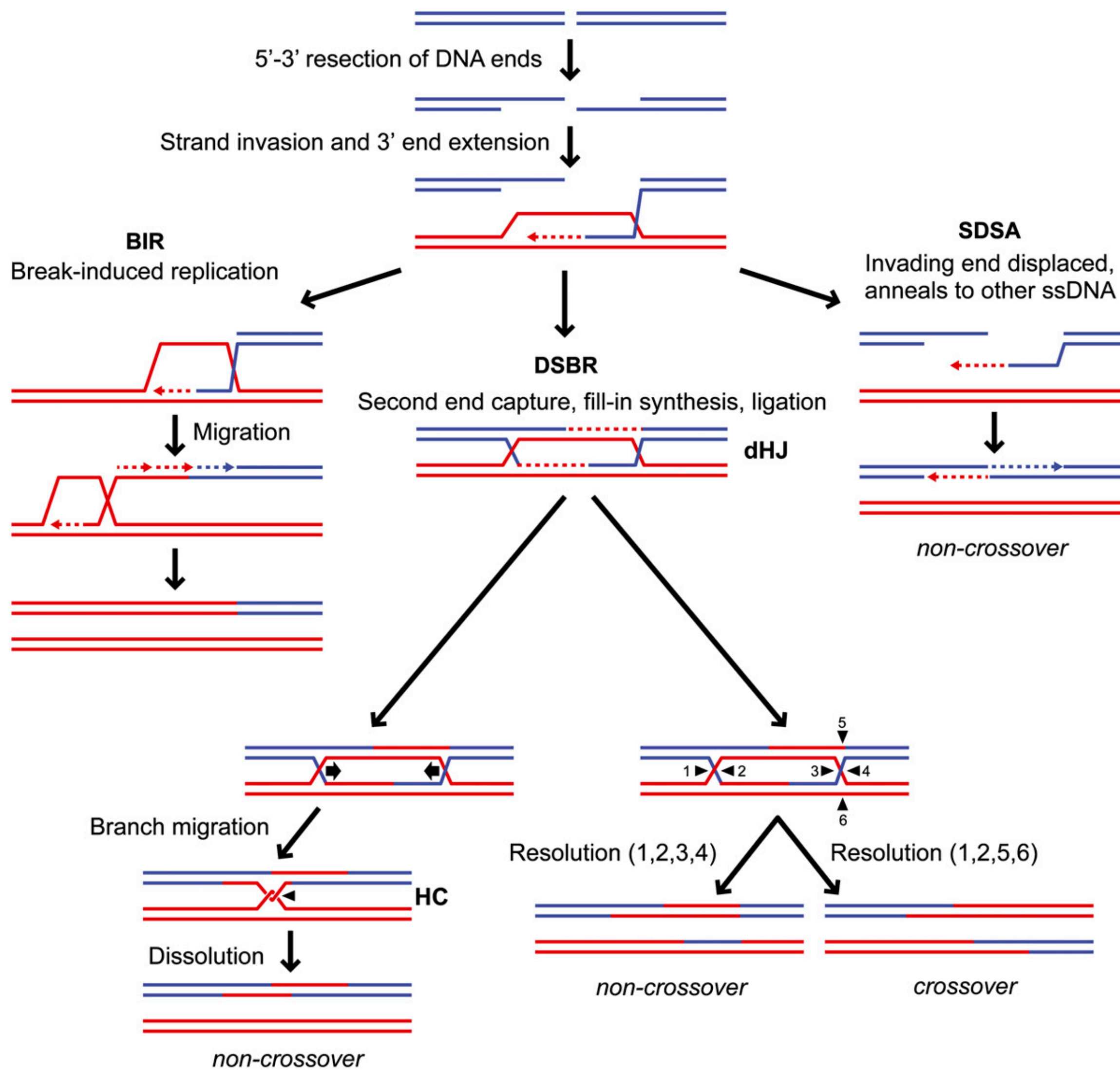


**Figure 2** Models for homology-dependent DSB repair. Recombinational repair of a DSB is initiated by 5′ to 3′ resection of the DNA end(s). The resulting 3′ single-stranded end(s) invades an intact homologous duplex (in red) to prime leading strand DNA synthesis. For one-ended breaks, a migrating D-loop is established to facilitate break-induced replication (BIR) to the end of the chromosome and the complementary strand is synthesized by conservative replication. For two-ended breaks, the classical double-strand break repair (DSBR) model predicts that the displaced strand from the donor duplex pairs with the 3′ ssDNA tail at the other side of the break and primes a second round of leading strand synthesis. After ligation of the newly synthesized DNA to the resected 5′ strands, a double Holliday junction intermediate (dHJ) is generated. The dHJ can be either dissolved by branch migration into a hemicatenane (HC) leading to noncrossover (NCO) products or resolved by endonucleolytic cleavage to produce NCO (positions 1, 2, 3, and 4) or CO (positions 1, 2, 5, and 6) products. In mitotic cells, the invading strand is often displaced after limited synthesis and the nascent complementary strand anneals with the 3′ single-stranded tail of the other end of the DSB and after fill-in synthesis and ligation generate exclusively NCO products (synthesis-dependent strand annealing, SDSA).

Indeed, the frequency of BIR at an endonuclease-induced DSB is <1%, but can be substantially higher if gene conversion is prevented by limiting homology to one side of the break (Malkova *et al.* 1996, 2005; Bosco and Haber 1998). The initial steps of BIR appear to be similar to DSBR and SDSA in requiring end resection, homologous pairing, and strand invasion (Davis and Symington 2004). Recent studies identified a migrating D-loop intermediate during BIR and demonstrated that synthesis is conservative (Donnianni and Symington 2013; Saini *et al.* 2013a), suggesting the “lagging” strand initiates on the nascent strand extruded from the trailing end of the D-loop. BIR can occur by several rounds of strand invasion, DNA synthesis, and dissociation, resulting in chromosome rearrangements when dissociation and reinvasion occur within dispersed repeated sequences (Smith *et al.* 2007; Ruiz *et al.* 2009). Thus, the highly mutagenic nature of BIR could contribute to genome evolution and disease development in humans.

***Single-strand annealing and microhomology-mediated end joining:*** The single-strand annealing (SSA) mechanism has been most extensively studied in the context of repair of an induced DSB formed between direct repeats (Paques and

Haber 1999). SSA might also be responsible for spontaneous deletions between direct repeats, but other mechanisms could operate. SSA efficiently repairs DSBs formed between repeats of >200 bp, but the frequency drops significantly for repeats of <50 bp (Sugawara *et al.* 2000). After resection of the DSB ends, the 3′ ssDNA tails can anneal when resection is sufficient to reveal complementary single-stranded regions corresponding to the repeats (Figure 3). Following annealing of the complementary ssDNA, heterologous flaps are formed if the repeats are separated from the break site by unique sequence. The flaps are removed by nucleases prior to gap filling and ligation (Fishman-Lobell *et al.* 1992; Ivanov and Haber 1995). Microhomology-mediated end joining (MMEJ) applies to an end joining mechanism that, like SSA, involves end resection and annealing between short (5–25 nt) direct repeats flanking a DSB (Ma *et al.* 2003; Decottignies 2007; Villarreal *et al.* 2012; Deng *et al.* 2014). These mechanisms are always mutagenic because they result in deletions, and MMEJ may be responsible for gross chromosome rearrangements (GCRs) that exhibit microhomologies at the junctions (Putnam *et al.* 2005).

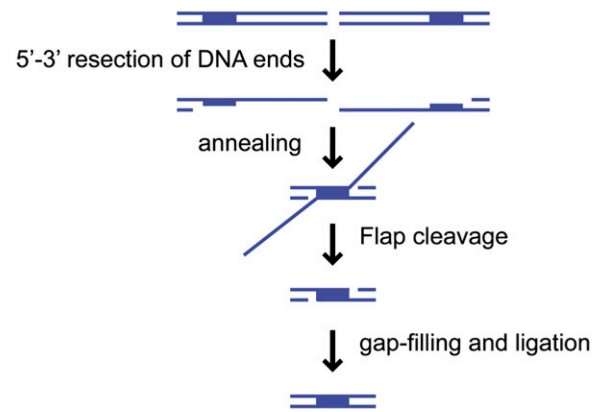


**Figure 3** Single-strand annealing. Repair of a DSB flanked by direct repeat sequences (boxes) can occur by single-strand annealing (SSA), if 5′ to 3′ resection is allowed to progress past the repeats. The complementary single-stranded repeats can anneal, leaving heterologous flaps to be removed by the Rad1–Rad10 endonuclease, before gap-filling and ligation completes the repair thereby deleting one of the repeats and the intervening sequence.

### *B. Proteins involved in homologous recombination*

Here we summarize the activities of proteins known to function at discrete steps of homology-dependent repair, as determined by biochemical analysis using defined DNA substrates and from the phenotypes of mutants in genetic and physical assays (for a list of human homologs and associated human diseases see Table 1). Physical assays, such as the *MAT* switching system, have been particularly useful to identify DNA intermediates formed during DSB repair and the genetic control of discrete steps in HR (Haber 2012). *MAT* switching is initiated by HO endonuclease cleavage of a specific site at the *MAT* locus and repair occurs by gene conversion using one of the transcriptionally silent donor cassettes, *HML* or *HMR*. The system has been adapted for studies of DSB repair by placing the *HO* gene under the control of the *GAL1* promoter to synchronously induce HO in a population of cells by addition of galactose to the growth medium (Jensen and Herskowitz 1984). The HO recognition site can be inserted at other genomic sites unrelated to *MAT* to induce recombination (Nickoloff *et al.* 1986). I-SceI, a site-specific endonuclease responsible for intron mobility in yeast mitochondria, has also been used to initiate DSB-induced recombination in the yeast nuclear genome by expressing an engineered version from the *GAL1* promoter (Plessis *et al.* 1992). The advantage of both systems is that 50–100% of the target sequences are cut within 1 hr following induction of the nuclease, and, by taking DNA samples at different times after DSB formation, intermediates in the process, for example, resected DNA ends and strand invasion intermediates, can be identified by Southern blot hybridization and/or PCR methods.

***DNA end resection:*** Homology-dependent DSB repair initiates by nucleolytic degradation of the 5′ strands to yield 3′ ssDNA tails, a process referred to as 5′–3′ resection (reviewed by Mimitou and Symington 2009) (Figure 4). The *RAD50* and *XRS2* genes were initially implicated in controlling end resection because the mutants show a marked delay in the initiation of resection at endonuclease-induced DSBs; however, recombination products are still produced, but with delayed kinetics and lower yield than wild-type cells (Ivanov *et al.* 1994). Since recombination is not completely defective, it is puzzling why *mre11*, *rad50*, and *xrs2* mutants exhibit extreme sensitivity to DNA damaging agents and could be due to other functions of the proteins, such as in telomere maintenance, processing of ends with adducts, and nonhomologous end joining repair. The Mre11, Rad50, and Xrs2 proteins form a stable heterotrimeric complex (MRX) that plays structural and catalytic roles in the initiation of end resection (Mimitou and Symington 2009). Mre11 contains conserved phosphoesterase motifs that are essential for 3′–5′ dsDNA exonuclease and ssDNA endonuclease activities *in vitro* (Furuse *et al.* 1998; Usui *et al.* 1998; Moreau *et al.* 1999; Trujillo and Sung 2001). Rad50 has a similar domain organization to the structural maintenance of chromosomes (SMC) family of proteins (Alani *et al.* 1989; Lammens *et al.* 2011; Lim *et al.* 2011; Williams *et al.* 2011). The integrity of the long coiled-coil domains, as well as the conserved Cys-X-X-Cys dimerization motif (Rad50 hook) at the apex of the coiled-coil domains, is important for all functions of the MRX complex, suggesting tethering of DNA ends or sister chromatids via MR binding and dimerization is critical for repair (De Jager *et al.* 2001; Hopfner *et al.* 2002; Wiltzius *et al.* 2005; Hohl *et al.* 2011). Mre11 binds to the base of the Rad50 coiled-coils forming a "head" region composed of the Mre11 nuclease and Rad50 ATPase domain that together provides DNA binding and end processing activities that are regulated by ATP binding and hydrolysis (Lammens *et al.* 2011; Lim *et al.* 2011; Williams *et al.* 2011). Interaction with Xrs2 is required for translocation of Mre11 to the nucleus,

**Table 1 Evolutionary conservation of homologous recombination proteins and examples of related human diseases**

| Functional class | *S. cerevisiae* | *H. sapiens* | Associated disease(s) | References |
|---|---|---|---|---|
| End resection | Mre11-Rad50-Xrs2 | MRE11-RAD50-NBS1 | Nijmegen breakage syndrome; AT-like disorder | (Varon et al., 1998) |
| | Sae2 | CtIP | | |
| | Exo1 | EXO1 | Colorectal cancer | (Yang et al., 2014) |
| | Dna2-Sgs1-Top3-Rmi1 | DNA2-BLM-TOP3-RMI1-RMI2 | Bloom syndrome | (Kaneko and Kondo, 2004) |
| Adaptors | Rad9 | 53BP1, MDC1 | Breast cancer | (Bartkova et al., 2007; Rapakko et al., 2007) |
| | - | BRCA1 | Breast cancer | (Petrucelli et al., 2010) |
| Checkpoint signaling | Tel1 | ATM | Ataxia-telangiectasia | (Gatti et al., 2001) |
| | Mec1-Ddc2 | ATR-ATRIP | Seckel syndrome | (O'Driscoll et al., 2003) |
| | Rad53 | CHK2 | | |
| | Rad24-RFC | RAD17-RFC | | |
| | Ddc1-Mec3-Rad17 | RAD9-HUS1-RAD1 | | |
| | Dpb11 | TOPBP1 | Breast cancer | (Karppinen et al., 2006) |
| Single-stranded DNA binding | Rfa1-Rfa2-Rfa3 | RPA1-RPA2-RPA3 | | |
| Single-strand annealing | Rad52 | RAD52 | | |
| | Rad59 | - | | |
| Mediators | Rad52 | - | | |
| | - | BRCA2-PALB2 | Breast cancer | (Petrucelli et al., 2010) |
| Strand exchange | Rad51 | RAD51 | Breast cancer | (Nissar et al., 2014; Sun et al., 2011) |
| | Rad54 | RAD54A, RAD54B | | |
| | Rdh54 | - | | |
| Rad51 paralogs | Rad55-Rad57 | RAD51B-RAD51C-RAD51D-XRCC2-XRCC3 | Breast cancer | (Silva et al., 2010; Vuorela et al., 2011) |
| | Psy3-Csm2-Shu1-Shu2 | RAD51D-XRCC2-SWS1 | | |
| Anti-recombinases | Srs2 | FBH1, PARI | | |
| | Mph1 | FANCM | Fanconi Anemia | (Meetei et al., 2005) |
| | - | RTEL1 | Hoyeraal-Hreidarsson syndrome | (Ballew et al., 2013) |
| Resolvases and nucleases | Mus81-Mms4 | MUS81-EME1 | | |
| | Slx1-Slx4 | SLX1-SLX4 | Fanconi Anemia | (Crossan *et al*., 2011; Kim *et al*., 2011; Stoepker *et al*., 2011) |
| | Yen1 | GEN1 | | |
| | Rad1-Rad10 | XPF-ERCC1 | Xeroderma pigmentosum | (Gregg et al., 2011) |

See also http://www.malacards.org.

and Xrs2 is also thought to regulate the MR complex and to mediate DNA damage signaling via interactions with Tel1 and Sae2 (Nakada *et al.* 2003; Tsukamoto *et al.* 2005; Lloyd *et al.* 2009; Schiller *et al.* 2012).

MRX can act as an endonuclease with Sae2 to cleave oligonucleotides from the 5′ strands resulting in short (~100 nucleotides) 3′ ssDNA tails, or it can promote resection indirectly by recruitment of the Exo1 and/or Dna2 nucleases (Mimitou and Symington 2008; Zhu *et al.* 2008; Shim *et al.* 2010). The Mre11 nuclease activity and Sae2 are essential to remove covalent adducts, such as Spo11 (which forms a covalent attachment to 5′ ends to initiate meiotic recombination) or hairpin caps, from DNA ends, but not for resection of endonuclease-induced DSBs (Mimitou and Symington 2009). There is a delay of ~30 min before resection of an HO-induced DSB initiates in the *sae2* mutant and this results in an increased frequency of NHEJ repair (Lee *et al.* 2008; Mimitou and Symington 2008; Deng *et al.* 2014). Sae2 exhibits its endonuclease activity *in vitro* that is stimulated by MRX, but unlike Mre11 has no obvious nuclease motifs (Lengsfeld *et al.* 2007). Which of these two nucleases, or if both, contributes to the initiation of resection is currently unknown. A class of separation-of-function *rad50* alleles, *rad50S*, confers similar phenotypes to the *sae2* and *mre11* nuclease-defective mutants (Alani *et al.* 1990). One attractive model for resection that has emerged from studies of meiotic recombination is for MRX and Sae2 to incise the 5′ strand at a distance from the end, followed by bidirectional resection from the nick using the Mre11 3′–5′ exonuclease and Exo1 5′–3′ exonuclease (Zakharyevich *et al.* 2010; Garcia *et al.* 2011). This model rationalizes how the Mre11 3′–5′ exonuclease participates in end resection and how Exo1 can overcome the block imposed by Ku binding at DNA ends.

Extensive resection is catalyzed by the 5′–3′ dsDNA exonuclease, Exo1, or by the combined action of the Sgs1 helicase and Dna2 endonuclease (Gravel *et al.* 2008; Mimitou and Symington 2008; Zhu *et al.* 2008) (Figure 4). The Sgs1 interacting partners Top3 and Rmi1 are also required for end resection,

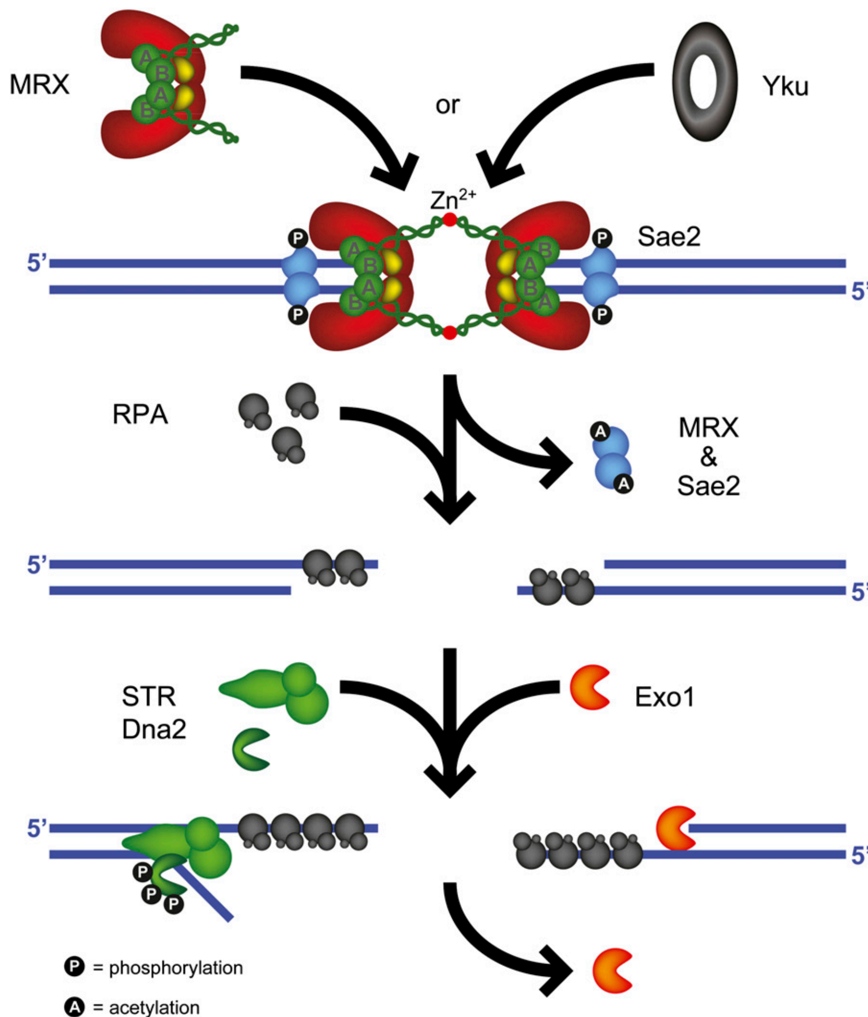


**Figure 4** Resection of DSB ends. Resection of DSB ends progress 5′ to 3′ and in two steps. First, MRX and Sae2 catalyze short-range resection of ~100 nt. The initial resection by MRX and Sae2 is particularly important for cleaning up "dirty" ends harboring chemical adducts, secondary structures, or covalently attached proteins. The Yku complex inhibits initial end resection by competing with MRX for binding to ends. Second, extensive resection for up to 50 kb is catalyzed by Exo1 and/or STR–Dna2. Phosphorylation of Sae2 at serine 267 is required for resection. Sae2 is degraded upon acetylation. Dna2 nuclear localization and recruitment to DSBs require its phosphorylation at threonine 4 and serines 17 and 237 (see text for details).

but their roles appear to be structural rather than catalytic (Zhu *et al.* 2008; Niu *et al.* 2010). *In vitro* reconstitution of the Sgs1-Dna2 reaction revealed an essential role for the heterotrimeric replication protein A (RPA, Rfa1–Rfa2–Rfa3) to stimulate Sgs1 unwinding and Dna2 cleavage of the 5′ strand (Cejka *et al.* 2010a; Niu *et al.* 2010). Depletion of RPA from cells at the time of HO cleavage results in a block to extensive resection and failure to recruit Dna2 to DSBs. RPA is also required to prevent formation of secondary structures within the 3′ ssDNA tails that can generate hairpin capped ends or can be degraded by MRX and Sae2 (Chen *et al.* 2013). Although Exo1 or Sgs1–Dna2 can directly process free DNA ends with no covalent adducts, they are unable to remove end-blocking lesions, such as Spo11. One possible reason for the greatly delayed initiation of resection observed for the *mre11*, *rad50*, or *xrs2* mutants is the poor recruitment of Exo1 and Sgs1–Dna2 in the absence of the MRX complex. The other possible reason is the presence of Ku, a heterodimeric dsDNA end binding protein essential for NHEJ, which inhibits Exo1-mediated resection and is greatly enriched at DSBs in the absence of the MRX complex (Ivanov *et al.* 1994; Clerici *et al.* 2008; Mimitou and Symington 2010; Nicolette *et al.* 2010; Shim *et al.* 2010). Indeed, the resection defect of the *mre11* mutant is suppressed by elimination of Ku or overexpression of *EXO1* (Bressan *et al.* 1999; Tsubouchi and Ogawa 2000; Moreau *et al.* 2001; Lewis *et al.* 2002). Similarly, the resection defect of the *sae2* mutant is suppressed by *yku70* in an Exo1-dependent manner (Limbo *et al.* 2007; Mimitou and Symington 2010).

The rate of extensive end resection is ~4 kb/hr and can remove up to 50 kb of DNA if there is no donor sequence to template repair of the DSB (Zhu *et al.* 2008). However, extensive resection is unlikely to be necessary for HR, and even the short 3′ ssDNA tails resulting from MRX–Sae2-dependent cleavage in the absence of Exo1 and Sgs1–Dna2 are sufficient for Rad51-dependent recombination (Mimitou and Symington 2008; Zhu *et al.* 2008). Although extensive resection is not required for HR, resection of >10 kb is needed to activate the DNA damage checkpoint (Gravel *et al.* 2008; Zhu *et al.* 2008; Roberts *et al.* 2012). Elimination of MRX, the Mre11 nuclease or Sae2 in the *exo1 sgs1* background results in a complete block to end resection and lethality (Mimitou and Symington 2008).

***Homologous pairing and strand invasion:*** The critical step of HR is pairing between the ssDNA formed by end resection and one strand of the donor duplex to form hDNA, a reaction

catalyzed by the Rad51/RecA family of recombinases (Shinohara *et al.* 1992; Symington 2002). Although Rad51 can catalyze DNA strand exchange under certain conditions *in vitro*, efficient exchange requires the activity of several other proteins, including RPA, Rad52, Rad54, Rad55, and Rad57 *in vivo*. Rad51 binds to ssDNA and dsDNA in an ATP-dependent fashion to form right-handed helical nucleoprotein filaments that can span thousands of nucleotides (Ogawa *et al.* 1993; Sung 1994; Sehorn *et al.* 2004; Sheridan *et al.* 2008; Ferrari *et al.* 2009). The contour length of DNA within the nucleoprotein filaments is extended by 50% relative to B-form DNA. Although Rad51 binds dsDNA *in vitro*, only the Rad51–ssDNA filament is active for homologous pairing and strand exchange. Rad51 has two DNA binding sites, one of which is required for high-affinity DNA binding and filament formation, and the second is required for homologous pairing (Cloud *et al.* 2012). Alone, Rad51 exhibits weak strand exchange activity that is stimulated by RPA, but this stimulation only occurs if Rad51 is allowed to nucleate on single-stranded DNA prior to the addition of RPA (Sung 1994, 1997a; Sugiyama *et al.* 1997). RPA is thought to stimulate Rad51 binding to ssDNA by removing secondary structures to allow assembly of a contiguous Rad51 nucleoprotein filament. If RPA and Rad51 are added simultaneously to ssDNA then RPA inhibits the reaction. This inhibition can be overcome by the addition of mediator proteins, such as Rad52 (Sung 1997a; New *et al.* 1998; Shinohara and Ogawa 1998). RPA also stimulates Rad51-mediated strand transfer by sequestering the displaced ssDNA that can inhibit the pairing reaction (Eggler *et al.* 2002).

***Rad51 mediators:*** Rad51 mediators include proteins that enable loading of Rad51 on RPA-coated ssDNA, stabilize Rad51 nucleoprotein filaments and/or promote strand exchange by Rad51 (Sung *et al.* 2003). Rad52 interacts directly with both RPA and Rad51 and is thought to mediate Rad51 filament assembly by delivering Rad51 to RPA-bound ssDNA where it binds cooperatively to DNA, displacing RPA (Sung 1997a; New *et al.* 1998; Krejci *et al.* 2002; Sugiyama and Kowalczykowski 2002) (Figure 5). These *in vitro* studies are consistent with Rad52 being required for Rad51 recruitment to an HO-induced DSB *in vivo* as measured by chromatin IP and fluorescence microscopy (Sugawara *et al.* 2003; Lisby *et al.* 2004). Rad52 interaction with Rad51 occurs through a domain in the nonconserved C-terminal region of the protein (Milne and Weaver 1993; Krejci *et al.* 2002). Mutants expressing Rad52 C-terminal truncations that remove the Rad51 interacting domain exhibit intermediate IR sensitivity as compared to wild type and *rad52* mutants, and the IR sensitivity is suppressed by overexpression of *RAD51* or by deleting *SRS2*, which encodes a helicase that disrupts Rad51–ssDNA complexes *in vitro* (Boundy-Mills and Livingston 1993; Milne and Weaver 1993; Kaytor *et al.* 1995). An acidic region of Rad52, encompassing residues 308–311 is required for interaction with RPA and for DNA damage resistance (Plate *et al.* 2008).

Rad55 and Rad57 are referred to as Rad51 paralogs because they share ~20% identity to the Rad51/RecA core region (Kans and Mortimer 1991; Lovett 1994). Rad55 and Rad57 interact to form a stable heterodimer and Rad55 also interacts with Rad51 (Hays *et al.* 1995; Johnson and Symington 1995; Sung 1997b). The Rad55-Rad57 complex is implicated as a mediator of Rad51 filament assembly because it alleviates the RPA inhibition to Rad51-catalyzed strand exchange *in vitro* (Sung 1997b). Rad51 recruitment to an HO-induced DSB occurs more slowly in the absence of Rad55, consistent with a role for Rad55–Rad57 in Rad51 filament formation or stabilization (Sugawara *et al.* 2003). The IR sensitivity of *rad55* and *rad57* mutants is partially bypassed by overexpression of *RAD51*, or by *RAD51* gain-of-function alleles, such as *rad51-I345T*, that encode proteins with higher affinity for DNA than wild-type Rad51 (Hays *et al.* 1995; Johnson and Symington 1995; Fortin and Symington 2002). Deletion of *SRS2* also suppresses the IR sensitivity of *rad55* and *rad57* mutants (Fung *et al.* 2009). *In vitro* studies show the Rad55–Rad57 complex stabilizes Rad51 nucleoprotein filaments and counteracts Srs2-mediated displacement of Rad51 from ssDNA (Liu *et al.* 2011).

The Psy3, Csm2, Shu1, and Shu2 proteins (collectively referred to as the Shu proteins) are thought to function in early HR because mutation of any of the *SHU* genes suppresses the *top3* slow growth defect and *sgs1* HU sensitivity, similar to *rad51*, *rad55*, and *rad57* mutations (Shor *et al.* 2002, 2005). The *shu* mutants are not sensitive to IR, but do exhibit sensitivity to MMS, an alkylating agent that stalls replication, and show increased mutation rates dependent on the translesion synthesis (TLS) DNA polymerase, Polζ (Huang *et al.* 2003; Shor *et al.* 2005), which is consistent with a role in error-free postreplicative repair (Ball *et al.* 2009). The *shu* mutations partially suppress the accumulation of sister-chromatid joint molecules in the *sgs1* mutant, suggesting they function specifically in early HR events to fill gaps during replication (Mankouri *et al.* 2007). The Shu proteins appear to antagonize the activity of the Srs2 antirecombinase, possibly through Rad51 filament stabilization (Bernstein *et al.* 2011). The Shu proteins interact to form a complex and structural studies of the Psy3–Csm2 subcomplex indicate structural similarity to a Rad51 dimer, raising the possibility that the Shu complex is incorporated into the Rad51 filament, as suggested for Rad55–Rad57 (Liu *et al.* 2011; Tao *et al.* 2012; Sasanuma *et al.* 2013). The Psy3–Csm2 complex exhibits DNA binding *in vitro*, with a preference for forked and 3′ overhang DNA substrates, and is able to stabilize Rad51 binding to ssDNA (Godin *et al.* 2013; Sasanuma *et al.* 2013).

***Single-strand annealing:*** Rad52 also promotes annealing of ssDNA *in vitro* (Mortensen *et al.* 1996; Shinohara *et al.* 1998) and this activity is likely important for capture of the second end in DSBR and SDSA and for the SSA mechanism of recombination (Sugawara and Haber 1992; Sugiyama *et al.* 2006; Lao *et al.* 2008). However, Rad52 is not required for MMEJ unless the microhomologies are >14 bp in length (Villarreal *et al.* 2012). This Rad51-independent function of Rad52 may explain the greater defect in most recombination

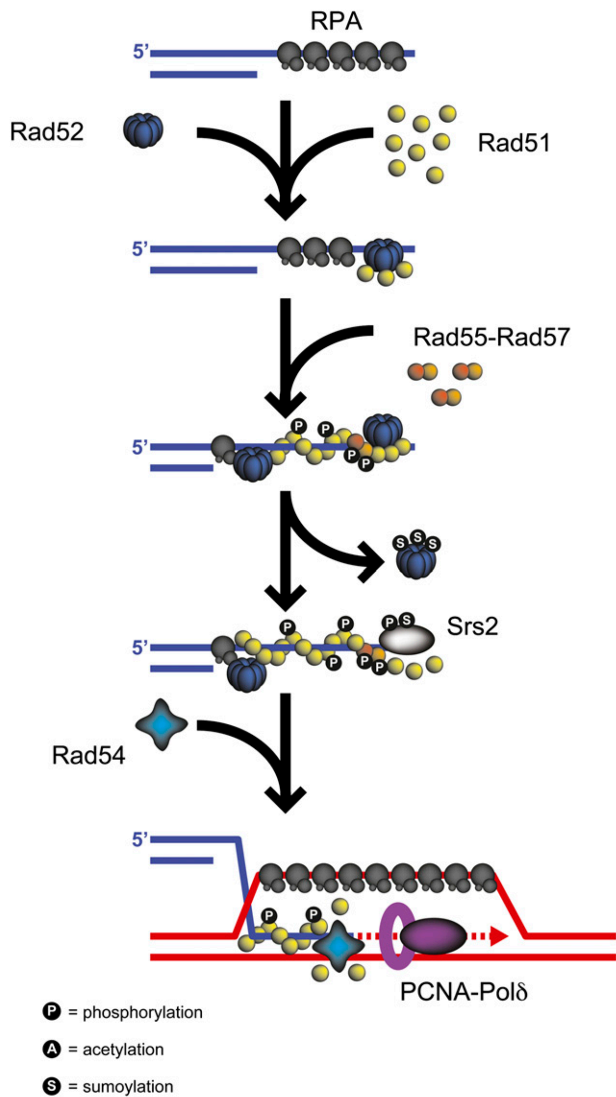


**Figure 5** Rad51 filament dynamics. During Rad51-catalyzed strand invasion, Rad52 mediates the loading of Rad51 onto RPA-coated ssDNA to facilitate formation of a Rad51 nucleoprotein filament. The Rad51 filament is further stabilized by Rad55–Rad57. In contrast, the Srs2 helicase counteracts the Rad51 mediators by displacing Rad51 from ssDNA to disrupt toxic recombination intermediates. Similarly, Rad54 can displace Rad51 from dsDNA to allow loading of PCNA–Polδ at the 3′ end of the invading strand. Phosphorylation of Rad51 at serine 192 is required for ATP hydrolysis and DNA binding. Rad55 is phosphorylated at serines 2, 8, and 14. Sumoylation of Rad52 at lysines 43, 44, and 253 mediates its dissociation from ssDNA. Phosphorylation and sumoylation of Srs2 have pro- and antirecombination functions, respectively (see text for details).

assays reported for *rad52* mutants compared to *rad51*, *rad54*, *rad55*, and *rad57* mutants (Symington 2002). RPA is inhibitory to ssDNA annealing *in vitro*; however, Rad52 is able to overcome this inhibition (Sugiyama *et al.* 1998). *In vivo*, RPA prevents annealing between microhomologies that are too short to be annealed by Rad52 and prevents SSA in the absence of Rad52 (Smith and Rothstein 1999; Villarreal *et al.* 2012; Deng *et al.* 2014). Rad59, a protein with homology to the N-terminal DNA binding domain of Rad52 (Bai and Symington 1996), interacts with Rad52 and augments the strand-annealing activity (Petukhova *et al.* 1999; Sugawara *et al.* 2000; Davis and Symington 2001; Wu *et al.* 2006). The N-terminal domain of Rad52 is required for strand annealing and multimerization of Rad52 and is essential for Rad52 function *in vivo* (Kagawa *et al.* 2002; Mortensen *et al.* 2002; Singleton *et al.* 2002).

***DNA translocases:*** Rad54 and Rdh54/Tid1, members of the Swi2/Snf2 family of chromatin remodeling proteins, stimulate homologous pairing by Rad51 *in vitro* (Petukhova *et al.* 1999, 2000). Rad54 stimulates D-loop formation between ssDNA and a homologous supercoiled plasmid, but also promotes dissociation of D-loop structures. The dissociation function is decreased when long ssDNA substrates are used or when duplex regions flank the invading ssDNA (Wright and Heyer 2014). Rad54 mediates chromatin remodeling and is able to promote Rad51-dependent D-loop formation on chromatinized templates (Alexiadis and Kadonaga 2002; Alexeev *et al.* 2003; Jaskelioff *et al.* 2003). Rad54 and Rdh54 exhibit dsDNA-specific ATPase activity and translocate on dsDNA to generate unconstrained negative and positive supercoils (Mazin *et al.* 2000; Van Komen *et al.* 2000). Strand separation at unwound regions is expected to facilitate the search for homology between dsDNA and the incoming Rad51 nucleoprotein filament. Interestingly, Rad54 enables Rad51-dependent D-loop formation between ssDNA and linear duplex DNA, a reaction not observed for the *Escherichia coli* RecA protein (Wright and Heyer 2014). Since homologous pairing between recipient and donor sequences is still observed *in vivo* in *rad54* mutants, it has been suggested that the primary function of Rad54 is postsynaptic rather than during the search for homology (Sugawara *et al.* 2003). Indeed, Rad54 is important to remove Rad51 from the end of strand invasion intermediates to permit access to DNA polymerases to extend the invading end (Li and Heyer 2009) (Figure 5). Rad54 and Rdh54 displace Rad51 from dsDNA and this could be important to remove unproductive association of Rad51 with dsDNA during presynapsis, thus increasing the pool of Rad51 available for HR, for turnover of Rad51 upon completion of recombination, or to uncover the 3′ end of paired intermediates to allow initiation of DNA synthesis (Solinger *et al.* 2002; Holzen *et al.* 2006; Shah *et al.* 2010). Overexpression of *RAD51* in the *rdh54* mutant results in the formation of toxic Rad51 foci on undamaged chromatin, suggesting Rdh54 is the major translocase to remove Rad51 in undamaged cells, whereas Rad54 acts on damaged chromatin (Shah *et al.* 2010).

Srs2 is a DNA helicase that plays both positive and negative roles in recombination. Srs2 mutants show elevated levels of spontaneous recombination, but in some DSB-induced recombination assays, the recovery of recombinants is reduced (Rong *et al.* 1991; Vaze *et al.* 2002). *In vitro*, Srs2 translocates on ssDNA, displacing Rad51 (Krejci *et al.* 2003; Veaute *et al.* 2003). The current view is that Srs2 prevents

initiation of recombination events presynaptically by disrupting the Rad51 nucleoprotein filament, and this activity could be important to prevent unwanted recombination at replication forks, where Srs2 colocalizes with PCNA (Pfander *et al.* 2005; Burgess *et al.* 2009). A direct interaction of Srs2 with PCNA may also regulate DNA synthesis during HR to suppress formation of crossover products (Burkovics *et al.* 2013). Although Srs2 is able to disrupt D-loop intermediates *in vitro*, it is less effective than Mph1, and the pattern of hDNA products recovered from plasmid gap repair in the *srs2* mutant is not consistent with a simple D-loop dissociation mechanism (Sebesta *et al.* 2011; Mitchel *et al.* 2013).

***DNA synthesis during HR:*** DNA synthesis is essential to extend the 3′ end within the D-loop and is likely to be required after strand displacement to fill gaps adjacent to the annealed sequences, replacing the nucleotides lost by end resection. Genetic studies suggest these two phases of DNA synthesis may use different polymerases. The reversion frequency of a marker located 300 bp from an HO cut site was higher during DSB repair than during normal growth, and the mutagenesis was largely DNA Polζ dependent (Holbeck and Strathern 1997; Rattray *et al.* 2002). Polζ-dependent mutagenesis of nearby genes has also been reported for other recombinogenic initiating lesions, such as inverted repeats, GAA repeats, and interstitial telomere repeats (Shah *et al.* 2012; Aksenova *et al.* 2013; Saini *et al.* 2013b; Tang *et al.* 2013). In contrast, another study demonstrated that the mutagenic DNA synthesis associated with gap repair is independent of the TLS polymerases, Polζ or Polη (Hicks *et al.* 2010). The DNA synthesized in the context of the D-loop appears to be carried out by DNA Polδ operating at much lower fidelity and processivity than during S-phase synthesis (Maloisel *et al.* 2008; Hicks *et al.* 2010). Consistent with these findings, the Polδ complex is able to extend D-loop intermediates generated *in vitro* by Rad51 and Rad54 (Li and Heyer 2009). It is possible that long tracts of ssDNA formed by end resection are subject to base modification and gap-filling synthesis by DNA Polζ causing mutations in sequences close to the DSB, but not in the context of D-loop synthesis.

Conditional alleles of essential replication genes have been used to determine the role of replication proteins during DSBR by physical monitoring of *MAT* switching. Gene conversion of the *MAT* locus is independent of ORC, the Cdc7–Dbf4 kinase, the MCM complex, Cdc45, DNA Polα, and Okazaki fragment processing proteins, but requires PCNA, Dpb11, and either Polδ or Polε (Wang *et al.* 2004; Germann *et al.* 2011; Hicks *et al.* 2011). In contrast to gene conversion repair of a two-ended DSB, BIR requires lagging strand as well as leading strand synthesis (Lydeard *et al.* 2007). The extensive DNA synthesis associated with BIR needs the nonessential subunit of the Polδ complex, Pol32, and is also compromised by the *pol3-ct* mutation, which affects stability of the Polδ complex (Lydeard *et al.* 2007; Deem *et al.* 2008; Payen *et al.* 2008; Smith *et al.* 2009; Brocas *et al.* 2010). The Pif1 helicase is required for BIR and *in vitro* studies show Pif1 functions with the Polδ complex to extend the 3′ end of a Rad51-generated D-loop (Saini *et al.* 2013a; Wilson *et al.* 2013). Pif1 facilitates extensive DNA synthesis by liberating the newly synthesized ssDNA to establish a migrating D-loop, in agreement with the current model for BIR synthesis (Figure 2). All three replicative DNA polymerases are required for BIR, but the need for Polε occurs later than for Polα and Polδ (Lydeard *et al.* 2007).

***Resolution of recombination intermediates:*** Resolution of D-loop intermediates by displacement of the extended invading strand is the primary mode of DSB repair in mitotic cells, at least for events initiated by endonucleases (Mitchel *et al.* 2010) (Figure 2). The Mph1 helicase dissociates Rad51-generated D-loop intermediates *in vitro* and mutants show increased levels of COs during DSB-induced recombination, in agreement with a role in promoting SDSA repair (Sun *et al.* 2008; Prakash *et al.* 2009; Tay *et al.* 2010; Sebesta *et al.* 2011; Lorenz *et al.* 2012; Mazon and Symington 2013; Mitchel *et al.* 2013). The increased COs observed in the *mph1* mutant are dependent on *MUS81* (Mazon and Symington 2013). Joint molecules, detected by two-dimensional agarose gel electrophoresis, accumulate transiently in the absence of *MPH1*, and persist at high levels in the *mph1 mus81* double mutant (Mazon and Symington 2013). Dissociation of D-loop intermediates is expected to have a negative impact on BIR and indeed overexpression of *MPH1* reduces the frequency of BIR, while the *mph1* mutant exhibits an increased BIR frequency (Luke-Glaser and Luke 2012; Stafa *et al.* 2014). The *srs2* mutant also exhibits increased levels of COs associated with DSB-induced recombination, but, as noted above, the pattern of hDNA products observed is not consistent with D-loop dissociation.

The DSBR model predicts the formation of a dHJ intermediate, which must be resolved for segregation of the recombinant duplexes. The Sgs1–Top3–Rmi1 complex can resolve dHJ intermediates *in vitro* by a process called dissolution (Wu and Hickson 2003; Cejka *et al.* 2010b). The helicase activity of Sgs1 branch migrates the constrained HJs and the topoisomerase activity of Top3 is thought to remove the supercoils between the two HJs eventually leading to NCO products (Figure 2). Consistent with the *in vitro* studies, *sgs1* and *top3* mutants show increased levels of COs associated with spontaneous recombination and DSB-induced gene conversion (Wallis *et al.* 1989; Watt *et al.* 1996; Ira *et al.* 2003). Furthermore, joint molecules (JMs) containing a dHJ intermediate are detected at higher levels during DSB-induced interhomolog recombination in *sgs1* diploid cells than in wild type (Bzymek *et al.* 2010).

X-shaped JMs are detected in the highly repetitive ribosomal DNA (rDNA) locus during S-phase and their abundance increases when DNA Polα/primase is limiting (Zou and Rothstein 1997). Replication-dependent X-structures at unique sequences that are independent of Rad51 and Rad52 have been found in unperturbed cells. These structures

are thought to be hemicatenanes due to their physical properties and resistance to cleavage by HJ resolvases *in vitro* (Lopes *et al.* 2003). Late forming replication-dependent X-structures accumulate in the absence of the Sgs1 helicase when cells are treated with MMS and in this case require HR functions, as well as the template-switching branch of post-replication repair for their formation, suggesting they are formed at ssDNA gaps as a means to bypass lesions (Figure 6) (Liberi *et al.* 2005; Branzei *et al.* 2008).

The alternative means to remove HJ-containing intermediates is through endonucleolytic cleavage. Several structure-selective nucleases (Mus81–Mms4 heterodimer, Yen1 and Slx1–Slx4 heterodimer) have been shown to cleave branched DNA structures, including HJs, *in vitro* (Boddy *et al.* 2001; Kaliraman *et al.* 2001; Fricke and Brill 2003; Ip *et al.* 2008). Interestingly, *mus81*, *mms4*, *slx1*, and *slx4* mutations were all identified on the basis of synthetic lethality with *sgs1* (Mullen *et al.* 2001). Because Mus81–Mms4 (Eme1 in *Schizosaccharomyces pombe*) exhibits higher cleavage activity on D-loops and nicked HJs than intact HJs, Mus81–Mms4/Eme1 most likely processes an early strand exchange intermediate, prior to ligation to form a dHJ intermediate, to generate crossover products (Kaliraman *et al.* 2001; Osman *et al.* 2003; Mazon and Symington 2013; Mukherjee *et al.* 2014). Ho *et al.* (2010) found a significant decrease in the formation of DSB-induced CO products between homologs in the *mus81* diploid and a greater decrease in the *mus81 yen1* double mutant, suggesting Mus81–Mms4 is the primary activity to resolve recombination intermediates with Yen1 serving as a back up function. The partial redundancy between these activities is also observed for DNA damage sensitivity, but Yen1 is not able to counteract the lethality of the *mus81 sgs1* mutant unless it is constitutively activated (Blanco *et al.* 2010; Ho *et al.* 2010; Tay *et al.* 2010; Matos *et al.* 2013). Surprisingly, COs between ectopic repeats are only reduced by 50% in the *mus81 yen1* double mutant (Agmon *et al.* 2011). Persistent JMs containing a single HJ (sHJ) connecting the ectopic sequences were identified in the *mus81 yen1* mutant, and their formation, as well as generation of CO products, was dependent on *RAD1* (Mazon *et al.* 2012; Mazon and Symington 2013). However, Rad1–Rad10 has no apparent role in the formation of COs between chromosome homologs (Mazon *et al.* 2012). Rad1–Rad10 is proposed to facilitate ectopic CO formation by cleaving the leading edge of the captured D-loop at the heterology boundary creating a substrate for subsequent cleavage by Mus81–Mms4 or Yen1 (Mazon *et al.* 2012).

In contrast to *mus81*, the synthetic lethality of *slx1* or *slx4* with *sgs1* is not suppressed by *rad51* mutation, suggesting the lethality might be due to problems other than, or in addition to, unresolved recombination intermediates (Fabre *et al.* 2002; Bastin-Shanower *et al.* 2003). *In vitro*, the yeast Slx1–Slx4 complex preferentially cleaves 5′ flap structures; however, the human SLX1–SLX4 complex is reported to cleave intact HJs (Schwartz and Heyer 2011). The *slx1* mutant has no obvious defect in the formation of mitotic or meiotic crossovers, however, in an *slx4* mutant, spontaneous mitotic crossovers are reduced (Ho *et al.* 2010; De Muyt *et al.* 2012; Zakharyevich *et al.* 2012; Gritenaite *et al.* 2014).

Removal of heterologous flaps that can form during strand invasion or following strand annealing by the SDSA and SSA models requires the Rad1–Rad10 endonuclease, which cuts branched DNA structures at the transition between dsDNA and ssDNA (Fishman-Lobell and Haber 1992; Ivanov and Haber 1995; Mazon *et al.* 2012). The flap cleaving activity of Rad1–Rad10 requires Slx4, but not Slx1, and the mismatch repair proteins, Msh2 and Msh3 (Fishman-Lobell and Haber 1992; Ivanov and Haber 1995; Sugawara *et al.* 1997; Flott *et al.* 2007). Saw1, which was identified in a genome-wide screen for SSA defects, binds specifically to 3′ flap structures *in vitro* and recruits Rad1–Rad10 to ssDNA flaps *in vivo* (Li *et al.* 2013, 2008). Although Rad1–Rad10 is generally considered to be essential for heterologous flap removal, in an assay that detects chromosomal translocations formed by SSA, the *rad1* defect was suppressed by *rad51* (Manthey and Bailis 2010). This result raises the possibility that Rad51 binds to unrepaired ssDNA flaps and prevents access to nucleases other than Rad1–Rad10.

## III. Genetic Assays of Mitotic Recombination

In this section, we describe the various assays that have been used over the years to both define mitotic recombination as well as to aid in the isolation of mutations in genes that affect the process. Further insight has been gained by the introducing of site-specific lesions in these assays, such as inserting the HO-cut site or site-specific nicking site (Nickoloff *et al.* 1986; Galli and Schiestl 1998; Cortes-Ledesma and Aguilera 2006; Nielsen *et al.* 2009). Concomitant expression of these nucleases has allowed researchers to induce DSBs and nicks to evaluate their role in many of these assays.

### *A. Allelic recombination in diploids*

Allelic recombination refers to events that occur at allelic positions between homologous chromosomes. As shown in Figure 1C and Figure 7A, most recombination events occur in the G2 phase of the cell cycle and, although there is a strong preference for recombination between sister chromatids, numerous genetic studies have documented recombination between homologs. Conversion events can be selected by generation of a functional copy of a gene from different mutant alleles (heteroalleles). Use of heterozygous markers centromere (*CEN*) proximal to the recombining locus allows identification of associated crossovers by LOH, though half of the potential CO events are not detected because of random segregation of chromatids at mitosis (Figure 1C and Figure 7) (Chua and Jinks-Robertson 1991; Ho *et al.* 2010). The rate of spontaneous gene conversion is generally $\sim 10^{-6}$ events/cell/generation and 10–20% of events are associated with COs (Haber and Hearn 1985). Allelic recombination is stimulated by several orders of magnitude if cells are irradiated with IR or UV (Manney and Mortimer 1964; Esposito and Watsgaff

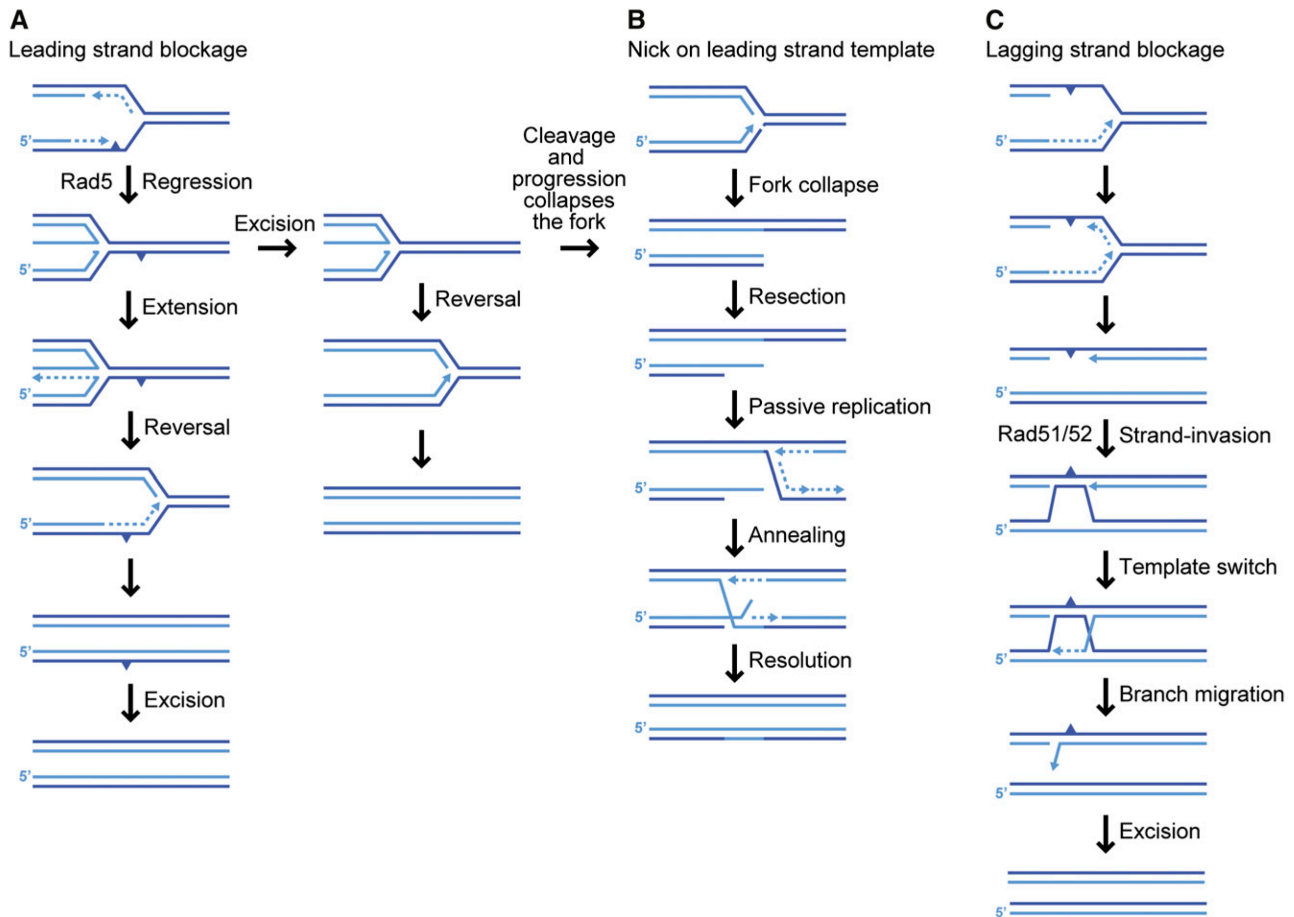


**Figure 6** Recombination at replication forks. Parental strands are shown in dark blue and nascent strands in light blue. Polymerase-blocking DNA lesion indicated by a filled triangle. (A) Error-free bypass of leading strand blockage. The replication fork stalled at a DNA lesion on the leading strand template may be regressed in a Rad5-dependent manner to expose the lesion for excision repair after which the regressed fork is reversed and replication resumed. Alternatively, leading strand synthesis may transiently switch templates within the regressed fork. Upon fork reversal and reanneling of the extended leading strand to its parental template, the DNA lesion is bypassed and can subsequently be repaired by excision repair. (B) Fork collapse and rescue by passive replication. Fork collapse may result if the replication fork encounters a nick on the leading strand template or if a regressed fork is endonucleolytically cleaved to form a one-ended DSB, which is most often rescued by passive replication from an adjacent replication fork that can anneal to the end and be resolved into two intact sister chromatids. (C) Error-free bypass of lagging strand blockage. Lagging strand synthesis can be completed by postreplicative recombination to reestablish strand continuity at the lesion using the nascent sister chromatid as a template. The remaining lesion on the parental lagging strand can subsequently be removed by excision repair.

1981), or if one of the recombining loci has the recognition sequence for the HO or I-SceI rare-cutting endonucleases (Nickoloff *et al.* 1999; Palmer *et al.* 2003; Mozlin *et al.* 2008). The frequency of recombinants is sufficiently high when induced by a targeted DSB to analyze unselected events and to distinguish between a CO and BIR (Malkova *et al.* 1996; Ho *et al.* 2010) (Figure 7B).

Because spontaneous gene conversion events occur at low frequency and only one recombinant daughter cell is selected, reciprocal COs cannot be distinguished from BIR. Petes and colleagues developed a clever genetic assay to detect spontaneous reciprocal crossovers that occur between *CEN5* and the *CAN1* locus (Barbera and Petes 2006). The rate of spontaneous reciprocal exchange is $4 \times 10^{-5}$ within this 120-kb interval. By using haploid strains with 0.5% sequence divergence to create the diploid, single nucleotide polymorphisms (SNPs) were used to map the site of exchange between *CEN5* and *CAN1* and determine the length of the conversion tracts associated with crossovers (Lee *et al.* 2009; Lee and Petes 2010). Unlike meiotic recombination, which occurs at nonrandom positions, the mitotic crossovers were evenly distributed and the median length of conversion tracts was ~12 kb, much longer than meiotic conversion tracts (Lee *et al.* 2009). Surprisingly, many of the conversion tracts associated with spontaneous crossovers showed 4:0 segregations of the heterozygous markers or hybrid tracts consisting of 3:1 adjacent to 4:0 segregations. Such events are best explained by the presence of a DSB in a G1 cell, replication of the broken chromosome and repair of the two broken sisters from the nonsister chromatids in G2, where one event would have to be associated with a CO to generate a sectored colony (Esposito 1978; Lee and Petes 2010).

The rate of spontaneous gene conversion is reduced >20-fold in *rad51* and *rad52* mutants, and by 5- to 10-fold in *rad54*

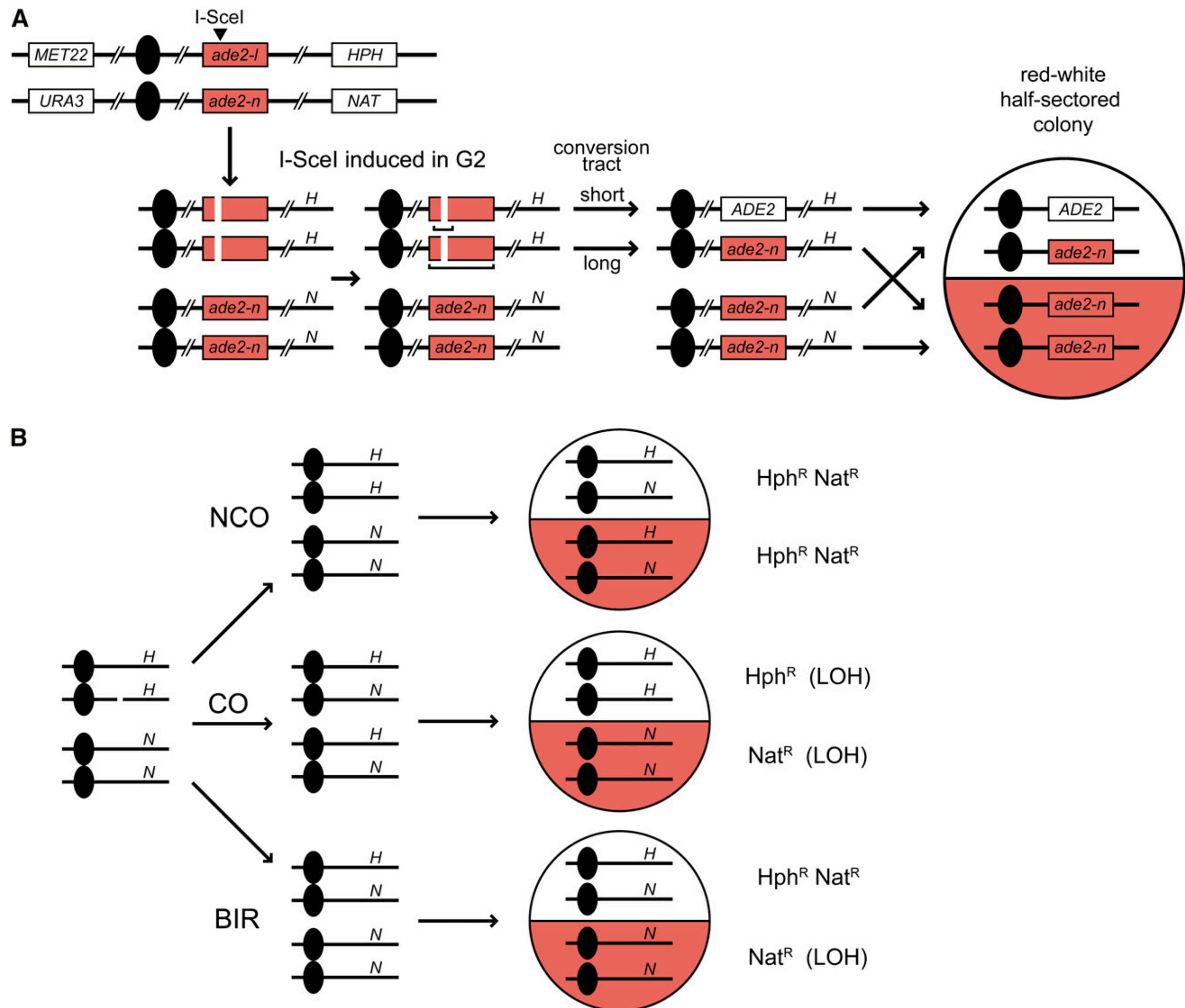


**Figure 7** Assay for heteroallelic recombination. Nonfunctional *ade2-I* and *ade2-n* heteroalleles and wild-type *ADE2* give rise to red and white colonies, respectively. (A) I-SceI-induced heteroallelic recombination. A DSB induced by the I-SceI endonuclease in the *ade2-I* allele in G2 can be repaired from the intact homolog by short-tract or long-tract gene conversion to give rise to *ADE2* and *ade2-n*, respectively. Red-white half-sectored colonies are indicative of a recombination event that occurred in the first generation after plating. Markers *MET22* and *URA3* on the other side of the centromeres (filled circles) facilitate the scoring of chromosome nondisjunction events. Markers *HPH* and *NAT* adjacent to the *ade2* locus facilitate the scoring of CO events. (B) Scoring CO, NCO, and BIR events associated with gene conversion. Genotyping of red-white half-sectored colonies with respect to the *HPH* (H) and *NAT* (N) markers described in panel A allows the distinction of CO and BIR events as reciprocal and nonreciprocal LOH, respectively. The remaining events are NCOs.

and *rdh54* mutants as compared to wild type (Bai and Symington 1996; Klein 1997; Petukhova *et al.* 1999; Mortensen *et al.* 2002). In the absence of Rad52, aberrant products are recovered that are associated with chromosome loss and are thought to occur by a half crossover mechanism (Haber and Hearn 1985; Coic *et al.* 2008). Mutation of *MRE11*, *RAD50*, or *XRS2* results in higher rates of allelic recombination in diploids, but a slight reduction in the recombination rate between repeats (Alani *et al.* 1990; Ajimura *et al.* 1993; Rattray and Symington 1995). Cohesin loading at DSBs and stalled replication forks is dependent on MRX, suggesting recombinogenic lesions are channeled from sister chromatids to homologs in the absence of this complex (Strom *et al.* 2004; Unal *et al.* 2004; Tittel-Elmer *et al.* 2012). Allelic recombination is also higher in *rad59* diploids compared to wild type (Bai and Symington 1996). Rad59 physically interacts with the RSC chromatin-remodeling complex, which is required for cohesin loading at DSBs, and is important for sister chromatid recombination (Oum *et al.* 2011); thus, Rad59 may function to promote sister-chromatid recombination and in its absence, lesions might be channeled to nonsisters as suggested for the MRX complex.

### B. Recombination between dispersed repeats

Ectopic or nonallelic homologous recombination (NAHR) refers to events occurring between homologous sequences located at

nonallelic positions (Figure 1, E and F). Naturally occurring repeats (for example, the rDNA array and Ty elements) and artificial duplications have been used as substrates for ectopic recombination, mainly in haploid cells. The repeated sequences can be located on different chromosomes or within the same chromosome. Closely spaced repeats in the same orientation (tandem or nontandem) or inverted relative to each other generally exhibit higher rates of recombination than repeats present on different chromosomes (dispersed) (Liefshitz *et al.* 1995). To a first approximation, the rate of spontaneous recombination between dispersed repeats and its genetic control are more similar to allelic recombination than to direct or inverted repeat recombination, suggesting heterologous chromosomes interact as frequently as homologous chromosomes in mitotic cells (Lichten and Haber 1989). However, global analyses of the yeast genome three-dimensional organization by chromosome conformation capture indicate that some loci are more prone to contact than others and these restricted chromosome territories influence the frequency of ectopic recombination (Burgess and Kleckner 1999; Duan *et al.* 2010; Agmon *et al.* 2013). Reciprocal exchange between dispersed repeats yields chromosome translocations that can only be detected if the orientation of the repeats with respect to their centromeres is the same. About 10–50% of gene conversion events between dispersed repeats have an associated crossover (Jinks-Robertson and Petes 1986; Liefshitz *et al.* 1995; Robert *et al.* 2006). However, the frequency of associated COs is generally less for DSB-induced ectopic recombination (Inbar and Kupiec 1999; Ira *et al.* 2003).

***Direct-repeat recombination:*** Gene conversion between direct repeats can be detected using heteroalleles of a selectable gene, and can occur by intrachromatid or sister-chromatid interactions (Figure 8A). Gene conversion between misaligned sister chromatids can generate a triplication or deletion on one chromatid while retaining the direct repeat on the other, whereas a crossover between misaligned sister chromatids generates triplication and deletion products (Klein 1988) (Figure 1B). Intrachromatid gene conversion associated with a crossover generates a chromosomal deletion and an episomal circular product. Although deletions are formed at high frequency between direct repeats, the reciprocal product is only associated with ~7% of deletions, suggesting they arise primarily by a nonconservative mechanism (Schiestl *et al.* 1988; Santos-Rosa and Aguilera 1994).

Gene conversion events between direct repeats that maintain the intervening sequence require *RAD51*, *RAD54*, *RAD55*, and *RAD57*, whereas deletions arise independently of these genes, consistent with their formation by SSA (McDonald and Rothstein 1994; Liefshitz *et al.* 1995; Petukhova *et al.* 1999). By contrast, *rad52* mutants exhibit reduced frequencies of conversion and deletion events (Klein 1988; Thomas and Rothstein 1989b) likely due to the role of Rad52 as a Rad51 mediator and as a strand annealing protein, respectively.

The ribosomal genes in yeast naturally occur as a multiple tandem array (150–200 copies). Recombination within and between the repeats is important to maintain homogeneity of the cluster and is restricted to mitosis (Petes and Botstein 1977). Repeat homeostasis of rDNA is measured by determining the repeat length of the native array, which reveals either gain or loss of repeats. Recombination between repeats can also generate rDNA circles and the accumulation of rDNA circles in mother cells has been implicated in cellular aging (Sinclair and Guarente 1997; Kobayashi 2008). Due to their repetitive nature, rDNA have been excellent substrates for studying recombination. The insertion of selectable and counterselectable markers allows measurements for the effects of mutations on recombination outcomes—Rad51 is necessary for both deletion and duplication of the marker and Rad52 is necessary for duplication (Szostak and Wu 1980; Gangloff *et al.* 1996). In addition, the multiple copies of rDNA permit the detection of recombination intermediates after two-dimensional gel electrophoresis (Brewer and Fangman 1988; Zou and Rothstein 1997). Circles of rDNA are formed at high rates in mutants that are defective in DNA topoisomerases Top1 and Top2, indicating that regulating DNA topology is important for the stability of the array (Christman *et al.* 1988; Kim and Wang 1989). In addition, mutation of Top3, a eukaryotic type IA topoisomerase, also leads to increased rDNA instability (Wallis *et al.* 1989).

Importantly, to maintain sequence homogeneity, there is a genetic system in place that ensures recombination between the rDNA repeats. This system, controlled by the Fob1 replication block protein, is thought to help avoid collisions between transcription of rDNA and the bidirectional DNA synthesis that can be potentially initiated in every repeat (Takeuchi *et al.* 2003). At each repeat, Fob1 binds and inhibits DNA synthesis in one direction so that there are very few replication/transcription collisions. However, it is likely that the accumulation of blocked forks actually increases the amount of recombination that takes place naturally within this array, since in the absence of Fob1 protein, spontaneous recombination is reduced fivefold (Defossez *et al.* 1999). The increased recombination between the repeats stimulates homogenization of this multiple tandem array. The recent finding that spontaneous chromosomal fragile sites in yeast are enriched for motifs that correlate with paused replication forks supports the view that the Fob1 sites are playing this role in the rDNA array (Song *et al.* 2014).

***Inverted-repeat recombination:*** Substrates with inverted repeats were designed to avoid formation of recombinants by SSA (Figure 8B). Gene conversion between inverted repeats retains the original configuration, whereas intrachromatid gene conversion associated with a CO or long tract conversion between misaligned sister chromatids results in inversion of the intervening sequence (Rothstein *et al.* 1987; Rattray and Symington 1994; Chen *et al.* 1998). Spontaneous recombination between inverted repeats is highly dependent on *RAD52*, but reduced only 5- to 10-fold

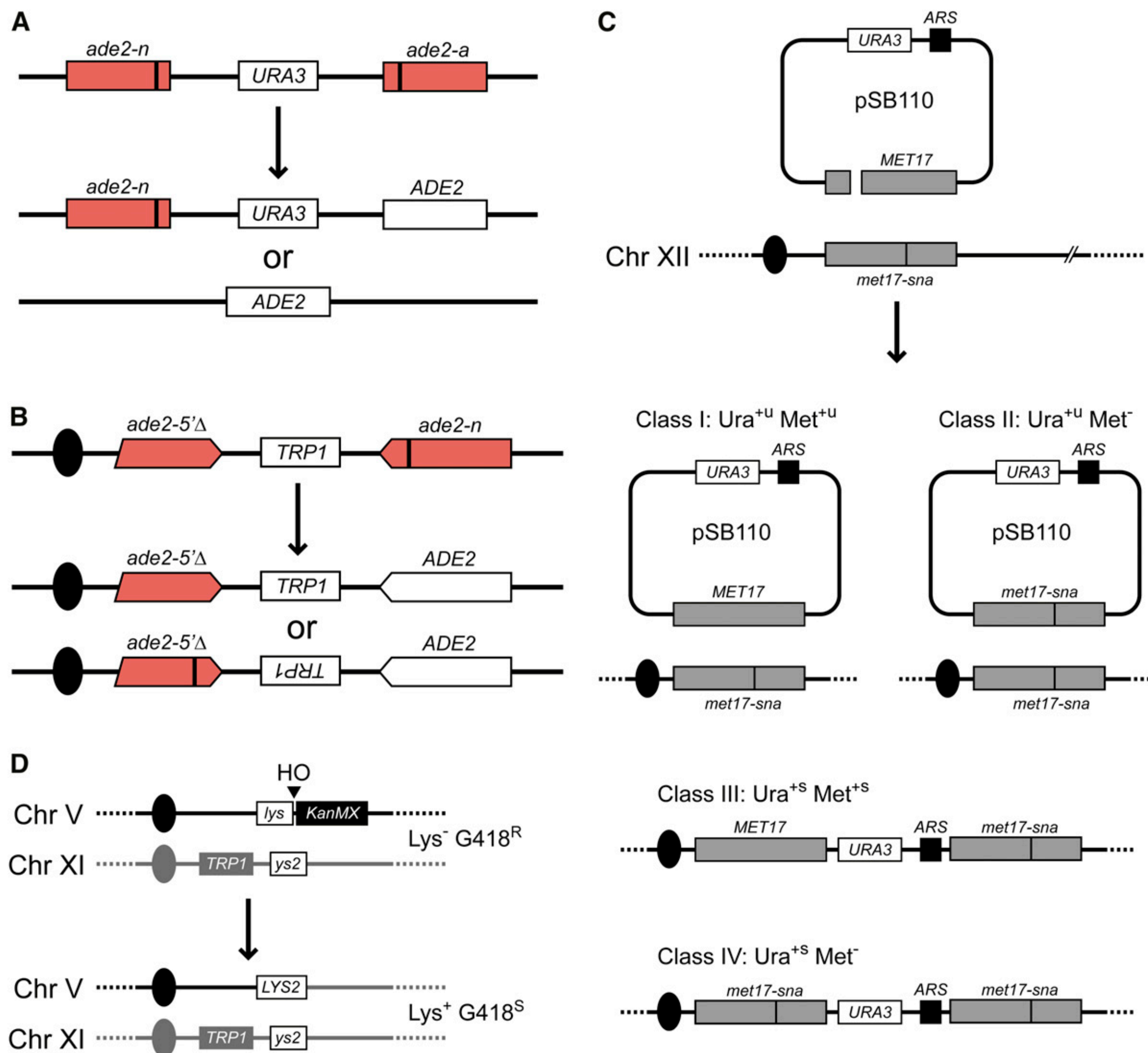


**Figure 8** Genetic assays. (A) Direct-repeat recombination. Spontaneous homologous recombination between *ade2-n* and *ade2-a* alleles can occur by gene conversion to produce Ade$^+$ Ura$^+$ cells or by SSA to produce Ade$^+$ Ura$^-$ cells (Fung *et al.* 2009). (B) Inverted repeat recombination. Inverted repeat recombination assays exclusively Ade$^+$ recombinants arising from gene conversion since SSA will not produce viable recombinants. CO and NCO events will lead to inversion and noninversion of the *TRP1* marker, respectively (Mott and Symington 2011). (C) Plasmid gap repair assay. The efficiency of plasmid–chromosome recombination, crossover frequency, and conversion tract length is assayed by transformation of the gapped pSB110 plasmid into yeast containing the chromosomal *met17-sna* mutant allele in which a SnaBI site is eliminated 216 bp downstream of the gap in the plasmid (Symington *et al.* 2000). When the plasmid gap is repaired by noncrossover gene conversion, the result is unstable (u) Ura$^+$ transformants, which will be Met$^+$ (class I) or Met$^-$ (class II), depending on the absence or presence of co-conversion of the *met17-sna* mutation, respectively. If the gene conversion event is associated with a crossover, the result is a stable (s) Ura$^+$ phenotype (classes III and IV). ARS, autonomously replicating sequence. (D) Break-induced replication. In this assay, BIR is initiated by induction of an HO-mediated DSB adjacent to a 3′ truncated *lys2* gene (*lys*) on chromosome V. The *lys* fragment has 2.1 kb of homology to a 5′ truncation of *lys2* (*ys2*) close to the telomere on chromosome XI (Donnianni and Symington 2013), which serves as a donor for BIR. BIR results in deletion of the *KanMX* gene and all nonessential genes telomere proximal to the HO cut site and loss of G418 resistance (G418$^R$). The strain has the *MAT***a***-inc* allele to prevent cleavage at the endogenous *HO* cut site.

in the *rad51* mutant (Dornfeld and Livingston 1992; Rattray and Symington 1994; Gonzalez-Barrera *et al.* 2002). DSB-induced recombination between chromosomal inverted repeats is reduced by >1000-fold in the *rad51* mutant, but plasmid-borne inverted repeats exhibit less of a requirement for *RAD51* (Gonzalez-Barrera *et al.* 2002; Ira and Haber 2002; Rattray *et al.* 2002). *RAD59* is required for spontaneous *RAD51*-independent events, suggesting they occur by a strand annealing mechanism, possibly by template switching during DNA replication (Bai and Symington 1996; Gonzalez-Barrera *et al.* 2002; Mott and Symington 2011). The DSB-induced *RAD51*-independent events in the plasmid context might occur by BIR and SSA or by some other type of nonconservative event (Kang and Symington 2000; Ira and Haber 2002).

Naturally occurring inverted repeats, such as Ty elements and delta sequences, are also substrates for spontaneous recombination that can lead to genome instability and gross chromosomal rearrangements (Rothstein *et al.* 1987; Argueso *et al.* 2008; Casper *et al.* 2009; Paek *et al.* 2009; Chan

and Kolodner 2011). Many of these events are dependent on DSB repair pathways; however, notably, fusion of inverted repeats that lead to chromosome rearrangements are replication dependent (Paek *et al.* 2009). The rearrangements involving Ty elements and delta sequences are almost completely dependent on the *RAD51* and *RAD52* gene products (Rothstein *et al.* 1987; Liefshitz *et al.* 1995).

### *C. Plasmid gap repair*

Transformation-based assays using plasmids linearized *in vitro* have been used extensively to study the mechanism and genetic control of DSB repair (Orr-Weaver *et al.* 1981, 1983; Bartsch *et al.* 2000; Mitchel *et al.* 2010; Tay *et al.* 2010). A plasmid containing a DSB or double-stranded gap within sequences that have homology to a chromosomal locus are introduced to cells by transformation and repair of the DSB or gap is templated by the homologous chromosomal sequence. In most assays, a second marker on the plasmid is used to select for transformants. In some respects, plasmid–chromosome recombination resembles ectopic recombination between dispersed repeats because both involve limited homology. If the plasmid contains no origin of replication, then only CO recombinants (integration of the plasmid at the chromosomal locus) are recovered; however, if the plasmid has an origin to allow stable maintenance as an episome then both NCO and CO products can be detected (Figure 8C). Use of a *CEN ARS* vector restricts events to NCOs (Bartsch *et al.* 2000). The frequency of COs recovered from an *ARS*-containing plasmid varies between assays, ranging from 20 to 50%, significantly higher than observed for DSB-induced chromosomal ectopic recombination (Orr-Weaver and Szostak 1983; Inbar and Kupiec 1999; Bartsch *et al.* 2000; Ira *et al.* 2003; Welz-Voegele and Jinks-Robertson 2008). Use of a plasmid substrate with SNPs located ~100 bp apart to detect hDNA intermediates that persist in a mismatch repair defective background revealed that most NCO products formed by SDSA and only a few events were diagnostic of dHJ dissolution. Furthermore, the CO products were most consistent with resolution of a sHJ intermediate (Mitchel *et al.* 2010).

Early studies showed an essential role for *RAD52* in plasmid gap repair, and subsequently, *rad51*, *rad55*, and *rad57* mutations were shown to reduce the frequency of gap repair by >50-fold (Orr-Weaver *et al.* 1981; Bartsch *et al.* 2000). Elimination of *RAD1* reduces integration of an *ARS*-containing plasmid by 5- to 10-fold, whereas *mus81* and *yen1* mutations do not decrease integration. These confusing data were rationalized by studies showing the *RAD1*-dependent accumulation of a sHJ intermediate between ectopic sequences in the *mus81 yen1* mutant leading to the hypothesis that Rad1–Rad10 clips the D-loop intermediate when it encounters the heterology barrier creating a sHJ intermediate linking the plasmid to the chromosome; replication through the sHJ would then generate CO and NCO products (Mazon *et al.* 2012; Mazon and Symington 2013). This model explains the pattern of hDNA observed in CO recombinants and also the high frequency of plasmid integration (Figure 9).

### *D. Assays for BIR*

BIR is most easily studied by creating a DSB where just one of the two ends can undergo homology-dependent strand invasion. Telomeres are a natural source of one-ended DSBs and maintenance of telomeres in the absence of telomerase provides a convenient genetic assay for BIR (see Wellinger and Zakian 2012 for review). Cells senesce in the absence of telomerase but survivors can arise by Rad52- and Pol32-dependent recombination (Lundblad and Blackburn 1993; Lydeard *et al.* 2007). Two pathways for generation of survivors have been defined: type I survivors are due to amplification of the Y′ subtelomeric repeats, have very short telomere repeat tracts, and are dependent on Rad51, Rad52, Rad54, Rad57, and Pol32; type II survivors have long heterogeneous telomere tracts and require the MRX complex, Pol32, Rad52, Rad59, and Sgs1 for their formation (see Wellinger and Zakian 2012).

To force repair of a chromosome-internal DSB by BIR, most assays restrict homology to only one side of the DSB to prevent gene conversion repair. Several systems have been developed using HO to create a chromosomal DSB, and a transformation-based system utilizing linear plasmid vectors has also been described (Morrow *et al.* 1997; Bosco and Haber 1998; Davis and Symington 2004; Malkova *et al.* 2005; Lydeard *et al.* 2007; Donnianni and Symington 2013). Malkova and colleagues use a haploid strain disomic for chromosome III to study BIR (Malkova *et al.* 2005; Deem *et al.* 2011; Saini *et al.* 2013a). The *MAT***a**-*inc* allele, which is refractory to HO cleavage, is present on one homolog and the HO-induced DSB on the other is forced to repair by BIR due to deletion of homology on the *CEN*-distal side of the DSB. Heterozygous markers present on both chromosome arms are used to differentiate between BIR, chromosome loss, and half COs. Two systems to study BIR in haploids make use of truncated partially overlapping fragments of the *CAN1* or *LYS2* gene to regenerate a wild-type copy of the gene by BIR (Lydeard *et al.* 2007; Donnianni and Symington 2013). The recipient cassette has an HO cut site between at the border of homology and a selectable marker located in a nonessential region of chromosome V; the donor cassette is located on another chromosome. After induction of the DSB, the recipient sequence invades the donor copying to the end of the chromosome, and the nonessential sequences *CEN*-distal to the DSB are lost (Figure 8D). Although BIR was originally reported to be very slow by physical monitoring assays, when the donor is close to the telomere and only 15–20 kb of DNA needs to be synthesized, the efficiency and kinetics are similar to other ectopic recombination assays (Jain *et al.* 2009; Donnianni and Symington 2013). Furthermore, cells synchronously released from G1 show a higher BIR efficiency than cells arrested in G2/M at the time of DSB induction.

BIR has also been proposed to explain Fob1-stimulated recombination in rDNA (Kobayashi *et al.* 1998). Strand invasion events occurring at the matching repeat on the sister chromatid preserves copy number while those occurring at

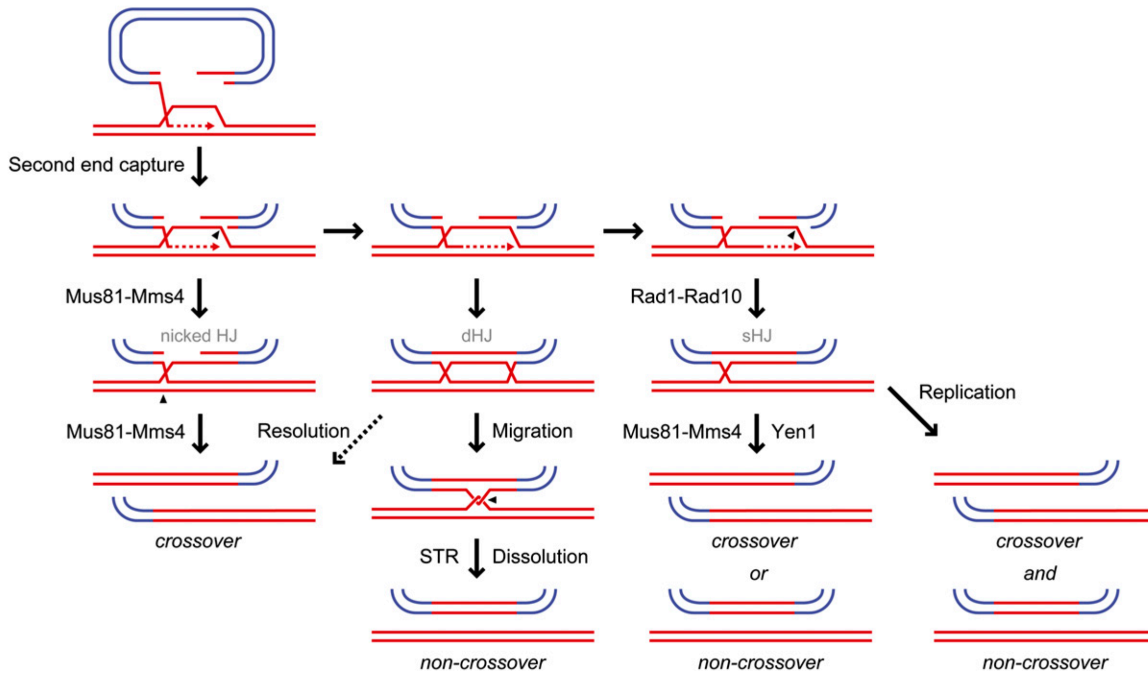


**Figure 9** Model for the role of endonucleases in the resolution of recombination intermediates. Invasion by a 3′ end of a gapped vector into a chromosomal donor sequence generates a D-loop, which is extended by DNA synthesis. Initially, second end capture results in a structure that is a potential substrate for Mus81–Mms4 cleavage to produce a nicked HJ and subsequently a CO upon further cleavage. If, on the other hand, the captured D-loop is gap filled and ligated, a dHJ is formed, which in most cases is converted to a hemicatene and dissolved by STR to yield a NCO, but could also be resolved by Mus81–Mms4 to produce a CO or NCO. Alternatively, if resection and DNA synthesis proceed beyond the heterology boundary, the D-loop can branch migrate to create a region of single-stranded DNA adjacent to the branch point, which could be cleaved by Rad1–Rad10 to generate a single HJ (sHJ). The sHJ can be resolved by either Mus81–Mms4 or Yen1 cleavage to produce a CO or NCO, or converted to a CO and a NCO product during the next S phase.

unmatched repeats cause rDNA copy number changes. Cohesin restricts recombination to matched repeats and is regulated by transcription of non-coding RNA sequences near the replication fork block (Kobayashi and Ganley 2005). Transcription of these RNAs is regulated by the Sir2 histone deacetylase, explaining the increased rates of rDNA recombination observed in *sir2* mutant cells (Gottlieb and Esposito 1989; Kobayashi and Ganley 2005).

Although most of the genetic requirements for BIR are similar to other recombination reactions, *POL32*, which encodes a nonessential subunit of DNA Polδ, and *PIF1* are required for BIR but not for short tract gene conversion (Lydeard *et al.* 2007; Deem *et al.* 2008; Smith *et al.* 2009; Saini *et al.* 2013a; Wilson *et al.* 2013; Stafa *et al.* 2014). Interestingly, half crossovers are recovered at high frequency from *pol3-ct*, *pol32*, and *pif1* mutants (Deem *et al.* 2008; Smith *et al.* 2009; Saini *et al.* 2013a; Wilson *et al.* 2013), consistent with defective extension of the invading strand followed by cleavage of the D-loop intermediate. Half crossover products are also elevated in checkpoint mutants in agreement with the role of the DNA damage checkpoint in suppressing activation of structure-selective nucleases (Vasan *et al.* 2014).

## IV. The Nature of the Recombinogenic DNA Lesion

This section describes the current thinking on the nature of the DNA lesion that results in defined recombination events. Many of our views on the exact kind of lesion that occurs have been influenced by studies of defined site-specific recombination events and the analysis of recombination mutants. Mating type switching is the "poster child" for this kind of study, having contributed greatly over the years to our understanding of the process.

### *A. Induction of recombination by DSBs and nicks*

As described above, DSBs made by endonucleases serve as potent initiators of recombination. Furthermore, treatment of cells with ionizing radiation or radiomimetic drugs stimulates mitotic recombination. Testing the role of nicks as recombination initiators is more difficult because they can be healed by direct ligation, and any stimulation observed could be due to conversion to a DSB during replication (Figure 6). Insertion of the bacteriophage f1 gene *II* nick site between *trp1* and *his3* heteroalleles was shown to stimulate interchromosomal recombination when the gene II protein was expressed. Interestingly, there was a bias favoring conversion of the marker to the 5′ side of the nick site, in contrast to DSB initiated events that stimulate bidirectionally from the break site (Strathern *et al.* 1991). Conversion of the nick to a one-ended DSB during S-phase could account for the directionality of gene conversion observed. Aguilera and colleagues have shown a minimal HO site of 21 bp is cut by HO on one strand more frequently than on both strands, and the resulting nicks are converted to DSBs

as cells transition through S-phase (Cortes-Ledesma and Aguilera 2006). Although the frequency of cutting is lower than at the optimal HO cut site, it is sufficient to detect intermediates by physical methods. This system has proven extremely useful to study replication-associated DSBs and their repair by sister-chromatid recombination (Gonzalez-Barrera *et al.* 2003; Cortes-Ledesma and Aguilera 2006).

Studies with DNA damaging agents are used to gain insight in how particular lesions behave in different assays (reviewed in Kupiec 2000). For example, γ-rays and UV induce mainly DSBs or single-stranded gaps (Ma *et al.* 2013), respectively, while CPT leads to covalently bound topoisomerase I to the 3′ phosphate end at a nick (Pommier 2009). Replication of the unremoved adduct leads to a DSB in the next round of replication. In addition, the absence of topoisomerases I, II, or III themselves leads to increased recombination, especially in the rDNA multiple tandem array as discussed above. In these cases, it is thought that the absence of the topoisomerase causes topological problems such as catenanes and hemicatenates resulting in broken chromosomes during mitosis.

### *B. Replication-coupled recombination*

Most spontaneous recombination is thought to occur during DNA replication, when the replisome encounters various challenges, such as reduced dNTP levels, DNA adducts on the template strand, DNA secondary structures, or tightly bound proteins that can stall the replication fork, and these impediments occasionally cause fork collapse resulting in a DSB (reviewed in Aguilera and Gomez-Gonzalez 2008). Fork collapse is thought to result from the replication fork running into a transient nick on the template strand, by fork reversal (pairing of the nascent strands) leading to the formation of Holliday junction or "chicken-foot" structure that can be cut by a HJ endonuclease or direct cleavage of the stalled fork by structure-selective nucleases, such as Mus81–Mms4 or Slx1–Slx4 (Figure 6). Fork regression requires Rad5 to facilitate either immediate excision repair or limited extension of the leading strand using the nascent lagging strand as a template (Sogo *et al.* 2002; Cotta-Ramusino *et al.* 2005; Blastyak *et al.* 2007), known as "template switching" (Higgins *et al.* 1976), followed by fork reversal and postreplicative excision repair (Figure 6A). Instead of fork reversal, the regressed fork may be cleaved, causing fork collapse (reviewed in Atkinson and McGlynn 2009). The one-ended DSB generated by fork collapse could be rescued by replication from an adjacent origin (Figure 6B). DNA adducts that block progression of the replicative polymerases result in ssDNA gaps on both lagging and leading strands, which can be acted on by TLS polymerases or by recombination (Figure 6C).

Most recombinogenic lesions formed during S-phase are expected to be repaired by sister-chromatid recombination; however, some lesions must be repaired by a nonsister to account for the increased interchromosomal recombination observed for mutants with replication defects or after treatment of cells with agents that stall replication, such as UV. These events are initiated from either ssDNA gaps or DSBs. Fabre and colleagues have argued against DSBs as the spontaneous recombination initiating lesion on the grounds that *rad52 yku70* double mutants (deficient for both HR and NHEJ) are viable, yet the *srs2 sgs1* double mutant is inviable but rescued by loss of HR function, suggesting lethal spontaneous recombination intermediates occur at high frequency (Fabre *et al.* 2002). Furthermore, certain *rad52* hypomorphic alleles confer high sensitivity to IR but are hyper-rec for spontaneous interchromosomal recombination (Lettier *et al.* 2006). Recently, whole chromosome analysis has revealed that single-stranded gaps are the intermediates of recombinational repair after UV irradiation (Ma *et al.* 2013). On the other hand, the pattern of conversion tracts associated with spontaneous mitotic crossovers is most compatible with initiation of recombination by a DSB present in a G1 cell (Lee and Petes 2010). Furthermore, analysis of conversion tracts associated with UV-induced mitotic crossovers showed they were similar to spontaneous events and those resulting from gamma-irradiation of G1 diploids (St Charles *et al.* 2012; Yin and Petes 2013).

### *C. Fragile sites and noncanonical structures*

Fragile sites were first defined in mammalian cells as sequences that show gaps and breaks following inhibition of DNA synthesis and are associated with hotspots for genome rearrangements. Reducing the levels of DNA Polα or Polδ created a fragile site on yeast chromosome III detected by chromosome rearrangements between a pair of inverted Ty elements and other Ty elements located on other chromosomes (Lemoine *et al.* 2005, 2008). DSBs were detected at the inverted Ty elements when replication was compromised, analogous to mammalian fragile sites (Lemoine *et al.* 2005). Moreover, this fragile site experiences a high frequency of spontaneous BIR events leading to LOH on the right arm of chromosome III (Rosen *et al.* 2013).

Fragile sites are usually associated with DNA sequences that are difficult to replicate and prone to form secondary structures, such as certain trinucleotide repeats (TNRs), AT rich sequences, inverted repeats, and sequences with the potential to form G quadruplexes. Moreover, genome-wide mapping of fragile sites revealed a nonrandom distribution correlating with motifs that pause DNA replication forks, including replication-termination sites and binding sites for the helicase Rrm3 (Song *et al.* 2014). Insertion of inverted Alu elements, but not Alu direct repeats, was shown to stimulate ectopic recombination by 1000-fold in an MRX- and Sae2-dependent manner (Lobachev *et al.* 2002). A chromosomal DSB induced by the inverted Alu elements was detected by PFGE and shown to be hairpin capped in the absence of the MRX complex, the Mre11 nuclease, or Sae2. It was postulated that the Alu elements extrude into a cruciform, and the base, resembling a HJ, is cleaved by a structure-selective nuclease converting it into two hairpin-capped ends that must be opened by MRX and Sae2 to initiate recombination.

TNRs that are capable of forming hairpins when present in ssDNA show orientation-dependent replication fork stalling, increased chromosome fragility, and contractions and expansions when present on the lagging strand template (Freudenreich *et al.* 1997; Miret *et al.* 1998). Insertion of a long TNR tract between *CEN5* and *CAN1* was shown to stimulate mitotic crossovers by 30-fold, but the local stimulation was much greater and the majority of crossovers and conversion tracts were close to the TNR tract (Tang *et al.* 2011).

G-quadruplex (G4) DNA induces recombination by interfering with both replication and transcription. Interestingly, G4-induced recombination is observed only when the G-rich strand is the template for leading strand synthesis (Lopes *et al.* 2011) or when transcription of the G-rich DNA is oriented with the C-rich strand as the transcription template (Kim and Jinks-Robertson 2011). The effect of transcription orientation was enhanced in the absence of the type IB topoisomerase Top1, possibly due to enhanced R-loop formation (Kim and Jinks-Robertson 2011). Pif1 unwinds G4 structures *in vitro* and prevents replication fork stalling and DNA breakage at G4 motifs *in vivo* (Paeschke *et al.* 2011), which likely explains its suppression of recombination triggered by G-quadruplex forming tandem repeats (Ribeyre *et al.* 2009). Finally, Mre11 binds and cleaves G4 DNA *in vitro* (Ghosal and Muniyappa 2005).

#### *D. Transcription-stimulated recombination*

Early studies searching for hotspots of genetic recombination showed that promiscuous transcription by RNA polymerase I stimulates mitotic recombination (Keil and Roeder 1984; Voelkel-Meiman *et al.* 1987). In addition, a high level of transcription by RNA polymerase II resulted in increased repeat recombination (Thomas and Rothstein 1989a,b; Saxe *et al.* 2000). Topological changes induced by transcription may be responsible for creating recombinogenic lesions since mutations in topoisomerase I and II also lead to increased recombination especially in rDNA (Christman *et al.* 1988; Kim and Wang 1989; Wallis *et al.* 1989; El Hage *et al.* 2010). Studies of the THO/TREX complex indicate that an increased frequency of R-loop formation during transcription is likely the cause of transcription-stimulated recombination (Huertas and Aguilera 2003). In many cases, increased recombination can be suppressed by overexpressing RNaseH, which preferentially removes the RNA from the DNA–RNA hybrid (Huertas and Aguilera 2003; El Hage *et al.* 2010). Interestingly, it was recently reported that the Rad51 protein is involved in the formation of RNA–DNA hybrids and that Srs2 normally counteracts their potential for genome instability (Wahba *et al.* 2013). Although the precise lesion involved in stimultating recombination is not known, multiple lines of evidence suggest that the intermediate is a DSB (reviewed in Aguilera and Garcia-Muse 2012). Recently, the DNA damage checkpoint has been linked to transcription-associated R-loops that impede DNA replication (Bermejo *et al.* 2011). It is thought that highly transcribed genes associate with the nuclear periphery to aid in RNA export. When a replication fork is encountered head on with the transcribed gene, the resulting collision collapses the fork. This action activates the DNA damage checkpoint to release the transcription/replication unit from the nuclear pore to allow relief of topological stress.

## V. Cell Biology of Recombination

Most recombination proteins can be expressed as functional fusions to genetically encoded fluorescent proteins such as GFP and mCherry (Lisby *et al.* 2004; Silva *et al.* 2012), which allows for the dynamic redistribution of these proteins to be monitored in real-time at the single-cell level during homologous recombination.

#### *A. Recombination foci*

Most homologous recombination proteins are recruited in many copies to the site of DNA damage during repair. The high local concentration of recombination proteins at the site of DNA damage can be visualized by fluorescence microscopy after immunostaining or by GFP-tagging of the proteins (Lisby *et al.* 2004; Eckert-Boulet *et al.* 2011; Silva *et al.* 2012). For example, a single DNA DSB is sufficient for the formation of a prominent focus containing 600–2100 molecules of Rad52 yielding a ≥50-fold higher local concentration of Rad52 at the DSB relative to the diffuse nuclear distribution in undamaged cells (Lisby *et al.* 2003b). Although the minimum number of Rad52 molecules required for mediating a single strand invasion is currently unknown, the high local concentration of recombination proteins within these foci may allow constitutively expressed proteins to be active only at the site of DNA damage, and therefore prevent untimely recombination or assembly of recombination complexes at undamaged DNA.

Recombination foci are highly dynamic in their protein composition and localization. Foci can assemble and disassemble within minutes. However, studies of Rad51, Rad52, and Rad54 foci in mammalian cells indicate that the residence time of individual molecules may vary between proteins and even subpopulations of proteins within foci (Essers *et al.* 2002). So far the dynamics of proteins within individual recombination foci has not been studied in yeast.

Although focus formation of recombination and checkpoint proteins is a useful tool for monitoring the cellular response to DSBs and a single DSB is sufficient to trigger focus formation (Lisby *et al.* 2003b), it is likely that some recombination events go undetected by this methodology. For example, recombinational restart of stalled replication forks, some sister chromatid events and intramolecular recombination may be too fast or require too few molecules of recombination proteins to be detected by current techniques.

#### *B. Choreography of focus formation*

HR starts with the recruitment of MRX, which binds directly to DNA ends (Chen *et al.* 2001; Hopfner *et al.* 2001; Lisby *et al.* 2004; Mimitou and Symington 2010). The two ends of

a DSB are held together by a mechanism that is partially dependent on MRX and Sae2 (Chen *et al.* 2001; Lisby *et al.* 2003a; Kaye *et al.* 2004; Lobachev *et al.* 2004; Clerici *et al.* 2005). For this reason, the two ends of a DSB give rise to a single Mre11 focus rather than two foci. Further, the MRX complex interacts with the Tel1 kinase and is required for its recruitment to foci at all phases of the cell cycle (Nakada *et al.* 2003; Lisby *et al.* 2004) (Figure 10). The Tel1 kinase phosphorylates histone H2A, which is a chromatin mark specific for damaged DNA in most eukaryotes (Rogakou *et al.* 1998, 1999; Redon *et al.* 2003). Importantly, the modification of chromatin by H2A phosphorylation facilitates binding of the checkpoint adaptor Rad9 to sites of DNA damage likely through a dual interaction of its BRCT domains with H2A-S129$^{P}$ and its Tudor domain with histone H3 methylated at lysine 79 (H3-K79$^{Me}$) leading to subsequent recruitment and activation of Rad53 (Giannattasio *et al.* 2005; Javaheri *et al.* 2006; Toh *et al.* 2006; Grenon *et al.* 2007; Hammet *et al.* 2007; Germann *et al.* 2011). Notably, Rad53 foci are faint and transient, which is consistent with the notion from mammalian cells that Rad53/CHK2 must redistribute from the site of DNA damage upon phosphorylation to mediate a pannuclear checkpoint response (Lukas *et al.* 2003).

The proteins involved in resection have very different focal appearances giving clues to their function and regulation. Mre11 and Sae2 form prominent foci at all phases of the cell cycle in response to DSBs (Lisby *et al.* 2004; Barlow *et al.* 2008). Dna2 shuttles between the nucleus and cytoplasm in a cell-cycle-dependent manner, residing in the cytoplasm during G1 phase and relocalizing to the nucleus in S/G2 upon phosphorylation by Cdc28 (CDK) (Kosugi *et al.* 2009; Chen *et al.* 2011). Dna2 forms Rad52-colocalizing foci after DSB formation (Zhu *et al.* 2008). Sgs1 is a low abundance nuclear protein, which forms foci in S/G2/M (Frei and Gasser 2000 and M. Wagner, personal communication). Exo1 levels are cell cycle regulated gradually increasing through G1 and peaking in late S/G2 phase before it is degraded in anaphase (M. Lisby, unpublished data). Resection is accompanied by the dissociation of MRX, Sae2, and Tel1 from the DSB and binding of RPA to the 3′ single-stranded overhangs (Figure 10) (Lisby *et al.* 2004; Barlow *et al.* 2008). The intensity of Rfa1 foci can be used to estimate the extent of resection. This approach was used to demonstrate at the single-cell level that the rate of DSB end resection increases at the G1–S transition (Barlow *et al.* 2008). RPA is necessary for recruiting a number of checkpoint and HR proteins including the Dna2, Mec1–Ddc2/Lcd1, Rad24–RFC and 9–1–1 (Ddc1–Mec3–Rad17) complexes. Notably, Tel1 and Mec1 have many of the same phosphorylation targets, including histone H2A. As a consequence, Tel1-dependent checkpoint signaling is likely replaced by Mec1-dependent signaling upon resection of DSB ends. Consistent with a functional crosstalk between the Ddc2–Mec1 and 9–1–1 complexes during checkpoint signaling, there is a reported requirement for the 9–1–1 complex to stabilize Ddc2 foci in irradiated G1 cells (Barlow *et al.* 2008). Further, in S and G2 phases, the 9–1–1 complex and the Cdc28 kinase both contribute to the stabilization of DNA damage-induced Ddc2 foci (Barlow *et al.* 2008), demonstrating how checkpoint signaling is coordinated with cell cycle phase. The multifunctional Dpb11 protein is recruited to foci by the 9–1–1 complex, reflecting its role in mediating the DNA damage checkpoint through activation of the Mec1 kinase (Puddu *et al.* 2008; Germann *et al.* 2011). In contrast, the DNA replication and recombination functions of Dpb11 are independent of focus formation (Germann *et al.* 2011).

In S and G2 phases, RPA facilitates the recruitment of Rad52 to resected DSBs, likely via a direct physical interaction (Hays *et al.* 1995; Lisby *et al.* 2001, 2004; Plate *et al.* 2008). The recruitment is independent of DNA replication and requires B-type cyclin/Cdc28 activity (Barlow and Rothstein 2009). However, the cell cycle regulation of Rad52 focus formation can be circumvented at high doses of ionizing radiation at which Rad52 also forms foci in G1 phase, although it is unknown if these foci are productive for recombination (Lisby *et al.* 2003a). Rad52 interacts with the Rad51 recombinase and Rad59 to recruit these proteins to foci (Milne and Weaver 1993; Davis and Symington 2003; Lisby *et al.* 2004). In addition, Rad59 also requires Rad52 for its nuclear accumulation (Lisby *et al.* 2004). However, recent data indicate that Rad59 has Rad52-independent functions, indicating that some Rad59 enters the nucleus in a *rad52* mutant (Coic *et al.* 2008; Pannunzio *et al.* 2008; Pannunzio *et al.* 2012). Rad51 foci form, but are dimmer in the absence of Rad55–Rad57, consistent with the role of the Rad51 paralogs to stabilize Rad51 filaments. Interestingly, Rad55 focus formation requires Rad51 (Lisby *et al.* 2004; Fung *et al.* 2009). Formation of DNA damage-induced foci by Rad54 requires both Rad55–Rad57 and Rad51, suggesting that Rad54 recruitment to the site of DNA damage requires Rad51 nucleoprotein filament formation (Lisby *et al.* 2004). Interestingly, the Rad54 homolog, Rdh54, is recruited both to DSBs and to the kinetochore, although the functional significance of this dual localization is unknown. The recruitment of Rdh54 to DSBs is Rad52- and Rad51 dependent, while its localization to the kinetochore is independent of the recombination machinery. Interestingly, Rad54, which does not localize to the kinetochore in wild-type cells, localizes to the kinetochore in an *rdh54* mutant (Lisby *et al.* 2004), possibly explaining some of the functional redundancy between these two proteins (Shinohara *et al.* 1997).

Additional proteins that are recruited to recombination foci include the Pif1 helicase, which forms Rad52-colocalizing foci (Wagner *et al.* 2006), and the Srs2 helicase and anti-recombinase, which is recruited to two distinct classes of foci (Burgess *et al.* 2009). During S phase, Srs2 is recruited to sumoylated PCNA speckles, and in late S/G2 Srs2 is recruited to recombination foci marked by Rad52. The recruitment of Srs2 to recombination foci is independent of its SUMO-interacting motif (Burgess *et al.* 2009). The

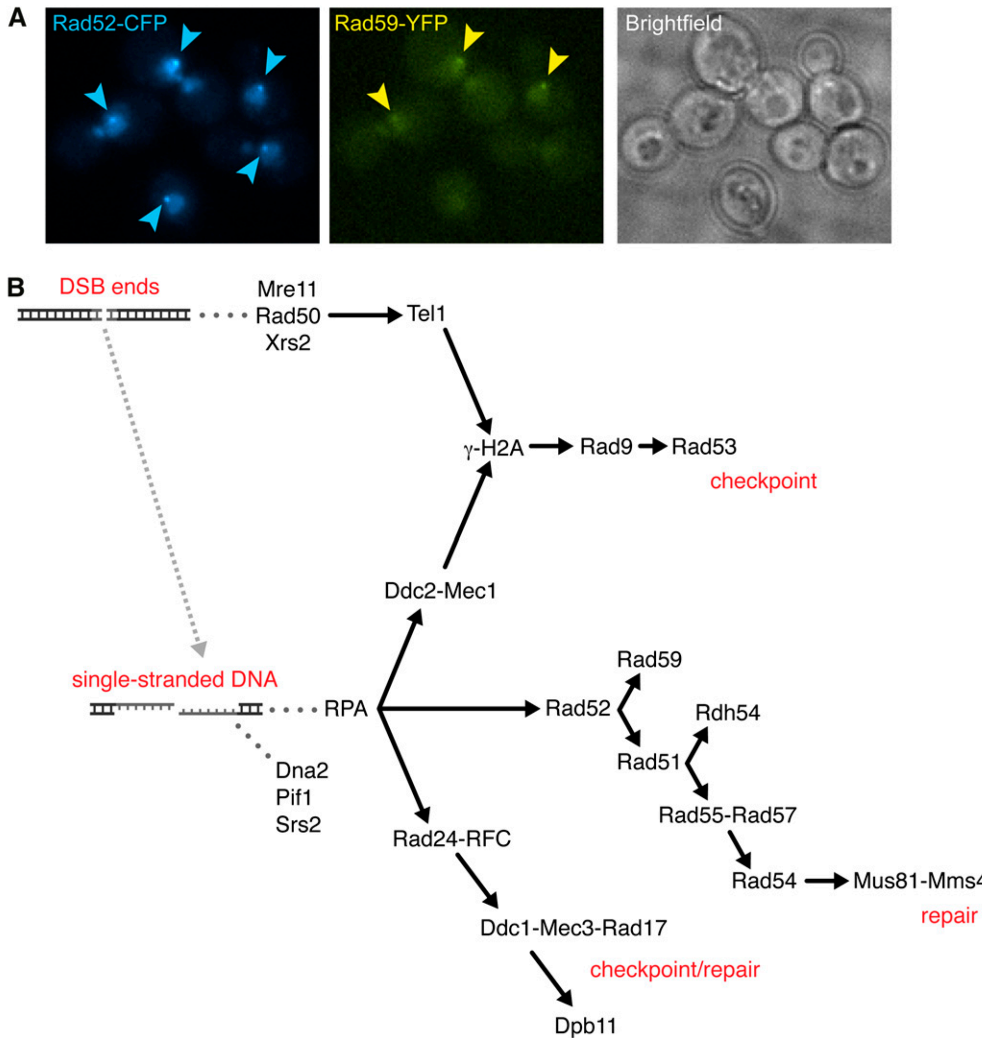


**Figure 10** Choreography of HR focus assembly. (A) Focus formation of HR proteins. The high local concentration of Rad52 and Rad59 at DSBs induced by treatment with 200 μg/ml zeocin for 2 hr at 25°. Strain NEB110-25B is a *MAT***a** haploid containing *RAD52-CFP* and *RAD59-YFP*. Arrowheads mark foci. (B) Order of assembly of HR proteins at foci. Proteins are recruited from the left to right starting with MRX binding at DSB ends and later replaced by proteins recruited to ssDNA at resected DSBs.

Mus81–Mms4 structure-selective endonuclease forms foci, which are largely dependent on Rad54, consistent with Mus81–Mms4 acting downstream of the strand-invasion step of homologous recombination (Matulova *et al.* 2009). The SUMO-targeted ubiquitin ligase Slx5–Slx8 forms foci that partially overlap with Rad52 and Rad9 foci in response to DNA damage (Cook *et al.* 2009).

### *C. DSB dynamics and recombination centers*

Work in both haploid and diploid yeast cells has found that, after DNA damage, the volume of the nucleus explored by the broken chromosome more than doubles from that seen in the absence of DSBs (Dion *et al.* 2012; Mine-Hattab and Rothstein 2012). The dynamics of unbroken chromosomes also increase depending on the number of DSBs (Mine-Hattab and Rothstein 2012; Seeber *et al.* 2013). The pairing of the homologs in diploid cells takes ∼20 min before the repair center disassembles and the loci separate again (Mine-Hattab and Rothstein 2012). These studies suggest that increased chromosomal mobility facilitates the homology search, which is otherwise restricted by the proximity of donor and recipient loci (Agmon *et al.* 2013). Genetics and cell biological studies, in both haploid and diploid yeast cells, showed that increased DNA mobility depends on the Rad51 recombinase (Dion *et al.* 2012; Mine-Hattab and Rothstein 2012). In haploid yeast, increased mobility also depends on Rad54 and two checkpoint proteins, Rad9 and Mec1 (Dion *et al.* 2012; Seeber *et al.* 2013). In a *sae2* mutant, which has delayed appearance of single-stranded DNA, increased chromosome mobility is also delayed (Mine-Hattab and Rothstein 2012). In contrast to the increased mobility observed after IR or enzymatically induced DSBs, spontaneous Rad52 foci are constrained, which may reflect recombination between sister chromatids in the context of DNA replication (Dion *et al.* 2013).

The mobilization of DSBs may also allow multiple DSBs to interact. In fact, it has been shown that multiple DSBs in the same cell often come together at a single Rad52 focus (Lisby *et al.* 2003b). These recombinational repair centers are observed in both haploid and diploid cells. The aggregation of multiple DSBs takes place subsequent to recognition by the MRX complex and prior to recruitment of Rad52, which is indicated by the observation that cells exposed to 40 krad of ionizing radiation (equivalent to 20 DSBs per haploid cell) initially exhibit up to 20 Mre11 foci within

5 min, which transitions to 1–2 Rad52 foci within 20–30 min (M. Lisby and R. Rothstein, unpublished data). Most likely multiple DSBs are held together by the same scaffolding processes that hold together the two ends of a single DSB, but the molecular components of the scaffold have not been fully described, although a partial dependency on Sae2 and the MRX complex for tethering ends has been reported (Chen *et al.* 2001; Lisby *et al.* 2003a; Kaye *et al.* 2004; Lobachev *et al.* 2004; Clerici *et al.* 2005). Thus, the tethering of DNA ends may facilitate DSB repair but at the same time pose a risk for translocation between clustered DSBs.

### *D. Nuclear compartments*

Some regions of the genome are more susceptible to deleterious recombination including repetitive elements such as the centromeres, telomeres, and Ty elements, and the highly transcribed rDNA and tRNA genes. Untimely recombination at these loci is prevented by compartmentalization of the nucleus into regions that suppress recombination and regions that allow or even stimulate recombination. The most prominent example is the nucleolus from which late-acting recombination and checkpoint proteins such as RPA, Rad52, Rad51, Rad59, Rad55, Rad24–RFC, 9–1–1 (Ddc1), Ddc2, and Rad9 are largely excluded even in the absence of DNA damage (Torres-Rosell *et al.* 2007). Although DSBs in the rDNA are initially recognized by the MRX complex within the nucleolus and resected (Torres-Rosell *et al.* 2007), they are only bound by Rad52 and downstream factors after exiting the nucleolus. The relocalization of rDNA breaks from the nucleolus to the nucleoplasm requires the Smc5–Smc6 complex and SUMO modification of Rad52 (see below), and mutants that disrupt the Smc5–Smc6 complex or prevent Rad52 sumoylation lead to Rad52 focus formation inside the nucleolus and rDNA instability (Torres-Rosell *et al.* 2007).

Similar to the rDNA, telomeres are compartmentalized. Telomeres associate into 6–8 clusters (Gotta *et al.* 1996), which are largely refractory to recombination and the DNA damage checkpoint response in general (Khadaroo *et al.* 2009; Ribeyre and Shore 2012). Importantly, all telomeres are generally in the vicinity of the nuclear envelope over long periods of time (Hediger *et al.* 2002). A component of the nuclear envelope in *Saccharomyces cerevisiae* is the SUN domain protein Mps3 (Antoniacci *et al.* 2007; Bupp *et al.* 2007). Several studies indicate that Mps3 is involved in anchoring telomeres at the nuclear envelope and shielding telomeres against spontaneous recombination (Antoniacci *et al.* 2007; Bupp *et al.* 2007; Schober *et al.* 2009) (reviewed in Taddei and Gasser 2012). Nevertheless, anchoring of telomeres at the nuclear periphery is essential for efficient DSB repair in subtelomeric DNA (Therizols *et al.* 2006).

In contrast to the nucleolus and telomere clusters, homologous recombination appears to be enhanced in the vicinity of nuclear pore complexes (NPCs). Persistent DSBs, collapsed replication forks, and eroded telomeres relocalize to NPCs, which stimulates recombinational repair at those loci (Nagai *et al.* 2008; Khadaroo *et al.* 2009). In an independent study, unrepaired DSBs were found to be enriched at the nuclear periphery in an Mps3-dependent but NPC-independent manner (Oza *et al.* 2009). Further, it was reported that spontaneous gene conversion is enhanced in a Nup84- and Slx8-dependent manner by tethering of a donor sequence to the nuclear pore complex (NPC) (Nagai *et al.* 2008). It was suggested that desumoylation of repair proteins by the SUMO-specific protease Ulp1, which associates with the NPC (Takahashi *et al.* 2000), could be responsible for the observed stimulation of gene conversion (Nagai *et al.* 2008) (see below).

## VI. Regulation of Homologous Recombination

Homologous recombination is tightly regulated according to the type of DNA lesion, cell cycle phase, ploidy, and other environmental and development cues. The regulation of HR serves to ensure that the HR machinery does not interfere with DNA transactions such as transcription and replication on undamaged chromosomes and to fine tune the fidelity of repair. In budding yeast, the regulation of HR takes place mainly at the transcriptional level and by post-translational modification of HR proteins, while there is so far little evidence for post-transcriptional regulation.

### *A. Transcriptional regulation of homologous recombination*

Many recombination proteins are constitutively expressed with some exceptions where expression is regulated with cell cycle or in response to DNA damage. Presumably, a constitutive basal expression of many recombination and checkpoint proteins allows for a rapid response to DNA damage. DNA damage-induced genes with relevance to mitotic recombination are the ribonucleotide reductase genes *RNR1–4*, which are required for reduction of ribonucleotides to their corresponding 2′-deoxyribonucleotides (dNTPs) necessary for DNA synthesis during recombination, the DNA damage checkpoint genes *RAD53* and *MEC1*, which form a positive feedback loop to increase their own expression, and the recombination genes *RFA1*, *RFA2*, *RFA3*, *RAD50*, *SRS2*, *RAD54*, and *RAD51* which are also induced in a *MEC1*-dependent manner (Cole *et al.* 1987; Elledge and Davis, 1989, 1990; Yagle and McEntee 1990; Basile *et al.* 1992; Kiser and Weinert 1996; Jelinsky and Samson 1999; Vallen and Cross 1999; Gasch *et al.* 2001; Mercier *et al.* 2001; Benton *et al.* 2006) (reviewed in Fu *et al.* 2008). In addition, many recombination genes exhibit cell cycle regulated expression, which peaks in late G1 to early S phase (Basile *et al.* 1992; Spellman *et al.* 1998 and reviewed in Mathiasen and Lisby 2014), although the functional importance of this regulation remains to be determined.

### *B. Regulation of homologous recombination by post-translational modifications*

Homologous recombination proteins are acted upon by most known post-translational modifications (PTMs) including

phosphorylation, ubiquitylation, sumoylation, and acetylation, which are all reversible. Since the PTMs and their consequences are context dependent, this section will review the regulation by PTMs during DNA double-strand break repair and during recombinational restart of DNA replication (Table 2), while the regulation of specialized recombination events such as alternative lengthening of telomeres is described elsewhere (Wellinger and Zakian 2012). The following section will focus on the PTMs, for which a biological function has been described.

***Regulation of DSB repair by PTMs:*** The initiating step of recombinational repair of a DSB end, resection to produce 3′ single-stranded overhangs, is regulated by cyclin-dependent kinase Cdc28 (CDK). Inhibition of Cdc28 using an analog-sensitive allele of *CDC28* or by overexpression of Sic1, an inhibitor of Cdc28, results in greatly reduced end resection (Aylon *et al.* 2004; Ira *et al.* 2004). As a consequence, resection of DSBs induced in G1 cells is greatly reduced compared with cycling or G2-arrested cells. Resection is regulated by PTMs at multiple levels. Initially, binding of the Ku complex to DSB ends blocks resection and its binding is inhibited by Cdc28 during S and G2 phase of the cell cycle (Clerici *et al.* 2008). G1 cells deficient for Ku show greater recruitment of Mre11 to an endonuclease-induced DSB and increased resection. As a consequence, HR can occur in G1 in *yku* mutants (Zhang *et al.* 2009; Trovesi *et al.* 2011). Overexpression of Exo1 is also able to overcome the inhibition to resection in G1 cells, consistent with other studies showing Ku is a barrier to Exo1-mediated end resection. The inhibitory effect on end resection was observed to a lesser extent in the *dnl4* (ligase IV deficient) mutant, suggesting the end binding function of Ku and ligation both contribute to protecting ends from degradation in G1 (Clerici *et al.* 2008; Zierhut and Diffley 2008). Interestingly, inhibition of CDK in G2 *yku80* cells fails to block short-range resection, similar to the situation in G1 *yku80* cells, and activation of CDK in G1 by overexpression of Clb2 restores both initiation and extensive resection (Clerici *et al.* 2008). Together, these results suggest that Ku (and to a lesser extent NHEJ) is the primary rate-limiting factor for the initiation of end resection in G1 by competing with MRX and Exo1 for end binding.

Resection is positively regulated by phosphorylation of Sae2 at serine 267 by Cdc28 during S and G2 phases (Huertas *et al.* 2008). Mutation of this site to a nonphosphorylatable residue, S267A, phenocopies *sae2*, including hypersensitivity to camptothecin, defective sporulation, reduced hairpin-induced recombination, impaired DSB processing, persistent Mre11 foci, and delayed Rad52 recruitment. Sae2 is phosphorylated at additional (S/T)Q motifs by Mec1 and Tel1 in reponse to DNA damage and mutation of these phosphorylation sites also impairs DNA repair (Baroni *et al.* 2004). These phosphorylation events activate Sae2 through a transition from an insoluble oligomeric state to active monomers/dimers, which allow the protein to be recruited to sites of DNA damage (Fu *et al.* 2014). Further, the stability of Sae2 is regulated by acetylation and treatment with the histone deacetylase inhibitor valproic acid causes accumulation of acetylated Sae2 and degradation of Sae2 (Robert *et al.* 2011). The mechanism for acetylation of Sae2 remains to be determined. Extensive resection is promoted in S and G2 phases by Cdc28-dependent phosphorylation of Dna2 at threonine 4 and serines 17 and 237, which is required for its recruitment to DSBs (Chen *et al.* 2011). Further, Dna2 shuttles from the cytoplasm to the nucleus upon phosphorylation on serine 17 by Cdc28 (Kosugi *et al.* 2009). Interestingly, resection in G2 is more dependent on MRX than in cycling cells, suggesting replication forks could serve to recruit Exo1 and/or STR–Dna2 in lieu of the MRX complex, possibly through RPA, which interacts directly with Dna2 (Bae *et al.* 2003; Chen *et al.* 2013). Loss of Rad9 can partially bypass the Cdc28 requirement for resection, suggesting that Rad9 could also be a target of the Cdc28-dependent regulation of resection (Lazzaro *et al.* 2008).

The nucleolytically produced 3′ single-stranded DNA ends are bound by RPA (Alani *et al.* 1992). The recruitment of Rad52 to ssDNA by RPA in S/G2 phase requires Cdc28 activity (Alabert *et al.* 2009; Barlow and Rothstein 2009), however the responsible phosphorylation sites have not been identified. Rad55 is subject to Mec1-dependent phosphorylation on serines 2, 8, and 14 in response to MMS or an HO-induced DSB (Bashkirov *et al.* 2000; Herzberg *et al.* 2006), and a *rad55-S2,8,14A* mutant exhibits reduced survival after DNA damage although the underlying mechanism remains to be established. The Rad51 recombinase itself is phosphorylated in a Mec1-dependent manner at serine 192 in response to DNA damage (Flott *et al.* 2011). Biochemical analysis indicates that serine 192 is required for Rad51 ATP hydrolysis and DNA binding, whereas mutation of serine 192 does not interfere with Rad51 multimerization. Srs2 is inhibited by Cdc28 phosphorylation at multiple sites to allow Rad51-dependent DSB repair via SDSA by controlling turnover of Srs2 at the invading strand (Saponaro *et al.* 2010).

Some aspects of recombination are also likely to be regulated by sumoylation of RPA, Rad52, Rad59, and Srs2 (Sacher *et al.* 2006; Burgess *et al.* 2007; Ohuchi *et al.* 2008; Saponaro *et al.* 2010; Cremona *et al.* 2012; Psakhye and Jentsch 2012). For Rad52, it was shown that sumoylation on lysines 43, 44, and 253 inhibits its ssDNA binding and annealing activities without affecting its interaction with Rad51 and RPA (Altmannova *et al.* 2010), and sumoylation of Rad52 protects it from proteasomal degradation (Sacher *et al.* 2006). Moreover, stimulating Rad52 sumoylation by overexpression of the Siz2 SUMO ligase or by fusing SUMO to the C terminus of Rad52 suppressed the DNA damage sensitivity of *srs2* cells (Esta *et al.* 2013). As a consequence, sumoylation of Rad52 improves the fidelity of recombinational repair by shifting DSB repair from SSA to gene conversion (Sacher *et al.* 2006; Altmannova *et al.* 2010). Sumoylation of Srs2 on lysines 1081, 1089, and 1142 appears to inhibit recombinational repair as mutation of these lysines to arginine partially suppresses the DNA repair

**Table 2 Regulation of HR proteins by post-translational modifications**

| Target | PTM | Modifier(s) | Function | References |
|---|---|---|---|---|
| Sae2 | S267$^{P}$ | Cdc28 | Promotes resection | Aylon *et al.* (2004); Huertas *et al.* (2008); Ira *et al.* (2004); Zierhut and Diffley (2008) |
| | P | Tel1/Mec1 | Activation through solubilization | Baroni *et al.* (2004); Fu *et al.* (2014) |
| | Ac | ? | Degradation | Robert *et al.* (2011) |
| Dna2 | T4$^{P}$, S17$^{P}$, S237$^{P}$ | Cdc28 | Promotes long-range resection | Chen *et al.* (2011) |
| | S17$^{P}$ | Cdc28 | Nuclear localization | Kosugi *et al.* (2009) |
| Rad55 | S2$^{P}$, S8$^{P}$, S14$^{P}$ | Mec1 | ? | Bashkirov *et al.* (2000); Herzberg *et al.* (2006) |
| Rad51 | S192$^{P}$ | Mec1 | ATPase regulation | Flott *et al.* (2011) |
| Srs2 | P | CDC28 | Inhibits displacement of Rad51 | Saponaro *et al.* (2010) |
| | K1081$^{S}$, K1089$^{S}$, K1142$^{S}$ | ? | Promotes displacement of Rad51 | Saponaro *et al.* (2010) |
| Rad52 | K43$^{S}$, K44$^{S}$, K253$^{S}$ | Siz2 | Inhibits DNA binding and annealing of ssDNA | Altmannova *et al.* (2010); Sacher *et al.* (2006) |
| | Ub | Slx5–Slx8 | ? | (Ii *et al.* (2007) |
| Slx4 | T113$^{P}$ | Mec1/Tel1 | Activation of Rad1-Rad10 nuclease | Toh *et al.* (2010) |
| | P | Mec1/Tel1 | Interaction with Dpb11 | Ohouo *et al.* (2010) |
| Mms4 | P | CDC28, Cdc5 | Activation in M phase | Loog and Morgan (2005); Matos *et al.* (2011) |
| Yen1 | P | CDC28, Cdc5 | Inhibition outside of anaphase | Loog and Morgan (2005); Matos *et al.* (2011) |
| | S655$^{P}$, S679$^{P}$ | CDC28 | Nuclear localization | Eissler *et al.* (2014); Kosugi *et al.* (2009) |
| Rad1 | K32$^{S}$ | Siz1, Siz2 | Inhibition of DNA binding | Sarangi *et al.* (2014) |
| PCNA | K127$^{S}$, K164$^{S}$ | ? | Binding of Srs2 | Hoege *et al.* (2002); Papouli *et al.* (2005) |
| | K164$^{Ub}$ | Rad6–Rad18 | Promotes translesion synthesis | Hoege *et al.* (2002) |
| | K164$^{Ub}{}_{n}$ | Rad5–Ubc13–Mms2 | Promotes error-free lesion bypass by template switching | Blastyak *et al.* (2007); Hoege *et al.* (2002) |
| Rtt107 | P | Mec1 (Slx4) | Replication restart | Roberts *et al.* (2006) |
| Mcm2–7 | S164$^{P}$, S170$^{P}$ | Mec1 (Mrc1) | Replication restart | Randell *et al.* (2010); Stead *et al.* (2012) |

P, phosphorylation. Ac, acetylation. S, sumoylation. Ub, ubiquitylation. Parentheses indicate partial dependency. ?, unknown.

defect of a Cdc28-phosphorylation deficient *srs2* mutant (Saponaro *et al.* 2010), suggesting that phosphorylation and sumoylation may have counteracting effects on Srs2 activity. Furthermore, sumoylation of Srs2 decreases its interaction with sumoylated PCNA and at the same time sumoylated PCNA inhibits Srs2 sumoylation (Kolesar *et al.* 2012). This observation suggests that sumoylation could make Srs2 association to the replication fork more dynamic. The effects of RPA and Rad59 sumoylation on recombination remain to be established in yeast. However, in human cells, RPA1 sumoylation facilitates recruitment of Rad51 to DNA damage-induced foci to initiate DNA repair through homologous recombination (Dou *et al.* 2010). It has also been suggested that desumoylation of repair proteins by the SUMO-specific protease Ulp1, which associates with the NPC (Takahashi *et al.* 2000), is responsible for the stimulation of spontaneous gene conversion observed at a locus artificially tethered to the nuclear envelope or to the NPC (Nagai *et al.* 2008). This notion is supported by changes in sumoylation patterns of RPA, Rad52, and Rad59 observed in nucleoporin mutants and in *slx8* (Burgess *et al.* 2007; Palancade *et al.* 2007).

The role of Rad1–Rad10 in processing heterologous flaps in collaboration with Slx4 requires phosphorylation of Slx4 on threonine 113 by Mec1 or Tel1 (Toh *et al.* 2010). Rad1 is sumoylated at lysine 32, which decreases the affinity of the Rad1–Rad10 for DNA without affecting its other activities, suggesting that Rad1 sumoylation promotes its disengagement from DNA after nuclease cleavage (Sarangi *et al.* 2014). Mus81–Mms4 and Yen1 activity are restricted to the G2/M transition and anaphase, respectively, by Cdc5- and Cdc28-dependent phosphorylation (Loog and Morgan 2005; Matos *et al.* 2011; Matos *et al.* 2013; Saugar *et al.* 2013). Premature activation of Mus81–Mms4 using a phosphomimetic Mms4 allele or by untimely activation of Cdc5 increases crossover-associated recombination events (Matos *et al.* 2013; Szakal and Branzei 2013). Yen1 is inactivated by Cdc28-dependent phosphorylation and activated at anaphase by the Cdc14 phosphatase (Blanco *et al.* 2014; Eissler *et al.* 2014). Further, to provide another level of regulation, Yen1 relocalizes from the cytoplasm to the nucleus upon phosphorylation on serines 655 and 679 by Cdc28 in G2/M phase (Kosugi *et al.* 2009; Blanco *et al.* 2014; Eissler *et al.* 2014).

***Regulation of recombinational restart of replication by PTMs:*** A range of DNA lesions may cause stalling of DNA replication. Replication fork blockage activates the replication checkpoint, which is responsible for slowing of S-phase and cell cycle progression, down-regulation of late origin firing, activation of DNA repair proteins, and stabilization of replication forks (reviewed in Friedel *et al.* 2009). The replication checkpoint is mediated by the Mec1 kinase and its downstream effector kinase Rad53. In the absence of Mec1 or Rad53, stalled replication forks collapse and the replisome dissociates (Tercero and Diffley 2001; Cobb *et al.* 2003), which is likely due to the failure to phosphorylate functional targets at the replication fork such as Mrc1, Pol31, Rtt107, Dbf4, and Pol1 (Osborn and Elledge 2003; Roberts *et al.* 2006; Chen *et al.* 2010; Randell *et al.* 2010). In contrast to active or stalled replication forks, which are refractory to

recruitment of Rad52 (Lisby *et al.* 2004; Alabert *et al.* 2009), collapsed replication forks readily recruit Rad52 into foci.

PCNA is the master regulator of DNA damage tolerance pathways at the replication fork. During S phase and in response to replication stress by hydroxyurea, PCNA is sumoylated at lysines 127 and 164 (Hoege *et al.* 2002; Papouli *et al.* 2005). Sumoylated PCNA is bound by the Srs2 helicase (Papouli *et al.* 2005), which acts as an anti-recombinase by displacing Rad51 from single-stranded DNA (Krejci *et al.* 2003; Veaute *et al.* 2003). Accordingly, Rad51 is enriched at the replication fork in a nonsumoylatable *pol30-K127,164R* mutant (Papouli *et al.* 2005). In contrast, when DNA damage such as UV- or MMS-induced lesions are encountered by the replication fork, PCNA is first monoubiquitylated at lysine 164 by the Rad6–Rad18 pathway (Hoege *et al.* 2002), which promotes translesion synthesis by a number of error-prone polymerases (reviewed in Finley *et al.* 2012). Sumoylation and ubiquitylation of PCNA are independent processes (Papouli *et al.* 2005). Lysine 164 of PCNA can be further modified by K63-linked polyubiquitylation through the Rad5–Ubc13–Mms2 pathway (Hoege *et al.* 2002), which facilitates error-free repair of lesions on the leading strand template by fork regression and template switching using the Rad5 helicase (Blastyak *et al.* 2007), while Rad52 mediates error-free repair of lesions on the lagging strand template (Prakash 1981; Zhang and Lawrence 2005; Gangavarapu *et al.* 2007) (Figure 6). However, the majority of error-free lesion bypass was reported to be *RAD52* independent (Zhang and Lawrence 2005).

The role of Mec1-dependent phosphorylation during the restart of stalled replication forks is still poorly understood. Mec1-dependent phosphorylation of Slx4 facilitates assembly of an Rtt107–Slx4–Dpb11 complex at stalled forks (Ohouo *et al.* 2010). Further, Slx4-dependent phosphorylation of Rtt107 by Mec1 is critical for replication restart after alkylation damage (Roberts *et al.* 2006). Possibly, the Rtt107–Slx4–Dpb11 complex acts as a scaffold for the assembly of additional fork stabilizing and repair factors such as the Smc5–Smc6–Mms21 SUMO ligase (Ohouo *et al.* 2010; Leung *et al.* 2011). The Smc5–Smc6–Mms21 complex was shown to promote sister-chromatid junction-mediated intra-S repair (Branzei *et al.* 2006; De Piccoli *et al.* 2006; Sollier *et al.* 2009), although the relevant sumoylation targets remain to be identified. Finally, Mrc1 facilitates Mec1 phosphorylation of the S/T-Q motifs of chromatin-bound Mcm2–7 during S phase to facilitate replication restart during replication stress (Randell *et al.* 2010; Stead *et al.* 2012).

The Slx5–Slx8 (SUMO-targeted ubiquitin E3 ligase) is localized to replication foci and is important for suppressing recombination during DNA replication (Burgess *et al.* 2007). This may be explained by the observation that deletion of *SLX5*–*SLX8* results in reduced levels of Rad52, Rad59, and RPA sumoylation. In the case of Rad52, its sumoylation inhibits recombination (Sacher *et al.* 2006; Altmannova *et al.* 2010). *In vitro*, Rad52 and Rad57 are targets of the ubiquitylation activity of Slx5–Slx8 (Ii *et al.* 2007).

### *C. Role of chromatin in controlling mitotic recombination*

Several lines of evidence suggest that homologous recombination is controlled by modification of chromatin structure (Chai *et al.* 2005; Shim *et al.* 2005; Tsukuda *et al.* 2005; Kent *et al.* 2007; Van Attikum *et al.* 2007; Sinha *et al.* 2009; Sinha and Peterson 2009; Tsukuda *et al.* 2009; Chen *et al.* 2012; Costelloe *et al.* 2012; Adkins *et al.* 2013). Similarly, capping of telomeres is likely a major barrier for recombination at telomere sequences either by inhibiting recombination proteins or by preventing resection of telomeres (Grossi *et al.* 2001; Dubois *et al.* 2002).

One of the principal and evolutionarily conserved chromatin marks associated with DNA damage that expose DNA ends or single-stranded regions is the phosphorylation of histone H2A on serine 129 by the Tel1 and Mec1 kinases (Rogakou *et al.* 1999; Downs *et al.* 2000; Shroff *et al.* 2004). An *hta-S129A* mutant is sensitive to DNA damage-inducing agents such as phleomycin, camptothecin, and methyl methanesulfonate (Redon *et al.* 2003; Downs *et al.* 2004). The modification of chromatin by H2A phosphorylation occurs preferentially at unresected DSB ends and in G1 phase, whereas the recruitment of chromatin modifiers NuA4, SWR1, RSC, SWI/SNF, and INO80 occurs in G2/M and correlates with homologous recombination (Downs *et al.* 2004; Morrison *et al.* 2004; Van Attikum *et al.* 2004; Bennett *et al.* 2013). The NuA4 complex contains an associated histone acetyltransferase, which targets histone H4 for acetylation and is important for DNA repair (Choy and Kron 2002; Downs *et al.* 2004). Histone H3-K56 acetylation, which is formed transiently by Rtt109 during DNA replication, is important for sister-chromatid repair of DSBs arising during replication (Munoz-Galvan *et al.* 2013). Other histone acetyltransferases, such as Gcn5 and Hat1, also contribute to the wave of chromatin acetylation that follows DSB formation (Qin and Parthun 2002; Tamburini and Tyler 2005). A number of histone deacetylases including Rpd3, Hda1, Sir2, and Hst1 are also recruited to sites of DNA damage presumably to remove DNA damage-induced chromatin marks after completion of repair (Robert *et al.* 2011; Tamburini and Tyler 2005). The acetylation marks may serve to stabilize the SWR1 complex at DSBs via binding of its Bdf1 subunit through its double bromodomain (Kobor *et al.* 2004). SWR1 mediates deposition of the histone variant Htz1 (H2A.Z) in place of H2A in chromatin (Kobor *et al.* 2004; Mizuguchi *et al.* 2004; Morillo-Huesca *et al.* 2010). The effects of these chromatin modifications are not fully understood, but it has been reported that deposition of Htz1 into chromatin is important for DSB end resection (Kalocsay *et al.* 2009). The checkpoint adaptor Rad9 is recruited to sites of DNA damage likely through a dual interaction of its BRCT domains with H2A-$S129^{P}$ and its Tudor domain with histone H3 methylated at lysine 79 ($H3\text{-}K79^{Me}$) (Giannattasio *et al.* 2005; Javaheri *et al.* 2006; Toh *et al.* 2006; Hammet *et al.* 2007; Germann *et al.*

2011). Finally, H2A-S129 phosphorylation is also required for loading cohesins at a defined DSB (Unal *et al.* 2004).

ATP-dependent chromatin remodeling is equally important for efficient homologous recombination especially in heterochromatin (Sinha and Peterson 2009; Sinha *et al.* 2009). The RSC complex is one of the earliest factors recruited to a DSB along with the MRX and Ku complexes (Shim *et al.* 2005), but RSC also appears to have a later role in recombination following synapsis (Chai *et al.* 2005). The RSC complex produces a histone-free region of a few hundred nucleotides immediately adjacent to a DSB to promote binding of the MRX and Ku complexes and to facilitate resection (Kent *et al.* 2007; Shim *et al.* 2007; Adkins *et al.* 2013). The later role of RSC in recombination may be linked to its interaction with Rad59 and/or its involvement in loading of cohesin at DNA breaks (see below) (Oum *et al.* 2011). In contrast, the SWI/SNF chromatin-remodeling complex was found to play a role at or preceding the strand-invasion step of HR (Chai *et al.* 2005). The Fun30 nucleosome-remodeling factor is important for extensive resection by Exo1 and Sgs1–Dna2 (Chen *et al.* 2012; Costelloe *et al.* 2012; Eapen *et al.* 2012). A fourth ATP-dependent chromatin remodeling factor, Rad54, enhances DNA strand invasion by Rad51 on chromatin substrates *in vitro* (Alexiadis and Kadonaga 2002), and further plays a crucial role *in vivo* for the initiation of DNA synthesis after strand invasion likely by enhancing the accessibility to DNA within nucleosomal arrays (Jaskelioff *et al.* 2003; Sugawara *et al.* 2003; Ceballos and Heyer 2011). Rad54 interacts directly with histone H3 and its chromatin-remodeling activity is stimulated by Rad51 (Jaskelioff *et al.* 2003; Kwon *et al.* 2007).

#### *D. Role of cohesin in regulating mitotic recombination*

Cohesin is loaded and maintained at double-strand breaks independent of global DNA replication and is required for efficient sister-chromatid recombination (Sjogren and Nasmyth 2001). Loading of cohesin at DSBs requires H2A-S129$^{P}$, Mre11, and Scc2, a component of the cohesin loading machinery (Strom *et al.* 2004; Unal *et al.* 2004), and is further aided by the RSC chromatin remodeler and Rad59 (Oum *et al.* 2011). Replication-independent cohesion is induced genome-wide by the Eco1 acetyltransferase, which targets Smc3 in response to DNA damage (Strom *et al.* 2007; Unal *et al.* 2007; Heidinger-Pauli *et al.* 2009). The establishment of damage-induced cohesion by cohesin acetylation is further aided by Ctf4, Ctf18, Tof1, Csm3, Chl1, and Mrc1 (Borges *et al.* 2013). In contrast, cohesion must be relieved locally by separase in order for efficient resection of DSBs during postreplicative repair (McAleenan *et al.* 2013). Another structural maintenance of chromosomes (SMC) factor, which is loaded at DSBs by Scc2 is the Smc5–Smc6 complex in a manner dependent on the BRCT domain-containing protein Rtt107/Esc4 (Lindroos *et al.* 2006; Leung *et al.* 2011). The Smc5–Smc6 complex is required for MMS-induced recombination and DSB repair (Onoda *et al.* 2004; De Piccoli *et al.* 2006).

#### *E. Ploidy/aneuploidy*

Mitotic recombination is more efficient in heterozygous *MAT***a**/*MAT*α diploids than in homozygous *MAT***a**/*MAT***a** or *MAT*α/*MAT*α cells, both spontaneously and after UV irradiation (Friis and Roman 1968; Hopper *et al.* 1975; Esposito and Watsgaff 1981). In diploid yeast, NHEJ is severely disabled through the repression of *NEJ1*, a key component of NHEJ, by the transcriptional repressor, Mata1–Matα2 (Heude and Fabre 1993; Frank-Vaillant and Marcand 2001). As expected, diploids are more radioresistant than haploids due to the extra copy of the genome (Mortimer 1958). However, a further increase in ploidy leads to increased radiosensitivity and reliance on homologous recombination for survival (Storchova *et al.* 2006). In response to replication stress, haploid cells use the Rad6-dependent pathways that resume stalled forks, whereas diploid cells use homologous recombination (Li and Tye 2011). Indeed, the DNA damage sensitivity of *rad6* and *rad18* mutants is suppressed by mating-type heterozygosity in a *RAD52*-dependent manner (Yan *et al.* 1995). The IR sensitivity of *rad55* and *rad57* mutants is also suppressed by *MAT* heterozygosity by an unknown mechanism. Although the mechanism was reported to be due to loss of NHEJ, in other strain backgrounds *rad55 yku70* or *rad55 dnl4* mutants retain IR sensitivity (Valencia-Burton *et al.* 2006; Fung *et al.* 2009). Mutation of *SRS2* suppresses the IR sensitivity of *rad55* and *rad57* mutants consistent with the model that Srs2 and Rad55–Rad57 have opposing roles in Rad51 nucleoprotein filament stability (Fung *et al.* 2009; Liu *et al.* 2011). *SRS2* transcript levels vary in response to ploidy and might contribute to the *MAT* heterozygosity suppression of *rad6*, *rad18*, *rad55*, and *rad57* mutants. The only recombination gene that is clearly regulated at the transcriptional level by mating type is *RDH54*. There is a Mata1–Matα2 binding site within the *RDH54* promoter and Rdh54 protein level is reduced fivefold in diploids compared with haploids (de Godoy *et al.* 2008; Galgoczy *et al.* 2004). Surprisingly, the phenotype of *rdh54* mutants is most apparent in diploids (lethality with *rad54* and *srs2*), suggesting the reduced levels are nevertheless required for HR (Klein 1997).

## VII. Postscript

The study of mitotic recombination in yeast has progressed immensely since the last time a chapter in the precursor of the YeastBook was written (Petes *et al.* 1991). Much has been learned about the many genes that act to preserve genome stability through genetics, biochemistry, and cell biology. However, there is still important work to be done. The *in vitro* reconstitution of many of the fundamental reactions has not yet been achieved. In addition, the emergence of single molecule techniques for defining biochemical reactions will greatly expand our detailed understanding of the steps for each of these reactions. Genetic analysis of combinations of mutants will be needed to push the boundaries to define new gene and pathway interactions. The underlying cell biology of these genes and pathways must also be

examined in detail. Finally, high throughput methods should be applied to more processes, which promises a greater depth of understanding of the relationships between the genetic, biochemical, and cell biological processes that are involved in this basic cellular function.

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