Template Switching During Break-Induced Replication Is Promoted by the Mph1 Helicase in *Saccharomyces cerevisiae*

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ABSTRACT Chromosomal double-strand breaks (DSBs) that have only one end with homology to a donor duplex undergo repair by strand invasion followed by replication to the chromosome terminus (break-induced replication, BIR). Using a transformation-based assay system, it was previously shown that BIR could occur by several rounds of strand invasion, DNA synthesis, and dissociation. Here we describe a modification of the transformation-based assay to facilitate detection of switching between donor templates during BIR by genetic selection in diploid yeast. In addition to the expected recovery of template switch products, we found a high frequency of recombination between chromosome homologs during BIR, suggesting transfer of the DSB from the transforming linear DNA to the donor chromosome, initiating secondary recombination events. The frequency of BIR increased in the mph1Δ mutant, but the percentage of template switch events was significantly decreased, revealing an important role for Mph1 in promoting BIR-associated template switching. In addition, we show that the Mus81, Rad1, and Yen1 structure-selective nucleases act redundantly to facilitate BIR.

CHROMOSOMAL double-strand breaks (DSBs) are cytotoxic DNA lesions and if left unrepaird, or repaired incorrectly, can result in a loss of genetic information, genome instability, or even cell death. There are two main pathways to repair DSBs: homologous recombination (HR) and nonhomologous end joining (NHEJ). HR uses a homologous duplex to template repair of the break and is generally considered to be an error-free process; however, repair from a nonister chromatid template can lead to loss of heterozygosity (LOH) or genome rearrangements (Symington 2002).

Initiation of HR requires degradation of the 5’-terminated strands to produce 3’-single-stranded DNA (ssDNA) tails that are first bound by replication protein A (RPA) and then replaced by Rad51 (Symington 2002). The Rad51 nucleoprotein filament searches for homologous sequences and promotes strand invasion to form a displacement loop (D-loop) (Figure 1). The 3’ end of the invading strand is used to prime DNA synthesis, templated by the donor duplex. The D-loop intermediate can be further channeled into one of several subpathways: synthesis-dependent strand annealing (SDSA), canonical double-strand break repair (DSBR), or break-induced replication (BIR). During SDSA, the invading strand that has been extended by DNA synthesis is displaced and anneals to complementary sequences exposed by 5’–3’ resection of the other side of the break. The remaining gaps can be filled by DNA synthesis and the nicks ligated to yield exclusively noncrossover products (Nassif et al. 1994; Ferguson and Holloman 1996). If the second end of the break is captured by the D-loop, a double Holliday junction (dHJ) can be generated after DNA repair synthesis and ligation (Szostak et al. 1983). dHJs can either be dissolved by the Sgs1–Top3–Rmi1 complex to form noncrossover products (Ira et al. 2003; Wu and Hickson 2003) or resolved by structure-selective nucleases to generate crossovers or noncrossovers. Mus81–Mms4 is the main nuclease responsible for mitotic crossovers in *Saccharomyces cerevisiae* with Yen1 serving as a back-up function (Blanco et al. 2010; Ho et al. 2010; Agmon et al. 2011; Muñoz-Galván et al. 2012). Mus81–Mms4...
is also proposed to act on the captured D-loop intermediate, prior to maturation to a dHJ, to form exclusively crossover products (Osman et al. 2003; Mazón and Symington 2013).

BIR is a one-ended double-strand break repair mechanism in which DNA synthesis primed within the D-loop proceeds to the chromosome end, copying tens of kilobases (Morrow et al. 1997; Malkova et al. 2005). BIR is far more mutagenic than normal S-phase DNA synthesis and recent studies have shown the nascent strands are synthesized by a conservative mechanism, similar to other gene conversion reactions (Arcangioli 2000; Ira et al. 2006; Deem et al. 2011; Donnianni and Symington 2013; Saini et al. 2013). BIR is the mechanism proposed for alternative lengthening of telomeres in the absence of telomerase and plays a minor role in repair of internal chromosome DSBs that have two ends with homology to a donor duplex (McEachern and Haber 2006; Ho et al. 2010). However, it is a preferential pathway to generate LOH in old yeast cells (McMurray and Gottschling 2003), and microhomology-mediated BIR (MMBIR) has been implicated in genomic rearrangements (Hastings et al. 2009).

BIR is most easily studied by creating a DSB where just one of the two ends can undergo homology-dependent strand invasion (Bosco and Haber 1998; Davis and Symington 2004; Malkova et al. 2005; Lydeard et al. 2007). In previous work from this laboratory, we described a plasmid-based assay to detect BIR in yeast. The chromosome fragmentation vector (CFV) contains the URA3 selectable marker, SUP11, CEN4, a tract of (G_{1,3}T)_{n}, to provide a site for telomere addition and a unique DNA segment to target invasion of chromosomal sequence (Morrow et al. 1997; Davis and Symington 2004). The CFV is linearized between the chromosome homology and telomere seeding sequence in vitro and used to transform yeast, selecting for Ura+ colonies. Most transformants arise by de novo telomere addition to heal one end of the CFV and strand invasion at the other end into the endogenous yeast locus to copy the entire chromosome arm, yielding a stable chromosome fragment (CF). When performed in diploid cells with polymorphic copies of chromosome III, ~15% of the CFs recovered contained sequences from both chromosome homologs, suggesting the replication intermediate formed during BIR is unstable and the invading end can switch to a different template—a process referred to here as “template switching” (Smith et al. 2007). Template switching was observed not only between chromosome homologs but also between ectopic homologies on different chromosomes, revealing the potential for this mechanism to generate genome rearrangements. Template switching has also been reported during BIR initiated at a chromosomai DSB, indicating that it is a general phenomenon and not restricted to the plasmid system (Ruiz et al. 2009; Pardo and Aguileria 2012). Template switching could be facilitated by helicases that displace the invading 3’ strand, as proposed for SDSA, or by cleavage of the D-loop intermediate by structure-selective nucleases (Pardo and Aguileria 2012). The obvious candidate for displacement of the 3’ strand during template switching is the Mph1 3’–5’ helicase, an ortholog of FANCM that displaces D-loops and extended D-loops following Rad51-mediated strand invasion (Sun et al. 2008; Prakash et al. 2009; Sebesta et al. 2011). Interestingly, when MPH1 is overexpressed in yeast, it inhibits BIR at a chromosomal DSB but not at telomeres (Luke-Glaser and Luke 2012). The Pif1 5’–3’ helicase is required for mitochondrial DNA maintenance and functions in nuclear genome stability by unwinding...
G quadruplexes and preventing de novo telomere addition at DSBs (Schulz and Zakian 1994; Ribeyre et al. 2009; Bochman et al. 2010; Paeschke et al. 2013). Recent studies identified a role for Pif1 during BIR (Hu et al. 2013; Saini et al. 2013; Wilson et al. 2013).

Here we describe a modification of the transformation assay to facilitate analysis of template switching associated with BIR in yeast. The assay is based on transformation of diploid yeast strains heteroallelic for ade2 or leu2 with CFVs that invade just upstream of the ade2 or leu2 locus. In addition to the expected recovery of CFs with a functional copy of ADE2 or LEU2 by template switching, recombination between the chromosome homologs targeted by the CFV occurred at a high frequency during BIR. The percentage of Ade+ transformants was significantly decreased in the mph1Δ and pif1-m2 mutants, and most of the Ade+ events recovered from the mph1Δ mutant were due to chromosomal recombination, revealing an important role for Mph1 in template switching associated with BIR.

Materials and Methods

Media, growth conditions, and genetic methods

Rich medium (1% yeast extract, 2% peptone, 2% dextrose) (YPD), synthetic complete medium (SC) lacking the appropriate amino acids or nucleic acid bases, sporulation medium, and genetic methods were as described previously (Sherman et al. 1986). Rich medium supplemented with 2% galactose instead of dextrose was used for induction of HO endonuclease. Standard procedures were used for genetic crosses and transformation (Ito et al. 1983; Sherman et al. 1986).

Yeast strains and plasmids

*S. cerevisiae* strains used in this study are RAD5 derivatives of W303 (Table 1). Strains containing ade2-I, ade2-n, his3Δ::hphMX4, his3Δ::natMX4, leu2ΔB, leu2ΔR, mph1Δ::kanMX6, mus81Δ::kanMX6, rad1::LEU2, yen1Δ::HIS3, and pif1-m2 alleles were described previously (Mortensen et al. 2002; Wagner et al. 2006; Ho et al. 2010). All deletion alleles are denoted by Δ in the text. The MET22 locus on the left arm of chromosome XV (Chr XV) was replaced by TRP1 in the ade2-n strains. Diploids were made by crossing appropriate haploids to generate strains that were MET22/met22::TRP1 ade2-I/ade2-n his3Δ::hphMX4/his3Δ::natMX4 or leu2ΔAR/leu2ΔB.

CFVs used in this study are ARS- derivatives of CFV/D8B-tg (Davis and Symington 2004; Marrero and Symington 2010). Vectors pLAG6 and pLAG10 contain inserts from the right arm of Chr XV (Saccharomyces Genome Database, SGD coordinates 558476–563456 and 551748–556242, respectively) while vectors pLS192 and pLAG8 contain sequences from the left arm of Chr III (SGD coordinates 96821–102096 and 93450–98535, respectively).

Transformation-based template switching assays

ade2 assay: A total of 500 ng of BgIII-digested pLAG6 or pLAG10 was used to transform competent yeast cells, selecting for Ura+ transformants. The percentage of Ura+ Ade+ (white) was determined from the total number of Ura+ transformants. All Ura+ Ade+ transformants were further analyzed by patching onto YPD medium to allow growth in nonselective conditions and then replica plating to 5-fluoroorotic acid (5-FOA)-containing medium to select for cells that became Ura- due to loss of the CF (Boeke et al. 1984). Cells that lost the CF were scored again for their color, allowing distinction between 5-FOA red (Ade-; W/R; template switching) and 5-FOA white (Ade+; W/W; BIR-associated chromosomal recombination) transformants, and scored on SC-Met, SC-Trp, YPD +nourseothricin (Nat), and YPD +hygromycin B (Hyg) for marker loss. Ura+ Ade- transformants derived from pLAG6 in wild type, mph1Δ, and mus81Δ strains were also scored for the heterozygous markers on the left and right arms of Chr XV after CF loss. Pulsed-field gel electrophoresis of intact chromosomal DNA prepared from Ura+ transformants was used to confirm formation of full-length CF products (Schwartz and Cantor 1984; Davis and Symington 2004).

leu2 assay: A total of 500 ng of linearized CFVs pLS192 and pLAG8, digested with SnaBI and BglII, respectively, was used to transform competent yeast cells, selecting for Ura+ transformants. Transformants were replica plated on SC-Leu medium to determine the percentage that were Leu+. Ura+ Leu+ transformants were further analyzed by patching on YPD, to allow growth in nonselective conditions and replica plated on 5-FOA to select for Ura- cells that lost the CF, followed by replica plating to SC-Leu to distinguish between Leu- (template switching) and Leu+ (BIR-associated chromosomal recombination) transformants.

Statistical significance was determined after totaling Ade+ and Leu+ transformants from several independent transformations using two-tailed Pearson’s chi-squared test ($\chi^2$; http://in-silico.net/). For the ade2 assay, the numbers of Ade+ Ura+ transformants were compared to Ade- Ura+ transformants (Figure 2D). To determine statistical significance of template switching vs. chromosomal recombination, the number of template switching transformants (W/R on 5-FOA) was compared to the number of BIR-associated chromosomal recombinants (W/W on 5-FOA; Figure 3A, Figure 4B, Figure 6B, and Figure S3). To determine the statistical significance of LOH for a specific transforming DNA and strain after growth of transformants on 5-FOA-containing medium, the number of transformants that lost Hyg or Nat resistance (LOH transformants) was compared to those with no LOH (Figure 3C and Figure S1).

BIR efficiency was determined using 500 ng of the specified linearized CFV or 100 ng of circular replicative plasmid pRS416 for each transformation, selecting for Ura+ transformants. BIR efficiency is expressed as the ratio of Ura+ transformants obtained per nanogram of CFV and per nanogram of CFV.
Table 1 Yeast strains

| Strain | Genotype | Source |
|--------|----------|--------|
| LSY2165-11C | MATa ade2-1 his3 Δ:: hpMX4 mus81Δ::kanMX6 | Ho et al. (2010) |
| LSY2165-30B | MATa ade2-1 his3 Δ:: hpMX4 mus81Δ::kanMX6 yen1Δ::His3 | Ho et al. (2010) |
| LSY2240 | MATa leu2Δ::EcclI lys2 | Mortensen et al. (2002) |
| LSY2241 | MATa leu2Δ::Stell trp1-1 | Mortensen et al. (2002) |
| LSY2332-28 | MATa ade2-1 his3 Δ:: hpMX4 mph1Δ::kanMX6 | Mazón and Symington (2013) |
| LSY2543 | MATa met22::KTRP1 ade2-n his3 Δ::natMX4 | This study |
| LSY2633 | MATa met22::KTRP1 ade2-n his3 Δ::natMX4 mus81Δ::kanMX6 | This study |
| LSY2638 | MATa ade2-1 his3 Δ:: hpMX4 | This study |
| LSY2650 | MATa ade2-1 his3 Δ:: hpMX4 mph1Δ::kanMX6 | This study |
| LSY2653 | MATa met22::KTRP1 ade2-n his3 Δ::natMX4 mph1Δ::kanMX6 | This study |
| LSY2708-1D | MATa ade2-1 his3 Δ:: hpMX4 pif1-m2 | This study |
| LSY2709-4C | MATa met22::KTRP1 ade2-n his3 Δ::natMX4 pif1-m2 | This study |
| LSY2738-15B, 16D | MATa ade2-1 his3 Δ:: hpMX4 mph1Δ::kanMX6 | This study |
| LSY2739-8B | MATa ade2-1 his3 Δ:: hpMX4 mph1Δ::kanMX6 pif1-m2 | This study |
| LSY2739-16A | MATa ade2-1 his3 Δ:: hpMX4 mph1Δ::kanMX6 pif1-m2 | This study |
| LSY2740-8B, 19B | MATa met22::KTRP1 ade2-n his3 Δ::natMX4 mus81Δ::kanMX6 yen1Δ::His3 | This study |
| LSY2802-5B | MATa met22::KTRP1 ade2-n his3 Δ::natMX4 mph1Δ::kanMX6 | This study |
| LSY2803-7C | MATa met22::KTRP1 ade2-n his3 Δ::natMX4 mflh1Δ::LEU2 | This study |
| LSY2804-5D | MATa met22::KTRP1 ade2-n his3 Δ::natMX4 mph1Δ::kanMX6 pif1-m2 | This study |
| LSY2804-17A | MATa met22::KTRP1 ade2-n his3 Δ::natMX4 mph1Δ::kanMX6 pif1-m2 | This study |
| LSY2950 | MATa/MATa leu2Δ::EcclII/leu2Δ::Stell lys2/lys2 trp1-1/1TRP1 | This study |
| LSY2951 | MATa/MATa ade2-lade2-n his3 Δ:: hpMX4/hs3 Δ::natMX4 | This study |
| LSY2951 | MET22l met22::KTRP1 | This study |
| LSY2332-28 × LSY2802-5B, LSY2650 × LSY2653 | Identical to strain LSY2951 except mph1Δ::kanMX6/mph1Δ::kanMX6 | This study |
| LSY2739-9B × LSY2804-17A | Identical to strain LSY2951 except pif1-m2/pif1-m2 | This study |
| LSY2739-16A × LSY2804-5D | Identical to strain LSY2951 except mph1Δ::kanMX6/mph1Δ::kanMX6 | This study |
| LSY2165-11C × LSY2633 | Identiﬁcal to strain LSY2951 except mus81Δ::kanMX6/mus81Δ::kanMX6 | This study |
| LSY2165-30B × LSY2740-8B, LSY2165-30B × LSY2740-19B | Identiﬁcal to strain LSY2951 except mus81Δ::kanMX6/mus81Δ::kanMX6 | This study |
| LSY2738-15B × LSY2803-7C, LSY2738-16D × LSY2803-7C | Identiﬁcal to strain LSY2951 except mflh1Δ::LEU2/mflh1Δ::LEU2 | This study |
| LSY2767-72D | MATa-inc lys2::natMX4 AVT2::lys3–3 HOcs::kanMX6 SYN8::TRP1-5’ Δ lys2 | Donninianni and (Chr. 1 128 kb) ade3::Pcan1-100 bar1::LEU2 | Synington (2013) |
| LSY2884-53D | Identiﬁcal to strain LSY2767-72D except mph1Δ::kanMX6 | This study |
| LSY2884-188C | Identiﬁcal to strain LSY2767-72D except pif1-m2 | This study |
| LSY2885-297B | Identiﬁcal to strain LSY2767-72D except URA3::GPD-TK7 | This study |
| LSY2885-160B, 248A | Identiﬁcal to strain LSY2767-72D except URA3::GPD-TK7 mus81Δ::kanMX6 | This study |
| LSY2885-121B, 127C | Identiﬁcal to strain LSY2767-72D except URA3::GPD-TK7 yen1Δ::His3 | This study |
| LSY2885-294A | Identiﬁcal to strain LSY2767-72D except mus81Δ::kanMX6 yen1Δ::His3 | This study |
| LSY2885-236A | Identiﬁcal to strain LSY2767-72D except mus81Δ::kanMX6 rad1::LEU2 | This study |
| LSY2886-1C, 51B | Identiﬁcal to strain LSY2767-72D except URA3::GPD-TK7 rad1::LEU2 | This study |
| LSY2921-108, 42B | Identiﬁcal to strain LSY2767-72D except mus81Δ::kanMX6 rad1::LEU2 | This study |
| LSY2921-93D | Identiﬁcal to strain LSY2767-72D except rad1::LEU2 yen1Δ::His3 | This study |

All strains are derived from the W303 background (ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1); only mating type and differences from this genotype are shown.

pRS416. The experiments were repeated at least three times for each CFV and strain. Mean BIR efficiencies were compared using Welch two-sample t-test from R Statistical software.

Chromosomal assay for BIR

Two-milliliter YPD cultures were grown overnight at 30°C. Cells were diluted and regrown to OD600 0.3–0.8 in YP with 2% raffinose and adjusted to an OD600 of 0.3. Aliquots of serial dilutions were plated on YPD, and SC –Lys and, for galactose induction, on YPGal plates and grown for 2–3 days. Colonies that grew on YPGal plates were replica plated to SC –Lys plates to ensure reconstitution of the functional LYS2 gene by BIR. The percent BIR rate was calculated as the ratio of colony-forming units (CFUs) on SC –Lys after HO induction and CFUs on YPD (Donninianni and Symington 2013). Significance was determined using the Student’s t-test.

Results

A genetic assay to detect template switching during BIR

We developed a simple genetic assay to screen candidate genes for their roles in template switching during BIR. Diploid yeast strains heteroallelic for ade2 or leu2 mutations
were transformed using linearized CFVs that invade upstream of the ade2 or leu2 loci and the resulting transformants were tested for adenine or leucine prototrophy, respectively. The strains for the ade2 assay contain ade2-I and ade2-n alleles on Chr XV homologs (Mozlin et al. 2008); the mutations are 0.95 kb apart and both inactivate ADE2, resulting in accumulation of a red pigment. CFVs pLAG6 and pLAG10 contain sequences homologous to Chr XV to promote strand invasion 1 kb and 8 kb upstream of the ade2 locus, respectively (Figure 2A). To confirm the generality of results from the ade2 assay, leu2 heteroalleles were used. The leu2ΔB and leu2ΔE mutations are 0.6 kb apart and inactivate LEU2 on Chr III (Mortensen et al. 2002). CFVs pLAG8 and pLS192, which invade 1 and 4 kb upstream of the leu2 region, respectively, were used for transformation (Figure 2A).

We first analyzed the BIR efficiency for both assays in cells with normal recombination functions (“wild type”); pLAG8 and pLAG192 have 30% higher BIR efficiency than pLAG6 and pLAG10 (P = 0.013 and P = 0.008, respectively). This difference could be explained by the shorter region of DNA that needs to be copied in the leu2 assay as compared to the ade2 assay (100 kb vs. 560 kb, respectively; Figure 2B) (Donnianni and Symington 2013).

Transformation of an ade2-I/ade2-n heteroallelic diploid with pLAG6 or pLAG10 results in generation of mainly red (Ade⁻) Ura⁺ transformants. The simplest mechanism to generate white Ade⁺ Ura⁺ transformants would be by invasion of one of the two Chr XV homologs by the CFV, and the generation of Ura⁺ Ade⁺ transformants could result from invasion of ade2-I, displacement, and invasion of the ade2-n allele. pLAG6, which invades closer to the ade2 locus than pLAG10, resulted in 2.6-fold higher frequency of Ade⁺ Ura⁺ transformants compared to pLAG10 (P < 0.0001). Figure 2C shows the simplest explanation for generation of Ura⁺ Ade⁻ transformants is by invasion of one of the two Chr XV homologs by the CFV, and the generation of white Ade⁻ Ura⁻ transformants could result from invasion of ade2-I, displacement, and invasion of the ade2-n allele.

Figure 2 Template-switching assays. (A) Schematic showing the invasion sites for pLAG6 and pLAG10 on Chr XV and pLAG8 and pLS192 on Chr III. (B) BIR efficiencies for the indicated vectors in wild type. Transformations were performed five to nine times and mean values are presented with standard deviation (SD). (C) The simplest explanation for generation of Ura⁺ Ade⁻ transformants is by invasion of one of the two Chr XV homologs by the CFV, and the generation of Ura⁺ Ade⁺ transformants could result from invasion of ade2-I, displacement, and invasion of the ade2-n allele. (D) Percentage of Ade⁺ or Leu⁺ transformants in wild type for both assays. pLS192 serves as control in the ade2 assay (invasion of a non-ade2-containing chromosome), and pLAG6 is a control in the leu2 assay (invasion of a non-leu2-containing chromosome).
which template switching occurs (Smith et al. 2007). In the \textit{leu2} assay, pLAG8 yielded 1.8-fold more \textit{Ura} \textit{Leu}+ transformants than pLS192 \textit{(P < 0.0001)}. The frequency of \textit{Ade} \textit{Ura}+ transformants using pLAG6 was 22-fold higher as compared to pLS192 \textit{(P < 0.0001)} while the frequency of \textit{Leu} \textit{Ura}+ transformants with pLAG6 was 28-fold higher than with pLAG6 \textit{(P < 0.0001)}, confirming that \textit{Ade} \textit{Ura}+ or \textit{Leu} \textit{Ura}+ transformants are a consequence of BIR upstream of the test locus and not caused by a genome-wide increase in spontaneous mitotic recombination during BIR (Figure 2D). Similarly, \textit{Ade} \textit{Ura}+ or \textit{Leu} \textit{Ura}+ transformants were only rarely recovered from transformation with the replicating plasmid pRS416. We acknowledge that only a small fraction of invasion, displacement, and reinvasion events are likely to generate \textit{Ade}+ or \textit{Leu}+ prototrophs and many template switch events will be genetically invisible.

\textbf{BIR-induced chromosomal recombination}

The \textit{Ade}+ or \textit{Leu}+ events due to template switching should have the \textit{ADE2} or \textit{LEU2} locus on the CF and after loss of the CF the cells would be expected to revert to an \textit{Ade}− or \textit{Leu}− phenotype. To test this prediction, \textit{Ade}+ \textit{Ura}+ or \textit{Leu}+ \textit{Ura}+ transformants were grown under nonselective conditions, replica plated to medium containing 5-FOA to select for \textit{Ura}− cells that had lost the CF, and then analyzed for adenine or leucine prototrophy (Figure 3A). Surprisingly, only 47% or 19% of \textit{Ade}+ transformants with pLAG6 or pLAG10, respectively, contained a CF-linked \textit{ADE2} gene indicative of template switching. For the \textit{leu2} assay, 70% and 49% of \textit{Leu}+ transformants with pLAG8 and pLS192, respectively, were \textit{Leu}− after CF loss (Figure 3B). Thus, a significant fraction of \textit{Ade}+ and \textit{Leu}+ transformants are due to BIR-induced recombination between chromosome homologs.

Induction of recombination between homologs was previously reported during plasmid gap repair and suggested to result from mismatch repair of a heteroduplex DNA intermediate (Silberman and Kupiec 1994). However, another possibility is that the D-loop intermediate is cleaved by a structure-selective nuclease effectively transferring the DSB from the linear fragment to the donor chromosome, initiating recombination with the homolog. The \textit{ade2-4/ade2-2} diploids have additional heterozygous markers on Chr XV to evaluate chromosome loss or LOH for markers on the left or right arm (Ho et al. 2010). The \textit{natMX4} and \textit{hphMX4} markers are inserted 150 kb centromere proximal to the \textit{ade2} locus. We expected heterozygosity for the flanking markers to be retained during BIR. However, if BIR-induced a chromosomal DSB, which initiated recombination between homologs, we would expect a significant fraction of the \textit{Ade}+ chromosomal recombinants to exhibit LOH for the \textit{natMX4} and \textit{hphMX4} markers (Nickoloff et al. 1999; Barbera and Petes 2006; Ho et al. 2010). Seven of 48 (14.6%) white 5-FOA resistant colonies (W/W) derived from pLAG6 exhibited LOH for \textit{Nat} or \textit{Hyg} resistance (Figure 3C). Only 2 of 40 red 5-FOA resistant colonies (W/R) exhibited LOH for \textit{natMX4} or \textit{hphMX4}, but due to the low sample size this is not significantly less than observed for the chromosomal \textit{Ade}+ events (W/W). Because the number of \textit{Ade}+ transformants was low, we also analyzed \textit{Ura}+ \textit{Ade}− transformants to determine whether there was a general increase in LOH involving Chr XV homologs during BIR, regardless of whether template switching or chromosomal recombination occurred within the \textit{ade2} locus. More than 500 \textit{Ade}− \textit{Ura}+ transformants derived from pLAG6 (R) were passaged on 5-FOA to lose the CF and then tested for LOH of the Chr XV markers. Of these, 31 (6.1%) exhibited LOH for \textit{Nat} or \textit{Hyg} resistance, one colony was \textit{Trp}− \textit{Nat}−, indicative of chromosome loss, and one colony was \textit{Met}−. The frequency of \textit{natMX4} or \textit{hphMX4} marker loss was significantly higher for the \textit{Ade}+ colonies due to chromosomal recombination than for \textit{Ade}− BIR events (R) \textit{(P = 0.036)}. \textit{Ura}+ colonies obtained from transformation with pRS416 were also analyzed for marker loss and only 2/1185 tested showed LOH. The significant increase in LOH for the \textit{natMX4} or \textit{hphMX4} markers following transformation with pLAG6 compared with pRS416 \textit{(P < 0.0001)} is consistent with induction of recombination between homologs during BIR.
**Mph1 contributes to template switching during BIR**

During SDSA, the extended invading 3’ end is displaced by helicases and, as proposed previously, displacement of the invading strand could facilitate template switching during BIR (Smith et al. 2007; Llorente et al. 2008). Therefore, we expected the inactivation of helicases that dissociate D-loop intermediates to increase the frequency of BIR, but to decrease the frequency of Ade+ Ura+ transformants. Mph1 has emerged as the main helicase responsible for SDSA in yeast since it is able to unwind Rad51 generated D-loops and DNA polymerase extended D-loops in vitro, and the mph1Δ mutant displays increased crossovers during mitotic recombination (Sun et al. 2008; Prakash et al. 2009; Tay et al. 2010; Sebesta et al. 2011; Mazón and Symington 2013; Mitchel et al. 2013). The percentage of Ade+ Ura+ transformants derived from plAG6 was reduced by 3.9-fold in the mph1Δ mutant as compared to wild type (0.68%; P < 0.0001; Figure 4A). Furthermore, analysis of CF-cured Ade+ Ura+ transformants from the mph1Δ mutant revealed that only 23.8% were due to template switching, 2-fold less than wild type (P = 0.0029; Figure 4B). These results are in agreement with the role of Mph1 in the displacement of D-loops (Figure 5A). To determine whether maintenance of heterozygosity during BIR was affected by the mph1Δ mutation, 542 Ade− Ura+ transformants derived from plAG6 were grown on 5-FOA-containing medium to lose the CF and then tested for loss of Nat or Hyg resistance. The frequency of LOH for the natMX4 and hphMX4 markers was 7.4%, not significantly different from wild type (Figure S1).

**Pif1 promotes BIR but does not affect template switching**

We measured template switching in the pif1-m2 mutant since previous studies have shown a decreased frequency of BIR in pif1 mutants (Chung et al. 2010; Hu et al. 2013; Wilson et al. 2013). The pif1-m2 allele expresses a form of Pif1 that retains mitochondrial activity, but is largely defective for nuclear function (Schulz and Zakian 1994). The percentage of Ade+ Ura+ transformants from the linear CFV leading to an artiﬁcial telomere healing at the telomere-seed sequence of the linear vector, independent Ura+ transformants were analyzed by pulsed-field gel electrophoresis and all were confirmed to have full-length CF products (Figure S2). The heterozygous markers on the left and right arms of Chr XV can be used to monitor half-crossover events as an alternative product to BIR. Surprisingly, of 23 Ade+ events analyzed from the pif1-m2 mutant, only one was consistent with formation by a half crossover; thus, most of the CFs recovered result from BIR and not from half crossovers.

We could not accurately assess the BIR frequency of the pif1-m2 mutant using the plasmid-based assay due to more efficient telomere healing at the telomere-seed sequence of the linear CFV leading to an artiﬁcially high transformation frequency (data not shown) (Schulz and Zakian 1994). To more accurately determine the BIR frequency of the mph1Δ
and pif1-m2 mutants, a chromosomal assay was used. The chromosomal assay is based on reconstitution of a functional *LYS2* gene by BIR (Donnianni and Symington 2013). A recipient cassette with a 3′-truncated *lys2* gene (designated *lysΔ3′*), a 36-bp HO endonuclease cut site (*HO cs*), and *kanMX6* to confer resistance to geneticin (*G418*) was inserted 34 kb from the left telomere of Chr V. There are no essential genes distal to the site of the insertion. A donor cassette composed of *TRP1* and a 5′ truncation of *lys2* (denoted by *5′Δys2*) was inserted 128 kb from the left telomere of Chr I (Figure 4C). The truncated *lysΔ3′* and *5′Δys2* genes share 2.1-kb homology. These donor and recipient cassettes were incorporated into a haploid strain with a deletion of the native *LYS2* locus, with the *MATα-inc* allele to prevent cleavage at the endogenous *HO cs* and which is capable of expressing *HO* from a galactose-regulated promoter. After DSB formation, the *lysΔ3′* sequence invades the donor cassette and copies to the end of Chr I, generating a *Lys+* nonreciprocal translocation product (Figure 4C). The other side of the Chr V DSB lacks homology to sequences in the yeast genome and is degraded, resulting in loss of the *kanMX6* gene and sensitivity to *G418*. The frequency of BIR was determined by the plating efficiency of cells on galactose-containing medium (*HO* constitutively expressed) that are *Lys+ G418* relative to medium with glucose (*HO* off). The BIR frequency was reduced by 1.9-fold in the *pif1-m2* mutant (*P = 0.0076*), whereas the BIR frequency increased by 2.4-fold in the *mph1Δ* background (*P = 0.0002*) (Figure 4D). These results confirm previous studies showing positive and negative roles of *PIF1* and *MPH1*, respectively, during BIR (Luke-Glaser and Luke 2012; Hu et al. 2013; Saini et al. 2013; Wilson et al. 2013).

**MUS81 is not required for BIR-associated template switching or chromosomal LOH**

The increased LOH of the *natMX4* and *hphMX4* markers during BIR is consistent with transfer of the DSB from the CFV to the chromosome to initiate interhomolog recombination. Structure-selective nucleases could cleave the strand invasion intermediate resulting in several possible outcomes. Mus81–Mms4 cleavage of the D-loop, followed by gap filling and ligation, would be predicted to covalently link the CFV to the donor chromosome (Figure 5B). Subsequent cleavage of the front end of the D-loop would result in a half crossover, creating a CF and truncated donor chromosome. The broken chromosome could then engage in BIR with the chromosome homolog, resulting in LOH for *natMX4* or *hphMX4* and the potential to form an Ade+ recombinant (Figure 5B). Alternatively, the truncated chromosome could invade the newly formed CF, retaining heterozygosity for *natMX4* and *hphMX4* after CF loss. The frequency of Ade+ recombinants and the percent of Ade+ events due to chromosomal recombination in the *mus81Δ* mutant were not significantly different from wild type (Figure 6). We also analyzed Ade− Ura+ transformants from the *mus81Δ* mutant after growth on 5-FOA-containing medium to lose the CF; the frequency of LOH for Nat or Hyg resistance was 4.1%, not significantly different from wild type (Figure S1).

The failure to detect a decrease in chromosomal Ade+ recombinants in the absence of *Mus81* could be due to Yen1 cleavage of the BIR intermediate (Blanco et al. 2010; Ho et al. 2010; Agmon et al. 2011; Muñoz-Galván et al. 2012). Because the *mus81Δ yen1Δ* diploid grows very slowly and has low transformation efficiency we were unable to generate sufficient Ura+ Ade+ transformants for meaningful analysis. Furthermore, the spontaneous chromosome instability of the *mus81Δ yen1Δ* resulted in a high frequency of LOH (~2%) even in cells transformed with the replicative plasmid, pRS416, making it difficult to identify events associated with BIR (data not shown). Since the growth defect conferred by *mus81Δ* and *yen1Δ* is less in haploids than in diploids (Ho et al. 2010), we measured the BIR frequency of *mus81Δ* and *yen1Δ* mutants using the haploid chromosomal system. The *mus81Δ* and *yen1Δ*
Presented.

formed the indicated strains using the chromosomal assay. Assays were performed for each strain is shown in parentheses. (C) BIR frequencies of strand from the CFV and one of the chromosome homologs DNA intermediate formed by pairing of an extended single through two rounds of mismatch repair of a heteroduplex Ade+ chromosomal recombinants could potentially occur or Ade+ chromosomal recombination.

Mismatch repair does not in mutations did not affect the BIR frequency and no significant decrease was observed for the mus81Δ transformants (A) and template switching (B) in the mus81Δ mutant transformed with linearized pLAG6. Total number of Ade+ Ura+ events analyzed for each strain is shown in parentheses. (C) BIR frequencies of the indicated strains using the chromosomal assay. Assays were performed five to six times for each strain and mean values with SD are presented.

**Discussion**

It has been previously shown that switching between DNA templates can occur during BIR and if it takes place between ectopic homologous sequences can result in genome rearrangements (Smith et al. 2007; Ruiz et al. 2009). The transformation-based assay using ade2 heteroallelic diploids described here allows simplified detection of template switching events by a change of phenotype. Still, only those events that occur by switching between the ade2-Δ and ade2-Δ mutations (located 0.95 kb apart) are detected as Ade+ recombinants and many template switch events are expected to be genetically invisible. In addition to template switching, we found a high frequency of events in which one of the chromosomal loci became Ade+, reminiscent of earlier studies reporting “tripartite” recombination induced by a DSB on an ectopic template (Ray et al. 1989; Silberman and Kupiec 1994). Here, we investigated the roles of the helicases encoded by MPH1 and PIF1 in template switching associated with BIR.

The Mph1 helicase is known to catalyze dissociation of the invading strand of Rad51-generated D-loops or extended D-loops in vitro and consequently would be predicted to facilitate template switching during BIR (Sun et al. 2008; Prakash et al. 2009; Sebesta et al. 2011). In agreement with a previous study (Luke-Glaser and Luke 2012) we found the frequency of BIR to be increased in the mph1Δ mutant, which we interpret to be due to Mph1-catalyzed dissociation of the invading 3’ end and the need for additional strand invasion events to complete BIR. The percentage of Ura+ transformants that were Ade+ was reduced in the mph1Δ mutant and template switching among the Ade+ events was also decreased, consistent with the need for Mph1 to dismantle the D-loop for template switching. In the mph1Δ mutant, the more stable D-loop intermediate might more readily transition to a migrating D-loop after recruitment of Pif1 (Wilson et al. 2013) or be cut by structure-selective nucleases leading to secondary recombination events. The Ade+ transformants recovered from the mph1Δ mutant were mainly due to chromosomal recombination, consistent with the idea that the stabilized D-loop is cleaved by structure-selective nucleases. If the D-loop were cut by nucleases to create a half crossover, linking the CFV to

Figure 6 Role of structure-selective nucleases in BIR. Percentage of Ura+ Ade+ transformants (A) and template switching (B) in the mus81Δ mutant transformed with linearized pLAG6. Total number of Ade+ Ura+ events analyzed for each strain is shown in parentheses. (C) BIR frequencies of the indicated strains using the chromosomal assay. Assays were performed five to six times for each strain and mean values with SD are presented.

**Mismatch repair does not influence template switching or Ade+ chromosomal recombination**

Ade+ chromosomal recombinants could potentially occur through two rounds of mismatch repair of a heteroduplex DNA intermediate formed by pairing of an extended single strand from the CFV and one of the chromosome homologs 

(Ray et al. 1989; Silberman and Kupiec 1994). Therefore, we tested if eliminating the Mlh1 protein, an essential component of the mismatch repair system, which plays no role in processing branched DNA intermediates (Harfe and Jinks-Robertson 2000), would change either the percentage of Ade+ transformants or the template switching/BIR-associated chromosomal recombination ratio. In both cases, data from the mlh1Δ mutant were the same as with wild type, with 1.78% Ade+ transformants and 47.02% due to template switching (Figure S3), indicating that mismatch repair has no apparent role in template switching or chromosomal recombination during BIR.
sequences downstream of the site of strand invasion, the fragmented chromosome would likely engage with the homolog to repair the one-ended DSB by BIR, resulting in homozygosis of the downstream marker. Indeed, we found that 6.1 and 7.4% of all Ura+ transformants derived from pLAG6 in the wild-type and mph1Δ strains, respectively, exhibited LOH for the natMX4 or hphMX4 markers after loss of the CF, suggesting secondary BIR events between a fragmented Chr XV and its homolog.

The frequency of BIR using a Chr III disome assay is decreased in the pif1Δ mutant and the products recovered are mainly due to half crossovers, similar to the phenotype observed for the pol32Δ mutant (POL32 encodes a nonessential subunit of DNA polymerase δ) (Deem et al. 2008; Smith et al. 2009; Chung et al. 2010; Saini et al. 2013; Wilson et al. 2013). Loss of Pif1 also prevents Rad51-dependent telomere elongation in the absence of telomerase (Hu et al. 2013). In a reconstituted in vitro assay, Pif1 was shown to function with the DNA polymerase δ complex to extend the 3’ end of a Rad51-generated D-loop (Wilson et al. 2013). Pif1 facilitated extensive DNA synthesis by liberating the newly synthesized ssDNA to establish a migrating D-loop. We could not accurately assess the BIR frequency in the pif1-m2 mutant using the CFV system because the increased efficiency of telomere elongation from the minimal telomere seed sequence of the CFV counteracted the decrease in BIR (data not shown).

A more accurate assessment of the BIR frequency was obtained using the chromosomal BIR assay, revealing a two-fold decrease in the pif1-m2 mutant. The pif1-m2 mutant is reported to retain some nuclear function and this might account for the higher BIR frequency than reported using the pif1Δ mutant (Schulz and Zakian 1994; Hu et al. 2013; Wilson et al. 2013). The fraction of Ade+ events among Ura+ transformants was significantly reduced in the pif1-m2 strain, but we did not observe a change in the ratio of template switching and chromosomal recombination associated with BIR, suggesting Pf1 plays no role in D-loop dissociation.

In agreement with a previous study (Silberman and Kupiec 1994), we observed a high frequency of recombination between chromosome homologs induced by the linear transforming DNA. Ray et al. (1989) also reported stimulation of triparental recombination by a chromosomal DSB. In the previous studies, chromosomal conversion was suggested to result from mismatch repair of an hDNA intermediate; however, we observed no effect on the frequency of this class of events in an mhl1Δ mutant. Instead, the high frequency of LOH for the natMX4 and hphMX4 markers is more consistent with transfer of the DSB from the CFV to the chromosome triggering secondary recombination events. A high frequency of LOH for Nat or Hyg resistance was observed for all Ura+ transformants derived from pLAG6, regardless of whether they were Ade+ or Ade−, suggesting a significant number of BIR intermediates are cleaved by structure-selective nucleases. Mus81–Mms4 cleaves model D-loop structures in vitro and could create the first cleavage necessary to form a half crossover with another nuclease cutting the 5’ flap represented by the leading edge of the D-loop to complete the event (Osman et al. 2003). Slx1–Slx4 is able to cleave a variety of branched structures, including 5’ flaps, and Yen1/GEN1 also cuts 5’ flaps in addition to HJs (Fricke and Brill 2003; Rass et al. 2010). The frequency of LOH for total Ura+ transformants (after selection for loss of the CF) from the mus81Δ mutant was not significantly less than observed for wild type, and chromosomal recombination was recovered at a similar frequency to wild type. Thus, Mus81–Mms4 either does not play a major role in formation of half crossovers or Yen1, Rad1–Rad10, and Slx1–Slx4 act redundantly with it. Pardo and Aguilera (2012) reported a decreased frequency of half crossovers to circularize Chr III in the absence of Mus81, Yen1, and Slx1, suggesting these nucleases function redundantly to process branched DNA structures.

Recent studies show BIR occurs by a migrating D-loop mechanism resulting in conservative inheritance of the nascent strands (Donnian and Symington 2013; Saini et al. 2013; Wilson et al. 2013). Structure-selective nucleases are not predicted to function in BIR by this mechanism, but could be required if progression of the D-loop stalls and requires restart by cleavage and reinvagination. Using the chromosomal assay, the frequency of BIR was unaffected in any of the nuclease single mutants, but we did find a significant decrease in the mus81Δ rad1Δ double mutant and a further decrease in the mus81Δ rad1Δ yen1Δ triple mutant, suggesting most of the BIR events detected in this system require cleavage of a branched DNA intermediate. The requirement for Rad1–Rad10 could be to remove the heterology presented by the HO cs sequence that would prevent pairing between the 3’ end and donor template or to cleave at the
heterology boundary if the D-loop intermediate branch migrated to the end of the homology shared by the lys2 sequences (Figure 7) (Fishman-Lobell and Haber 1992). Mus81 is in the same family of nucleases as Rad1 and there is some evidence for overlapping functions to process recombination intermediates between limited homology substrates (Schwartz and Heyer 2011; Mazón et al. 2012). Yen1 could potentially cut the front end of the D-loop or the cross strands resulting from strand invasion. The products of these cleavage events would necessitate a second cycle of BIR to form viable products.

In summary, our data support the hypothesis that Mph1 is the main helicase responsible for template switching during BIR. Unexpectedly, we observed a high frequency of LOH for Chr XV markers located 150 kb downstream of the site of strand invasion during BIR. We suggest these events are due to cleavage of the strand invasion intermediate, transferring the DSB from the linear CFV to the chromosome to initiate secondary BIR events, further increasing the genomic instability associated with this mutagenic DNA repair mechanism.

Acknowledgments

We thank L. Goss, H. Chen, and R. Rothstein for generous gifts of CFVs and yeast strains and members of the Symington lab for discussions and critical reading of the manuscript. This study was supported by a grant from the National Institutes of Health (R01 GM094386). R.A.D. is supported by an International Fellowship in Cancer Research cofunded by Associazione Italiana per la Ricerca sul Cancro and Marie Curie Actions.

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Communicating editor: N. Hollingsworth
Template Switching During Break-Induced Replication Is Promoted by the Mph1 Helicase in Saccharomyces cerevisiae

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Figure S1  Loss of heterozygosity during BIR. Ura' transformants derived from pRS416 or pLAG6 were tested for LOH of the \textit{natMX4} and \textit{hphMX4} markers by direct replica plating (pRS416), or after selection for loss of the CF (pLAG6).
Figure S2  Pulsed field gel electrophoresis (PFGE) analysis of CF and Chr. XV size in wild type and pif1-m2 mutant. Ura⁺ transformants obtained from pLAG6 were analyzed by PFGE and Southern blot hybridization using an ADE2 probe. Independent transformants from wild type (left panel) or pif1-m2 (right panel) are shown; C refers to the untransformed strains.
Mismatch repair is not required for chromosomal conversion. Percentage of Ura<sup>+</sup> Ade<sup>+</sup> transformants (A) and template switching (B) in the *mlh1Δ* mutant. Total number of Ade<sup>+</sup> Ura<sup>+</sup> events analyzed for each strain is shown in parenthesis.