Remarkably Long-Tract Gene Conversion Induced by Fragile Site Instability in *Saccharomyces cerevisiae*

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ABSTRACT Replication stress causes breaks at chromosomal locations called common fragile sites. Deletions causing loss of heterozygosity (LOH) in human tumors are strongly correlated with common fragile sites, but the role of gene conversion in LOH at fragile sites in tumors is less well studied. Here, we investigated gene conversion stimulated by instability at fragile site FS2 in the yeast *Saccharomyces cerevisiae*. In our screening system, mitotic LOH events near FS2 are identified by production of red/white sector colonies. We analyzed single nucleotide polymorphisms between homologs to determine the cause and extent of LOH. Instability at FS2 increases gene conversion 48- to 62-fold, and conversions unassociated with crossover represent 6–7% of LOH events. Gene conversion can result from repair of mismatches in heteroduplex DNA during synthesis-dependent strand annealing (SDSA), double-strand break repair (DSBR), and from break-induced replication (BIR) that switches templates [double BIR (dBIR)]. It has been proposed that SDSA and DSBR typically result in shorter gene-conversion tracts than dBIR. In cells under replication stress, we found that bidirectional tracts at FS2 have a median length of 40.8 kb and a wide distribution of lengths; most of these tracts are not crossover-associated. Tracts that begin at the fragile site FS2 and extend only distally are significantly shorter. The high abundance and long length of noncrossover, bidirectional gene-conversion tracts suggests that dBIR is a prominent mechanism for repair of lesions at FS2, thus this mechanism is likely to be a driver of common fragile site-stimulated LOH in human tumors.

KEYWORDS loss of heterozygosity; gene conversion; BIR; fragile site; homologous recombination

DNA integrity is frequently challenged by chemical, environmental, and physical agents, as well as by endogenous factors. Common fragile sites are one source of endogenous damage, causing DNA instability and breaks under conditions of replication stress when DNA replication is partially inhibited (Durkin and Glover 2007; Sarni and Kerem 2016). Replication stress has typically been induced experimentally by treating cells with the polymerase inhibitor aphidicolin (Glover *et al.* 1984) or by genetically lowering the levels of DNA polymerases (Lemoine *et al.* 2005; Lemoine *et al.* 2008). *In vivo*, replication stress has been observed early in the process of tumor development as a result of alteration of replication fork progression and nucleotide deficiency

caused by activation of oncogenes (Di Micco *et al.* 2006; Bester *et al.* 2011). In cancer cells, human common fragile sites are hotspots for chromosomal deletions, amplifications, and rearrangements (Burrow *et al.* 2009; Drusco *et al.* 2011; Ozeri-Galai *et al.* 2011, 2012), and a large-scale screen of deletions in 746 cancer cell lines reported that many areas of unexplained deletions are in fragile sites (Bignell *et al.* 2010).

The alterations at human common fragile sites in cancer cells are proposed to result from the processes of DNA repair at these sites. Homologous recombination repair pathways are stimulated by DNA breaks, single-strand gaps, and stalled replication forks (Symington *et al.* 2014); all of which have been proposed to be present at common fragile sites (Le Tallec *et al.* 2014). Although these pathways have the potential to accurately repair breaks, there are many opportunities for mutation (Guirouilh-Barbat *et al.* 2014). In particular, all homologous recombination pathways can result in gene conversion, which causes loss of heterozygosity (LOH) (Paques and Haber 1999). In the case of heterozygosity at a tumor-suppressor gene, an LOH event

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can result in loss of the functional allele, driving cancer progression.

In general, homologous recombination begins with 5' to 3' end resection at the break to expose single-stranded DNA (ssDNA) (Symington 2016). Mediator proteins, such as *Rad52p* in yeast, nucleate onto the ssDNA and assist in recruiting *Rad51p* to form a filament that searches for homology. Invasion of the 3' single-stranded tail into a region of homology in an intact duplex forms a D-loop, and at this point, homologous recombination pathways diverge (Mehta and Haber 2014; Symington *et al.* 2014). In canonical double-strand break repair (DSBR), the second 3' tail is captured and a double Holliday junction forms. The junctions are cleaved by a resolvase and, depending on the orientation of cleavage, either a reciprocal crossover (RCO) or noncrossover product is formed. Alternatively, helicase dissolution of the junctions can result in a noncrossover product. Repair of mismatches between the paired strands (Spies and Fishel 2015) or DNA synthesis during the process of repair (Wang *et al.* 2004) can generate areas of gene conversion. In the synthesis-dependent strand annealing (SDSA) pathway, the second 3' end tail is detected, but not fully captured as in DSBR (Jain *et al.* 2009). The intact duplex is used as a template for a short stretch of replication, then the invading strand dissociates and rejoins the other broken end. Again, gene conversion can result. In the break-induced replication (BIR) pathway, the second 3' end is either lost or was not present (such as in breaks that result from replication fork collapse) (Llorente *et al.* 2008). When a second 3' end is not detected, replication initiated by the invading 3' end proceeds to the end of the chromosome (Mehta and Haber 2014; Symington *et al.* 2014). It has been proposed that very long gene-conversion events may result from double BIR (dBIR), in which the homologous chromosome is initially used as a template for DNA replication and then the invading strand dissociates and uses the sister chromatid or the second end of the broken chromosome as a template to complete the replication process (Yim *et al.* 2014).

Previously we reported that homologous recombination resulting in LOH is a frequent outcome of fragile site instability in the yeast *Saccharomyces cerevisiae* (Rosen *et al.* 2013). Using *SUP4-o* as a marker gene to detect LOH events, we reported that instability at the native yeast fragile site FS2 strongly stimulates RCO and BIR events on the right arm of yeast chromosome III. However, because *SUP4-o* in that study was inserted near the telomere of chromosome III—105 kb centromere-distal from FS2—we could not detect local gene-conversion events at FS2 unassociated with a crossover. Here, we inserted *SUP4-o* close to FS2, which allows us to characterize the frequency of gene conversion stimulated by FS2 instability, the proportion of these gene-conversion events that are unassociated with crossover, and the lengths of these gene-conversion tracts.

We report that gene-conversion events unassociated with crossover represent 6–7% of mitotic LOH at the fragile site FS2, which is only a small fraction of all LOH events compared to

BIR, which is a major source of LOH stimulated by FS2 instability. Both unidirectional and bidirectional noncrossover gene-conversion tracts are observed at FS2 in cells under replication stress. The unidirectional tracts at the fragile site are generally very short and could result from SDSA or dBIR. Usually, bidirectional gene-conversion tracts are suggested to result from repair of mismatches between paired strands formed during canonical DSBR, and DSBR in yeast typically results in crossover events (San Filippo *et al.* 2008; Andersen and Sekelsky 2010; Mitchel *et al.* 2010; Symington *et al.* 2014; Yin and Petes 2014). Unexpectedly, we observed that bidirectional tracts across the fragile site are generally not associated with crossover. Also, these bidirectional noncrossover gene-conversion tracts are very long (median length of 40.8 kb) and may be better explained by a mechanism involving DNA replication than by repair of mismatches over a large region. These characteristics of noncrossover association and long length suggest that most bidirectional gene conversions at FS2 result from dBIR rather than canonical DSBR.

Materials and Methods

Strain construction

Four diploid strains were used for experiments: Y657, Y722, AMC355, and AMC358 (Figure 1). Each diploid was created by mating an MS71-derived haploid (Kokoska *et al.* 2000) with a YJM789-derived haploid (Wei *et al.* 2007). Diploid Y657 was created by mating haploids Y655 and Y325. Diploid Y722 was created by mating haploids Y651 and AMC273. Diploid AMC355 was created by mating haploids AMC356 and AMC353. Diploid AMC358 was created by mating haploids AMC350 and AMC342. Genotypes and construction details are in Supplemental Material, Table S1 for MS71-derived haploids and Table S2 for YJM789-derived haploids.

Growth media

All yeast strains were maintained at 30° on standard rich media (Guthrie 1991), with the exception that the medium contained 3% raffinose instead of dextrose. Raffinose was used because this carbon source does not affect the *GAL10* promoter. Galactose was added to the medium [no galactose, low galactose (0.005%), or high galactose (0.05%)] to control expression of the *GAL-POL1* construct. Low levels of galactose reduce *POL1* expression, which lowers the level of polymerase α in the cell, causing replication stress and instability at fragile site FS2 (Lemoine *et al.* 2005).

Induction of instability at FS2 and screening for LOH events

Diploid strains were inoculated in liquid-rich media with raffinose containing high galactose (0.05%) for overnight growth at 30°. Cells were washed with sterile water and resuspended in liquid rich media with raffinose containing either high galactose (0.05%) or no galactose, and the density was adjusted to achieve a starting OD₆₀₀ of 0.25–0.30. Cells were grown for 6 hr at 30°, then spread at low density to

Figure 1 Diploids for analysis of gene-conversion events near fragile site FS2. Four diploid strains were used to detect events that result in LOH near fragile site FS2. The MS71-derived homolog of chromosome III is shown in white and the YJM789-derived homolog of chromosome III is shown in pink. Ty1 elements are represented by black arrows. All diploids are homozygous for *ade2-1*. All diploids contain fragile site FS2 on the MS71-derived homolog of chromosome III. Both experimental diploids are homozygous for the *GAL-POL1* construct that permits induction of replication stress by low levels of polymerase α . Both control diploids are homozygous for the *POL1* gene under its native promoter. Experimental and control diploid #1 are hemizygous for *SUP4-o* inserted 150 bp centromere-distal to FS2. Experimental and control diploid #2 have full-length *ADE2* inserted 150 bp centromere-distal to FS2, and a 5' deletion allele of *ade2* inserted in the corresponding location on the opposite homolog.

achieve 150–350 colonies per plate on synthetic complete medium with low adenine and high galactose (0.05%). The synthetic medium was standard (Guthrie 1991) except that it contained 3% raffinose instead of dextrose, and 10 μ g/ml adenine (twofold less than standard synthetic medium). A total of 40–60 plates were spread per culture. Replicates of two or three independent cultures were done for each diploid under each condition. After 3 days of growth at 30°, plates were transferred to 4° for 24–48 hr to deepen red color development in colonies. Colonies were counted and totaled for each diploid in each condition, and all plates were screened for red/white or red/pink sectors. Any event during the first or second division at the time the diploid is plated that results in LOH at *III170045::SUP4-o* in Y657 and Y722, or LOH at *III170045::ADE2* in AMC355 and AMC358 will produce a sectorized colony in which the red portion is at least one-fourth of the colony. Thus, each such sectorized colony is an independent

event. We isolated a single cell from each half of each sectorized colony, purified it, and harvested genomic DNA for analysis.

Initial classification of events resulting in colony sectoring

In Y657 and Y722, sectorized colonies result both from point mutations in *SUP4-o* and from events that cause LOH at *SUP4-o*. To distinguish LOH from point mutation, we initially screen the red side of each sectorized colony from these strains by PCR using a pair of primers immediately surrounding the site where we inserted *SUP4-o* on chromosome III (Table S3). When *SUP4-o* is present, a 377-bp product is amplified. When the site is in its native state (lacking *SUP4-o*), a 130-bp product is amplified. Experimental diploid #1 and control diploid #1 are hemizygous for the *SUP4-o* insertion and both product sizes are produced. Sectorized colonies that result from point mutation in *SUP4-o* remain hemizygous for the *SUP4-o* insertion, therefore PCR on cells from the red side of these types of sectorized colonies produces both product sizes. Sectorized colonies that result from LOH by chromosome loss, BIR, RCO, or gene conversion have lost *SUP4-o*; thus PCR on cells from the red side of these types of sectorized colonies produces only the 130-bp product size.

Similarly, in AMC355 and AMC358, sectorized colonies result both from point mutations in the *ADE2* allele inserted on chromosome III and from events that cause LOH at this *ADE2* allele. To distinguish LOH from point mutation, we initially screen the red side of each sectorized colony from these by PCR using a pair of primers that produce a 407-bp product when the full-length allele of *ADE2* is present and a 177-bp product when the 5' deletion allele of *ade2* is present (Table S3). Experimental diploid #2 and control diploid #2 are hemizygous for the *ADE2* insertion and both product sizes are produced.

Detailed classification, analysis, and mapping of mitotic LOH events

All sectorized colonies with a change of zygosity indicating LOH at *SUP4-o* or *ADE2* on chromosome III were analyzed at additional polymorphic sites to determine the type and extent of the event responsible for LOH. This testing was done by PCR to amplify polymorphic sites on chromosome III which change a restriction enzyme site. For example, on chromosome III a single nucleotide polymorphism (SNP) at base 266,045 results in an *HpyCH4III* site on the YJM789-derived chromosome but not on the other homolog. We amplify the region by PCR, generating a 374-bp product (Table S3). If the site is heterozygous in the cell being examined, digestion of the amplified product with *HpyCH4III* followed by gel electrophoresis reveals three band sizes: the uncut 374-bp product and the cut 259-bp and 115-bp products. Primers and diagnostic restriction enzymes for all polymorphic sites tested on chromosome III are in Table S3. SNPs at bases 112,760 and 298,875 were the first SNPs analyzed for each sectorized colony. SNP 112,760 is on the left arm of chromosome III close to the centromere. Fragile site FS2 and the marker gene,

whether *SUP4-o* or *ADE2*, are on the right arm of chromosome III. SNP 298,875 is the most distal polymorphic site on the right arm of chromosome III. If these two SNPs remain heterozygous in both the red and white sides of the colony, the sectored colony is classified as a noncrossover gene-conversion event. If both SNPs remain heterozygous in the white side, and both SNPs are homozygous for the YJM789 allele in the red side, the sectored colony is classified as a chromosome loss event. If both SNPs remain heterozygous in the white side, and in the red side SNP 112,760 remains heterozygous while SNP 298,875 is homozygous for the YJM789 allele; the sectored colony is classified as a BIR event. If SNP 112,750 remains heterozygous on both the red and white sides, and SNP 298,875 is homozygous for the MS71 allele in the white site and homozygous for the JYM789 allele on the red side; the sectored colony is classified as RCO. Sectored colonies that were classified as noncrossover gene-conversion events or RCO events were analyzed using 30 additional polymorphic sites on the right arm of chromosome III to map the extent of the event (Table S3). Using this mapping information, the length of gene-conversion tracts was calculated using the same approach as Lee *et al.* (2009).

Statistical analysis

VassarStats was used to calculate 95% C.I. for the proportion (Newcombe 1998) of each type of mitotic event, for chi-square contingency tables to evaluate the significance of the difference between event frequencies, and for *t*-tests to compare the distributions of gene-conversion tract lengths.

Data availability

Strains are available upon request. Table S1 and Table S2 contain descriptions of all genotypes. Table S3 contains primers and diagnostic restriction enzymes for all polymorphic sites tested.

Results

Experimental system for identification and analysis of gene-conversion events near fragile site FS2

We constructed diploids to employ a screening system in which LOH in a mitotic division at the time of plating results in a red/white or red/pink sectored colony (Barbera and Petes 2006; Lee *et al.* 2009). We used this system previously to study RCO, BIR, and chromosome loss stimulated by instability at yeast fragile site FS2 (Rosen *et al.* 2013). Here, we have created diploid strains for the purpose of analyzing gene conversion at FS2 that is unassociated with crossover (Figure 1). All of these diploids are homozygous for the *ade2-1* allele in its native location on chromosome XV. This mutation is an ochre stop codon, and *ade2-1* yeast is red in color due to buildup of a red intermediate in the adenine biosynthetic pathway. Each experimental diploid is isogenic with its paired control diploid, except that the experimental diploids are homozygous for a construct that places the *POL1* gene under control of the *GAL10* promoter, while the control diploids

have *POL1* under their native promoters. The *GAL-POL1* construct enables us to place cells under replication stress, which stimulates instability at FS2. Cells grown in high-galactose medium (0.05%) have 300% of the normal amount of Pol1p, and those in low-galactose medium (0.005%) have 10% of normal Pol1p levels (Lemoine *et al.* 2005). All diploids are hemizygous for FS2: one homolog of chromosome III carries the pair of Ty1 elements in inverted orientation that have been characterized previously as fragile site FS2 (Lemoine *et al.* 2005), and the other homolog has a single Ty1 element in this location, in Crick orientation. Experimental diploid #1 and control diploid #1 both are hemizygous for *SUP4-o* inserted 150 bp centromere-distal to fragile site FS2. *SUP4-o* is an ochre suppressor transfer RNA (tRNA). In our system, diploids with one copy of *SUP4-o* are light pink and those with two copies are white. Experimental diploid #2 and control diploid #2 use the *ADE2* gene instead of *SUP4-o* as a marker for screening (Figure 1). Experimental diploids #1 and #2 and control diploids #1 and #2 are isogenic; however rather than *SUP4-o*, we inserted *ADE2* with its native promoter 150 bp centromere-distal to fragile site FS2. In comparison to the ~100-bp *SUP4-o* insertion in diploid #1, the *ADE2* insertion in diploid #2 is ~2 kb. To avoid differences in recombination resulting from the differing amounts of nonhomologous sequence inserted, in diploid #2 we inserted an *ade2* allele lacking its promoter and lacking the first 36 bp from the 5' end at the corresponding location on the YJM789-derived homolog of chromosome III.

After incubation in either replication-stress or nonstress conditions, cells are plated for single colonies. After growth, plates are screened for red/white or red/pink sectors. Gene-conversion tracts crossing *SUP4-o* or crossing *ADE2*, which form during a mitotic division at the time of plating, result in a sectored colony (Figure 2). Point mutation, loss of chromosome III, and BIR or RCO that cause LOH at *SUP4-o* or *ADE2* in a mitotic division at plating also result in sectoring (Figure 2) (Rosen *et al.* 2013).

Classification of events that result in sectoring

For analysis, a single cell is purified from each side of the sectored colony. We use PCR across the *SUP4-o* insertion site or *ADE2* insertion site to distinguish point mutation events from LOH events. In cells from sectored colonies from diploid #1, we used a pair of primers (Table S3) that generate two products (377 bp and 130 bp) when *SUP4-o* is hemizygous on chromosome III; in the case of LOH both copies of chromosome III in the red side of the sectored colony lack *SUP4-o* and only a 130-bp product is amplified. Similarly, in cells from diploid #2, we used a pair of primers (Table S3) that generate two products (407 bp and 177 bp) when chromosome III is heterozygous for both the full-length *ADE2* allele and the 5' deletion allele of *ade2*; in the case of LOH both copies of chromosome III in the red side of the sectored colony carry the 5' deletion allele of *ade2* and only a 177-bp product is amplified. Each sectored colony that resulted from an LOH event was further characterized through analysis of SNPs

Figure 2 Sectored colonies result from events that cause LOH near FS2. In our experimental system, LOH resulting from point mutation, gene conversion, RCO, BIR, and chromosome loss cause sectored colony formation (Rosen *et al.* 2013). In this figure the MS71-derived homolog of chromosome III is shown in white and the YJM789-derived homolog of chromosome III is shown in pink. (A) In experimental diploid #1 and control diploid #1: any gene-conversion event in which the homolog that does not contain *SUP4-o* is used as a template for copying, and which results in LOH at the *SUP4-o* locus at the time of plating, produces a pink/red sectored colony. (B) In experimental diploid #2 and control diploid #2: any gene-conversion event in which the homolog that does not contain the full-length *ADE2* is used as a template for copying, and which results in LOH at the *ADE2* locus at the time of plating, produces a white/red sectored colony. (C) An RCO event is diagrammed in diploid #1. In all diploids, a crossover that occurs centromere-proximal to the marker gene (*SUP4-o* or *ADE2*) will produce a sectored colony. (D) A BIR event is diagrammed in diploid #1. In all diploids, BIR that is initiated by a lesion on the chromosome III homolog carrying the fragile site and in which invasion of the opposite homolog occurs at a location centromere-proximal to the marker gene (*SUP4-o* or *ADE2*) will produce a sectored colony. (E) A chromosome-loss event is diagrammed in diploid #1. In all diploids, loss of the chromosome III homolog carrying the fragile site will produce a sectored colony.

between the homologous chromosomes. Our diploids were constructed by mating a YJM789-derived haploid with a haploid related to S288c; these haploids have $\sim 0.5\%$ sequence divergence (Wei *et al.* 2007). Using SNPs that change a restriction enzyme site, we mapped and classified LOH events through PCR and digested at 32 polymorphic sites on the right arm of chromosome III (Figure 3 and Table S3) (Rosen *et al.* 2013).

Frequency of gene-conversion events in cells under replication stress

The fragile site FS2 on yeast chromosome III is unstable in cells with low levels of DNA Pol1p, the catalytic subunit of polymerase α (Lemoine *et al.* 2005). Previously, we reported that breaks at FS2 are frequently repaired by homologous recombination resulting in LOH by BIR or RCO; using strains in which the reporter gene *SUP4-o* is inserted close to the telomere of chromosome III, 105 kb centromere-distal of FS2 (Rosen *et al.* 2013). However, because the placement of *SUP4-o* in this system is far away from FS2, we were not able to detect gene conversions at the fragile site that are unassociated with crossover. Here, in experimental diploid #1 we have inserted *SUP4-o* 150-bp centromere-distal to FS2, so that with this marker nearby we are able to detect and evaluate such gene-conversion events. We grew experimental diploid #1 in no galactose (replication stress conditions) to lower the level of DNA Pol1p and induce fragile site instability, then screened 10,896 colonies for sectoring. For each strain in each condition, the combined total frequency of LOH was calculated as: (sectored colonies due to BIR + sectors from chromosome loss + sectors from gene conversion unassociated with crossover + $2 \times$ sectors from RCO) $\div$ total colonies screened. RCO sectors were

doubled in this calculation because we can only detect crossovers that segregate both recombinant chromosomes into the same cell; however, the two possible segregation patterns are equally frequent (Chua and Jinks-Robertson 1991). The overall frequency of LOH in experimental diploid #1 in no galactose (replication-stress conditions) in mitosis was 2395×10^{-5} events per cell division (95% C.I. 2120–2690) (Figure 4 and Table 1). This frequency is 92-fold higher than the overall frequency of LOH in control diploid #1 in no galactose ($P < 0.0001$), an isogenic strain that has the native promoter on *POL1* ($n = 27,445$ colonies screened). The overall frequency of LOH in mitosis in experimental diploid #2 in no galactose (replication stress) conditions was similar to experimental diploid #1 ($P = 0.11$). High galactose conditions have been previously reported to cause some instability at FS2 (Lemoine *et al.* 2005), likely because the excess Pol1p present causes slight replication stress by interference with polymerase α holoenzyme formation. Consistent with this, we observe a modest increase in mitotic LOH in experimental diploid #1 when grown in high galactose ($P < 0.0001$) (Figure 4 and Table 1).

Combining gene conversions both with and without associated crossover; the frequency of LOH in mitosis due to gene conversion in experimental diploid #1 under no galactose conditions (replication stress) was 192×10^{-5} events per cell division (95% C.I. 120–290), and in experimental diploid #2 under replication stress was 250×10^{-5} events per cell division (95% C.I. 180–350) (Table 1). This is 48-fold and 62-fold higher, respectively, than the frequency of LOH due to gene conversion observed in control diploid #1 ($P < 0.0001$). However, in both experimental diploids under replication stress, gene conversion (both crossover- and non-crossover-associated) represents 8–9% of the mitotic LOH

Figure 3 Use of SNPs to map the location of mitotic recombination events. SNPs between the two homologs of chromosome III that alter restriction sites were used to evaluate the type of event responsible for sectoring and to map the location of each event. Experimental diploid #1 is shown. The gray chromosome represents the MS71-derived homolog and the red chromosome represents the YJM789-derived homolog. Centromeres are represented by large ovals and SNP sites by small ovals. FS2 is indicated by yellow bands and *SUP4-o* is represented by a gray rectangle. This strain is homozygous for the ochre-suppressible *ade2-1* mutation. (A) A BIR event that is stimulated by a lesion at FS2 is shown. The YJM789-derived homolog is used as a template for repair. After chromosome segregation in mitosis, the light pink cell remains heterozygous at all SNPs, while the red cell is homozygous for the YJM789 form of all SNPs distal to the invasion site. (B) A gene-conversion tract associated with RCO that occurs due to repair of a lesion at FS2 in S or G₂ phase is shown. The crossover location is indicated by a black X. Transfer of genetic information from the YJM789-derived homolog during repair resulting in 3:1 gene conversion is shown in the yellow box. After chromosome segregation in mitosis, the light pink cell is homozygous for the MS71 version of SNPs distal to the crossover, while the red cell is homozygous for the YJM789 form of SNPs distal to the crossover, and SNPs within the region of gene conversion are homozygous in the red cell but heterozygous in the light pink cell. If a crossover occurs at a more centromere-proximal location such that the *SUP4-o* gene is not included in a 3:1 conversion tract (not shown here), then two copies of *SUP4-o* could be segregated into the same cell during mitosis; resulting in red/white sectoring instead of red/light pink sectoring. (C) A noncrossover gene-conversion tract stimulated by a lesion at FS2 in S or G₂ phase is shown. Transfer of genetic information from the YJM789-derived homolog during repair resulting in 3:1 gene conversion of SNPs shown in the yellow box. After chromosome segregation in mitosis, the SNPs within the region of gene conversion are homozygous in the red cell but heterozygous in the light pink cell. Both the red cell and the light pink cell are heterozygous for SNPs centromere-distal to the gene-conversion tract.

observed; indicating that this is a more minor category of events stimulated by fragile site instability compared to the BIR and chromosome loss categories. In strains under replication stress, we observed more conversions unassociated with crossover than crossover-associated conversions (Figure

Figure 4 LOH and gene-conversion events on chromosome III are increased under conditions of replication stress. Experimental diploids #1 and #2 contain the *POL1* gene under control of the *GAL1/10* promoter, therefore growth of these strains in no-galactose medium results in replication stress. Control diploids #1 and #2 have the *POL1* gene under its native promoter, therefore growth of these strains in no-galactose medium does not cause replication stress. — indicates no replication stress, + indicates a low level of replication stress, and +++ indicates high levels of replication stress. (A) Total frequency of all events causing LOH on the right arm of chromosome III per mitotic division. The combined total frequency of LOH includes colonies observed due to BIR, chromosome loss, gene conversion unassociated with crossover, and RCO. Error bars represent 95% C.I. (B) Frequency of gene conversion on the right arm of chromosome III resulting in LOH during mitosis. Error bars represent 95% C.I. Gal, galactose; GC, gene conversion.

4 and Table 1). For each strain in each condition, the frequency of gene conversion unassociated with crossover was calculated as: total number of sectored colonies observed due to gene conversion unassociated with crossover ÷ total colonies screened. Gene conversion with crossover frequency was calculated as: (total number of sectored colonies observed due to gene conversion with crossover × 2) ÷ total colonies screened.

Locations and characteristics of gene-conversion tracts in cells under replication stress

We mapped and analyzed the locations of all gene-conversion events that were detected ($n = 136$) using 32 polymorphic sites on the right arm of chromosome III (Figure 5 and Table S3). From experimental diploid #1 under replication stress, there were 54 gene-conversion events (20 detected in the initial experiment, plus 34 collected among another set of

Table 1 Gene conversion and other events causing LOH in mitosis as a result of instability at fragile site FS2

Strain ^a	Description	Treatment	Replication stress level	Total colonies screened	Total sectored colonies	Frequency of mitotic events causing LOH ($\times 10^{-5}$)				
						All LOH events	Crossover ^b	Gene conversion ^c	BIR ^d	Chr loss ^e
Control diploid #1 (Y722)	Native <i>POL1</i> , isogenic with Y657	No gal	-	n = 27,445	n = 7	26 (20–60) [11] ^f	0 (0–10) [1]	4 (0–10) [1]	0 (0–10) [1]	22 (10–50) [1]
Experimental diploid #1 (Y657)	<i>GAL-POL1</i> , <i>SUP4-o</i> near FS2	High gal	+	n = 41,472	n = 86	207 (170–260) [8]	24 (10–40) [5]	41 (20–60) [10]	77 (60–110) [15]	65 (50–100) [3]
Experimental diploid #1 (Y657)	<i>GAL-POL1</i> , <i>SUP4-o</i> near FS2	No gal	+++	n = 10,896 ^g	n = 260	2395 (2120–2690) [92]	18 (10–70) [4]	174 (110–270) [44]	1019 (850–1230) [204]	1184 (990–1400) [54]
Control diploid #2 (AMC358)	Native <i>POL1</i> , isogenic with AMC355	No gal	-	n = 43,591	n = 1	2 (0–10) [0.1]	0	0	0	2 (0–10) [0.1]
Experimental diploid #2 (AMC355)	<i>GAL-POL1</i> , <i>ADE2</i> near FS2	No gal	+++	n = 12,416	n = 339	2730 (2470–3040) [105]	97 (70–190) [19]	153 (100–240) [38]	1442 (1240–1650) [288]	1039 (880–1230) [47]

– indicates no replication stress; + indicates a low level of replication stress, and +++ indicates high levels of replication stress. Chr, chromosome; gal, galactose.

^a All diploids result from mating a MS71-derived haploid (Kokoska *et al.* 2000) with a YJM789-derived haploid (Wei *et al.* 2007). Each haploid is isogenic with its parent strain, except for changes introduced by transformation (described in Table S1 and Table S2).

^b The frequency of RCOs was calculated as $2 \times$ (number of crossover events $\div$ total colonies). All of the crossovers that we observed were associated with gene conversion.

^c Only gene-conversion events unassociated with crossover are reported here.

^d Only BIR events that result from a break on the chromosome containing the *SUP4-o* allele or *ADE2* allele can be detected as a red/light pink or red/white sectored colony. Therefore, the BIR reported here results from breaks on the MS71-derived chromosome III homolog.

^e Only loss of the chromosome containing the *SUP4-o* allele or *ADE2* allele can be detected as a red/light pink or red/white sectored colony. Therefore, the loss here is of the MS71-derived chromosome III homolog.

^f The number in parenthesis is the 95% C.I. The number in square brackets is fold change from Y722 in no galactose.

^g In the low galactose treatment of Y647, 10,896 colonies were fully analyzed for crossovers, BIR, and chromosome loss. An additional 28,083 colonies were screened only to identify and collect additional gene conversion and crossover events and are not included in this frequency calculation.

28,083 colonies) and 22 events from this diploid when grown in high galactose. From experimental diploid #2 under replication stress there were 25 gene-conversion events. There was only one gene conversion detected from control diploid #1, and no gene-conversion events were detected from control diploid #2 (Figure 5).

All of the gene-conversion tracts from all strains were 3:1 (similar to the examples in Figure 3), except for one tract. The one unusual tract contained one SNP that was homozygous for the YJM789 form of the SNP in both the red side and the light pink side of the sector, next to a 3:1 region (Figure 5). Hybrid 3:1/4:0 conversion tracts of this type have been reported previously and are proposed to result from repair of two double-strand breaks that are generated when a chromosome is broken during G₁, then replicated during S phase to form two broken chromatids, both of which are repaired during S or G₂ by homologous recombination (Lee and Petes 2010). In contrast, 3:1 gene-conversion tracts are proposed to result from lesions formed during S phase on one of the two sister chromatids (Lee and Petes 2010). Therefore, the gene-conversion tracts we observed appear to result from lesions during S phase. All but two gene conversions had three copies of the sequence from the YJM789 homolog; indicating that they were initiated by a break on the MS71 homolog, which is the homolog containing fragile site FS2 (Nag and Petes 1990; Nickoloff *et al.* 1999; Merker *et al.* 2003). All of the crossover events we detected were associated with gene conversion. Of the noncrossover gene-conversion tracts at FS2 in cells under replication stress (no galactose): 42% ($n = 27$) were unidirectional, with one endpoint at the fragile site FS2 and extending only distally; and 58% ($n = 37$) were bidirectional, extending on both sides of the fragile site (Figure 5). Unidirectional noncrossover tracts extending only proximal to the fragile site cannot be detected in our system.

Of the 54 gene-conversion tracts collected from experimental diploid #1 under replication stress (no galactose), 16 (30%) had one endpoint between the *SUP4-o* marker inserted at base 170,045 on chromosome III and the next nearest centromere-distal SNP at base 175,324 (Figure 5). Similarly, of the 22 gene-conversion tracts collected from experimental diploid #1 in high galactose, eight (36%) had one endpoint at *SUP4-o* (Figure 5). The abundance of tracts with one endpoint at *SUP4-o* may simply result from selection for these events using *SUP4-o*, since the shortest tracts that can be detected in our system are those that cross this gene. Alternatively, *SUP4-o* may be specifically acting as an initiator or terminator of gene-conversion tracts. In yeast, tRNA genes can cause replication fork pausing (Deshpande and Newlon 1996; Dubarry *et al.* 2011), and can act as chromatin silencing barriers or insulators (Simms *et al.* 2004; Simms *et al.* 2008). tRNA gene sites are weakly recombinogenic when RNAP III collides with DNA polymerase (de la Loza *et al.* 2009); and when essential replication genes are downregulated, Ty retrotransposons and tRNA genes are frequently found together at the breakpoints of chromosome rearrangements

Figure 5 Gene conversions are stimulated by instability at fragile site FS2 during S phase. The MS71-derived homolog of chromosome III is shown in gray and the YJM789-derived homolog of chromosome III is shown in red. SNP markers used to map events are shown by ● and ▼ on the chromosome diagrams. ▼ indicates a restriction site exists, ● indicates lack of the site. Numbers are the approximate chromosome coordinate in kb. On the chromosome diagrams, ovals represent the centromere and black arrows represent Ty1 elements. The yellow band extending through all parts of the figure highlights the position of the fragile site and the marker gene. Gene-conversion tracts are represented by horizontal lines. Line color indicates which homolog was copied during gene conversion. Thin horizontal lines indicate 3:1 conversion tracts and thick lines indicate 4:0 tracts. Lines with an X at the end indicate crossover-associated gene conversions; we did not observe any crossover events without associated gene conversion. Lines lacking an X at the end are gene conversions that are unassociated with crossover. The initiation and termination of each gene-conversion tract are depicted in the middle between the closest flanking SNPs, but the actual location can be anywhere between the flanking SNPs. Tracts for each strain are shown in three groupings: crossover-associated tracts, noncrossover tracts with one endpoint at FS2, and noncrossover tracts that extend on both sides of FS2. (A) Locations of 54 gene conversions collected in experimental diploid #1 under replication stress (no galactose). These gene-conversion events were collected in two ways: 20 events were collected among the colonies in Table 1, and 34 events were collected among another set of 28,083 colonies. (B) Locations of 22 gene conversions collected in experimental diploid #1 in high galactose. (C) Location of the one gene conversion collected in control diploid #1 in no galactose. (D) Locations of the 25 gene conversions collected in experimental diploid #2 under replication stress (no galactose).

(Cheng *et al.* 2012). To evaluate the potential effect of *SUP4-o* either initiating or terminating gene-conversion tracts in our system, we compared these results to experimental diploid #2, in which we replaced the *SUP4-o* marker on chromosome

III with *ADE2*. There is no statistical difference in the frequency of mitotic gene-conversion tracts detected in experimental diploid #2 in no galactose (replication stress) conditions compared to experimental diploid #1 ($P = 0.8625$), suggesting that *SUP4-o* does not initiate gene conversions in our system. In experimental diploid #2 under replication stress (no galactose), 8 of the 25 tracts (32%) had one endpoint between the *ADE2* marker and the next nearest centromere-distal SNP at base 175,324 (Figure 5). There is no statistical difference between the proportion of tracts with one end at *SUP4-o* and the proportion of tracts with one end at *ADE2* ($P = 0.8415$), suggesting that *SUP4-o* does not terminate gene conversions in our system. We also evaluated an additional SNP at base 171,878 and found that 7 of the 24 tracts from experimental diploid #1 are homozygous for the YJM789 homolog at this SNP, indicating that these 7 tracts extend beyond this SNP and thus do not have an endpoint at *SUP4-o*. Therefore, the *SUP4-o* marker in our experimental system does not impact the extent of gene-conversion tracts, and results from our experimental diploids #1 and #2 can be evaluated jointly when characterizing gene conversion stimulated by FS2 instability.

Length of gene-conversion tracts in cells under replication stress

For each gene conversion, we calculated tract length as the average between the minimum possible size (distance from the first to last SNP included in the tract) and the maximum possible size (distance between the two nearest outside SNPs). The longest possible tract we can detect is 184 kb, the distance between our first and last SNPs on the right arm of chromosome III.

In cells under replication stress (no galactose), uni- and bidirectional noncrossover gene-conversion tracts differ significantly in length. Bidirectional noncrossover tracts, that is, tracts that extend on both sides across the fragile site, have a median length of 40.8 kb and a wide distribution of lengths (interquartile range of 29.2–78.8 kb, $n = 37$). There are no “hotspots” for the beginning or end of bidirectional tracts; and for each individual tract, the lengths extended to either side of the fragile site are unequal. Unidirectional tracts, that is, tracts that begin at the fragile site FS2 and extend only distally, are significantly shorter with a median length of 3.9 kb and a tighter distribution (interquartile range 3.9–16.8 kb, $n = 27$, $P < 0.0001$, t -test assuming unequal sample variances) (Figure 6). Of these 27 unidirectional tracts initiated at the fragile site, 17 (63%) were the shortest possible length that can be detected in our system; meaning that these tracts crossed only FS2 and the *SUP4-o* or *ADE2* marker gene.

Crossover-associated gene-conversion tracts in cells under replication stress were also significantly shorter in length than bidirectional noncrossover tracts, with a median length of 11.9 kb (interquartile range 6.2–23.4 kb, $P < 0.0001$, t -test assuming unequal sample variances) (Figure 6).

Ratio of crossover and noncrossover gene conversions

Only RCOs that segregate both recombinant chromosomes into the same cell can be detected in our system. However, the

Figure 6 Bidirectional noncrossover gene-conversion tracts are longer than unidirectional noncrossover tracts. (A) Comparison of tract length distribution, divided by type of tract, strain, and treatment. Bidirectional noncrossover tracts are those that extend on both sides of fragile site FS2. Unidirectional noncrossover tracts are those that begin at the fragile site FS2 and extend only centromere-distally. All gene-conversion tracts from experimental diploids #1 and #2 are plotted. Each round dot represents one tract. The length of each gene conversion was calculated as the average between the minimum possible size (distance from the first to last SNP included in the tract) and the maximum possible size (distance between the two nearest outside SNPs). (B) All noncrossover-associated gene-conversion tracts, both uni- and bidirectional ($n = 83$), binned by 5-kb length increments. GC, gene conversion.

alternate segregation pattern is equally frequent (Chua and Jinks-Robertson 1991). Thus, we make the assumption that the true number of crossover events is double what we detected in our screen. Of the 54 gene-conversion events collected from experimental diploid #1 grown in no galactose (replication-stress conditions), 8 were associated with crossover and 46 were

noncrossover. Thus, 26% of gene conversions were associated with crossover $[(8 \times 2)/(54 + 8)]$ and 74% were noncrossover. Of the 22 gene conversions from experimental diploid #1 grown in high galactose, 37% were crossover and 63% were noncrossover. Of the 25 gene conversions from experimental diploid #2 in no galactose, 39% were crossover and 61% were noncrossover.

Discussion

Replication stress from low levels of polymerase α causes breaks at the native yeast fragile site FS2, located on the right arm of yeast chromosome III (Lemoine *et al.* 2008). Instability at FS2 stimulates both RCOs and BIR events during mitosis (Rosen *et al.* 2013). This study is the first to detect and analyze mitotic gene-conversion events at FS2 that are unassociated with crossover. Our key findings are: (1) gene conversion is a relatively minor class of LOH events at FS2, (2) most (78%) of the unidirectional noncrossover tracts initiated at FS2 are the shortest possible length detectable in our screening system, and (3) bidirectional noncrossover tracts that cross FS2 are generally very long. These findings are discussed below.

Types of mitotic LOH stimulated by instability at fragile site FS2

In cells under replication stress resulting from low levels of polymerase α , the most frequent events responsible for mitotic LOH at fragile site FS2 are chromosome loss and BIR. Our data indicate that gene-conversion events unassociated with crossover account for 6–7% of mitotic LOH events stimulated by FS2 instability; and crossover events, both with and without gene conversion, are similarly infrequent. BIR is the homologous recombination repair pathway employed by yeast cells to repair one-end double-strand breaks (Llorente *et al.* 2008). Repair pathways that result in gene conversion, with or without associated crossover, typically require a second end. The fragile site FS2 consists of a pair of inverted Ty1 elements spaced ~ 280 bp apart (Lemoine *et al.* 2005). Under conditions of replication stress resulting from low levels of polymerase α , extended ssDNA on the lagging strand at the replication fork permits intrastrand base pairing between the two Ty1 elements forming a hairpin secondary structure (Figure 7). It is hypothesized that the double-strand breaks that have been observed at FS2 form as a consequence of hairpin cleavage by a nuclease (Lemoine *et al.* 2005; Casper *et al.* 2009; Rosen *et al.* 2013). Since our data indicate that instability at FS2 stimulates BIR much more than gene conversion, cleavage at the fragile site must typically collapse the replication fork to a one-end double-strand break (Figure 7). The replication origin closest to FS2 is **ARS 310**, which is located ~ 1 kb centromere-proximal to the fragile site. This origin initiates replication relatively early in 90% of cell cycles (Poloumienko *et al.* 2001). Centromere-distal to FS2, the nearest highly efficient replication origin is **ARS 315**, located ~ 55 kb away; and **ARS 313**, located ~ 25 kb distal, initiates

replication in only 10% of cell cycles (Poloumienko *et al.* 2001). Therefore under replication stress, a fork proceeding from *ARS 310* permits hairpin formation on the lagging strand when it reaches FS2, and cleavage of this hairpin generates a one-end double-strand break (Figure 7).

We propose that replication fork dynamics can explain relative infrequency of gene conversions at FS2. The converging replication fork from *ARS 315* (or perhaps *ARS 313*) must travel a relatively long distance and the low polymerase α conditions will slow the movement of this fork. Thus after replication fork collapse at FS2, there is likely to be a lag until a converging replication fork reaches the location of the break to form a second end, thus promoting BIR (Mayle *et al.* 2015). Alternatively, the replication fork from *ARS 310* may not collapse but instead proceed, leaving a single-strand gap on the lagging strand at the hairpin. In this case, template switching may be used to fill the gap, which results in gene conversion if the homologous chromosome is used as the template (Figure 7) (Carr and Lambert 2013; Rosen *et al.* 2013; Symington *et al.* 2014).

Unidirectional noncrossover gene-conversion tracts at FS2 are short

Gene-conversion tracts are typically reported to result from one of two homologous recombination pathways, either SDSA or canonical DSBR. The SDSA pathway results in only noncrossover events with gene conversion proceeding unidirectionally from the break (Figure 7). The DSBR pathway has the potential to result in either crossover or noncrossover events; but in yeast the outcome of DSBR is primarily RCO events, with gene conversion extending bidirectionally from the break (Mitchel *et al.* 2010; Yin and Petes 2014). In the models for these pathways, gene conversion results from mismatch repair of heteroduplex DNA (hDNA), and the unidirectional tracts from SDSA are expected to be shorter than bidirectional tracts from DSBR (Yin and Petes 2014).

The shortest tract that our system has the potential to detect is one that crosses only the marker gene (*SUP4-o* or *ADE2*). No tracts of this type were detected. The next shortest length that is possible to detect is one that crosses the marker gene plus one neighboring polymorphism. The nearest polymorphism is the Watson-orientation Ty1 of fragile site FS2, located centromere-proximal to the marker gene. We detected 17 noncrossover gene-conversion tracts in cells under replication stress (no galactose) that cross only the marker gene and the Watson-orientation Ty1. Thus, for these 17 tracts the initiating lesion is either at the marker gene or at the fragile site. In our comparison of the two experimental strains with different marker genes, we found no statistical difference between the proportion of tracts with one end at *SUP4-o* and the proportion of tracts with one end at *ADE2*; thus it is unlikely that either of these marker genes is a hot-spot for initiation or termination of gene-conversion tracts. We assume the 17 tracts that cross only the fragile site and the marker gene are unidirectional tracts that were initiated by a lesion at the fragile site. These tracts could be as short as

~161 bp, the distance from FS2 to the edge of the marker gene; or as long as ~5 kb, the distance from the fragile site to the nearest SNP on the opposite side of the marker gene. Ten additional noncrossover tracts that initiate at FS2 were detected, and the median length of these tracts was 18.7 kb. Thus in agreement with models of homologous recombination in the literature, unidirectional gene-conversion tracts are typically shorter than bidirectional tracts. If the replication fork does not collapse at FS2, these unidirectional tracts may result from postreplicative template switching to the homologous chromosome to bypass the hairpin on the lagging strand (Symington *et al.* 2014). The length of the region of bypass would be expected to be constrained to the length of an Okazaki fragment. If the replication fork does collapse, these unidirectional tracts likely result from SDSA, or as explained below, dBIR (Figure 7).

Bidirectional noncrossover gene-conversion tracts at FS2 are very long

Gene-conversion tracts that extend bidirectionally from a break are most often presented as resulting from hDNA formed during canonical DSBR (San Filippo *et al.* 2008; Andersen and Sekelsky 2010; Mitchel *et al.* 2010; Symington *et al.* 2014; Yin and Petes 2014). Although DSBR has the potential to result in either crossover or noncrossover events, in yeast the outcome of DSBR is primarily RCO (Mitchel *et al.* 2010). Therefore, we predicted that bidirectional gene conversions at the fragile site would be generally associated with crossovers; however, we observed the reverse—many more bidirectional noncrossover tracts than crossover-associated tracts. We also predicted that bidirectional noncrossover and crossover-associated tracts would have a similar distribution of lengths; instead, we observed that bidirectional noncrossover tracts are significantly longer than crossover-associated tracts.

Long mitotic gene-conversion tracts have been suggested to result from template switching during BIR, also called dBIR (Figure 7) (Llorente *et al.* 2008; Yim *et al.* 2014). Several studies have demonstrated that the invading 3' strand during BIR can be displaced and, after displacement, can reinvade at a region of homology and establish a second BIR process (Smith *et al.* 2007; Ruiz *et al.* 2009). In the dBIR mechanism, gene conversion results not from hDNA but rather from the amount of DNA synthesis during BIR completed prior to the template switch. Previously we demonstrated that BIR is strongly stimulated in cells under replication stress, and that many of these BIR events are initiated by breaks at FS2 (Rosen *et al.* 2013). BIR events can initiate centromere-proximal to the original break location due to extensive 5' to 3' end resection at DNA breaks to expose ssDNA (Chung *et al.* 2010; Symington 2016).

Both the extensive lengths and high abundance of bidirectional noncrossover tracts we observed can be explained if these tracts result not from canonical DSBR but from dBIR. The dBIR mechanism is also a better fit for our experimental system in which replication stress is likely to result in replication fork

Figure 7 Models of repair by homologous recombination at yeast fragile site FS2. (A) The right arm of one homolog of yeast chromosome III is shown. Drawing is not to scale. The centromere is depicted by a gray oval and the two Ty1 elements at fragile site FS2 are depicted by black arrows. The closest highly-active origins are ARS 310, located ~1 kb centromere-proximal to the fragile site; and ARS 315, located ~55 kb centromere-distal. (B) Under conditions of replication stress resulting from low levels of polymerase α , extended ssDNA on the lagging strand at the replication fork, which (C) permits intrastrand base pairing between the two Ty1 elements forming a hairpin secondary structure. (D) If replication continues, leaving a single-strand gap on the lagging strand, this could be repaired by template switching, which can result in gene conversion in regions of hDNA, highlighted in yellow. (E) If nuclease cleavage at the fragile site collapses the replication fork, this will result in a hairpin-capped double-strand break. (F) The arrival of a replication fork from a centromere-distal ARS will result in a two-end double-strand break. Homologous recombination repair pathways at the double-strand break can result in gene conversion in regions of hDNA, highlighted in yellow. (G) Prior to convergence with a fork from a distal ARS, a one-end double-strand break exists after nuclease cleavage. A one-end double-strand break is typically repaired by BIR. The invading 3' strand during BIR can be displaced and reinvade at a region of homology and establish a second BIR process, called dBIR. In the dBIR mechanism, gene conversion results from the amount of DNA synthesis completed prior to the template switch, highlighted in yellow. HJ, Holliday junction.

collapse to a one-end break. Of noncrossover gene-conversion tracts at FS2 in cells under replication stress (no galactose), 58% were bidirectional. If all bidirectional noncrossover gene-conversion events we observed are from dBIR, then the

frequency of dBIR we observed in these cells is 95×10^{-5} . However, the dBIR process need not be restricted to explanation of only bidirectional noncrossover tracts. Given the high frequency of BIR in our system, all noncrossover tracts at the fragile

site in cells under replication stress—whether unidirectional or bidirectional—could potentially be attributed to dBIR.

The dBIR events in this study result from template switching to the homologous chromosome III, but template switching to a nonhomologous chromosome is possible if the switch occurs at a dispersed repeated sequence, such as the Ty1 elements at FS2. In our analysis of polymorphic sites on the two homologs of chromosome III, template switching to a nonhomologous chromosome is indistinguishable from typical BIR events that extend to the end of chromosome III. However, by CHEF gel analysis and Southern blotting to determine the sizes of chromosome III in a subset of BIR events, we previously reported that 39% of BIR events initiated at FS2 switch to a nonhomologous template (Rosen *et al.* 2013).

Association of mitotic gene-conversion tracts at FS2 with crossover

The relative frequency of crossover-associated compared to noncrossover gene conversions varies widely in published studies and continues to be a topic of investigation. Since mitotic crossover can result in LOH throughout the region centromere-distal to the crossover, which can be detrimental, it seems logical that noncrossover repair pathways would predominate. This hypothesis has been supported by several studies that found crossover-associated conversions to be approximately one-quarter to one-third of all conversions (Haber and Hearn 1985; Chua and Jinks-Robertson 1991; Inbar and Kupiec 1999; Nickoloff *et al.* 1999; Yin and Petes 2013), but three studies reported that half or nearly half of all conversions were crossover-associated (Aguilera and Klein 1989; Welz-Voegele and Jinks-Robertson 2008; Yim *et al.* 2014). In this study, among the 79 gene conversions collected from cells grown under replication stress (no galactose), 30% were crossover-associated and 70% were noncrossover-associated. Based on characteristics of the noncrossover gene conversions, we hypothesize that at least half, and perhaps more, of the noncrossover events we observed are due to dBIR. We propose that the differences between studies is attributable to differences in the type and cell cycle timing of the initiating break, resulting in differences in the contribution of dBIR and SDSA to the production of noncrossover gene conversions.

Relevance to genomic alterations at human common fragile sites in cancer

Recently, a BIR-like mechanism that repairs damaged replication forks was described in human cells, and this mechanism is dependent on POLD3 (Costantino *et al.* 2014). Experimental depletion of *POLD3* suppressed changes in copy number in cells under replication stress, and *POLD3* was reported to be frequently amplified or overexpressed in tumors (Costantino *et al.* 2014). At an engineered replication fork stalling site in mammalian cells mutant for *BRCA1* or *BRCA2*, a high frequency of long-tract gene conversions, similar to the long-tract gene conversions reported here at fragile site FS2, was observed (Willis *et al.* 2014; Willis and Scully 2016). Replication

difficulty is a hallmark feature of human common fragile sites, and these sites are frequently implicated in genomic alterations in tumors (Burrow *et al.* 2009; Bignell *et al.* 2010; Ozeri-Galai *et al.* 2012). These data together with the results presented here suggest that BIR-like mechanisms are likely to be responsible for copy number changes and rearrangements at fragile sites in tumors, and we propose that instability at common fragile sites may also drive LOH in cancer through dBIR-mediated long-tract gene conversion.

Genomic sequencing has revealed that microhomology (5 bp or fewer) is frequently present at the junctions between segments in complex rearrangements in tumors, and preexisting low-copy repeats are frequently found close to these junctions (Stephens *et al.* 2009; Nik-Zainal *et al.* 2016). Multiple rounds of dissociation and reinvasion of the 3' end at short tracts of homology during BIR, termed microhomology-mediated BIR (MM-BIR), has been proposed as a mechanism for generating these complex rearrangements (Ira and Haber 2002; Anand *et al.* 2014). The mechanisms of BIR and MM-BIR are clearly distinct. BIR requires *RAD51* and long regions of homology at the 3' end invasion site, while MM-BIR is *RAD51*-independent, uses very short tracts of homology, and is enhanced by the presence of nearby islands of additional microhomology (Anand *et al.* 2014; Carvalho and Lupski 2016). Recently, it was shown in the yeast model system that BIR can switch to MM-BIR. The switch occurs when replication during BIR is impaired, and the translesion synthesis polymerases ζ and *Rev1p* are required for the DNA synthesis initiated at microhomologies (Sakofsky *et al.* 2015). Given that human common fragile sites are prone to replication difficulties and are enriched in Alu-family repeat sequences (Tsantoulis *et al.* 2008) that could serve as islands of microhomology, we speculate that MM-BIR may be a significant driver of genomic rearrangements at fragile sites. It will be of interest to investigate the role of MM-BIR in repair processes at yeast fragile site FS2. Our system has the potential to identify and evaluate complex genomic rearrangements generated by template switching to nonhomologous chromosomes after stimulation of BIR at FS2. Detailed evaluation of these events, and the effect of mutation in *Rev1p*, polymerase ζ , and other repair proteins could be analyzed.

In summary, gene conversion causes LOH, and instability at the yeast fragile site FS2 can stimulate repair processes that result in very long gene-conversion tracts. In human cells, LOH is an important contributor to the inactivation of tumor-suppressor genes, and tumor initiation and progression. Greater understanding of how large regions of LOH are generated is important. The characteristics of very long gene-conversion tracts at FS2 suggest dBIR is a prominent mechanism for repair of lesions at fragile sites. Thus, dBIR is likely to drive common fragile site-stimulated LOH in human tumors.

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Remarkably Long-Tract Gene Conversion Induced by Fragile Site Instability in *Saccharomyces cerevisiae*

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