A mutant allele of the TFIIH helicase gene, *RAD3*, promotes loss of heterozygosity in response to a DNA replication defect in *Saccharomyces cerevisiae*.

Michelle S. Navarro*, Liu Bi†, and Adam M. Bailis*,1

*Division of Molecular Biology, Beckman Research Institute, City of Hope National Medical Center, Duarte, California 91010-0269

†Department of Botany and Plant Sciences University of California, Riverside Riverside, CA 92521
Running head: LOH, DNA replication, and repair.

Five key words or phrases: DNA replication, mutation, loss of heterozygosity, recombination, helicase

1 Corresponding author: Adam M. Bailis, Division of Molecular Biology, Beckman Institute of the City of Hope, 1450 E. Duarte Rd., Duarte, CA 91010-0269. Phone: (626) 256-4673, ext. 64031. Fax: (626) 301-8271. E-mail: abailis@bricoh.edu.
Increased mitotic recombination enhances the risk for loss of heterozygosity, which contributes to the generation of cancer in humans. Defective DNA replication can result in elevated levels of recombination as well as mutagenesis, and chromosome loss. In the yeast *S. cerevisiae*, a null allele of the *RAD27* gene, which encodes a structure-specific nuclease involved in Okazaki fragment processing, stimulates mutation and homologous recombination. Similarly, *rad3-102*, an allele of the gene, *RAD3*, which encodes an essential helicase subunit of the core TFIIH transcription initiation and DNA repairosome complexes confers a hyperrecombinagenic and hypermutagenic phenotype. Combining the *rad27* null allele with *rad3-102* dramatically stimulated interhomolog recombination and chromosome loss but did not affect unequal sister-chromatid recombination, direct repeat recombination, or mutation. Interestingly, the percentage of cells with Rad52-YFP foci also increased in the double mutant haploids suggesting that *rad3-102* may increase lesions that elicit a response by the recombination machinery or, alternatively, stabilize recombinagenic lesions generated by DNA replication failure. This net increase in lesions led to a synthetic growth defect in haploids that is relieved in diploids, consistent with *rad3-102* stimulating the generation and rescue of collapsed replication forks by recombination between homologs.
INTRODUCTION

Genomic integrity and, ultimately, cell survival rely on the coordinated and accurate responses of various damage repair systems to insults incurred by the DNA. In their absence chromosomal instability, a hallmark of tumor cells is markedly increased (BISHOP and SCHIESTL 2001; FEITELSON et al. 2002; GRAY and COLLINS 2000; GUPTA et al. 1997; KAMB 2003; LENGUAER et al. 1998; LIN et al. 2003; MITELMAN et al. 1994; RADFORD et al. 1995; RAJAGOPALAN and LENGUAER 2004). Homologous recombination (HR) is a repair mechanism that is critical for repairing double-strand breaks (DSBs) created by DNA replication failure, ionizing radiation and other damaging agents (COX et al. 2000; DEBRAUWERE et al. 2001; GALLI et al. 2003; GAME and MORTIMER 1974; MICHEL et al. 2004; PAQUES and HABER 1999; RESNICK 1976; RESNICK and MARTIN 1976; SYMINGTON 1998; TISHKOFF et al. 1997). Many of the genes involved in HR, such as RAD50, RAD51, RAD52, RAD53, RAD54, RAD55, RAD56, RAD57, RAD59, RDH54/TID1, MRE11, and XRS2 (NBS1 in humans), were first identified through mutants sensitive to ionizing radiation (GAME and MORTIMER 1974). The HR proteins physically interact with and process DSBs to facilitate their repair (KROGH and SYMINGTON 2004; PAQUES and HABER 1999; SUGAWARA et al. 2003).

Repair by HR requires an initiating event, such as a DSB (PAQUES and HABER 1999; RESNICK 1976; RESNICK and MARTIN 1976; SZOSTAK et al. 1983), and a homologous donor sequence carrying sufficient genetic information to repair the break (BAILIS and ROTHSTEIN 1990; JINKS-ROBERTSON et al. 1993; RUBNITZ and SUBRAMANI 1984; SUGAWARA and HABER 1992). The donor sequences most commonly used to repair
DSBs are homologous sequences on the sister-chromatid or homologous chromosome. However, increased mitotic recombination with a homologous chromosome or non-allelic, ectopic sequences increases the risk for deleterious genome rearrangements or loss of heterozygosity (LOH), and paves the road for carcinogenesis or other diseases (BISHOP and SCHIESTL 2001; GUPTA et al. 1997). Several studies have demonstrated that the development of many cancers involves the loss or gain of information by interhomolog recombination mechanisms like gene conversion and break-induced replication (BISHOP and SCHIESTL 2001). For example, one study reported that 81.3% of colorectal adenocarcinomas exhibited LOH (LIN et al. 2003), while, in another study, up to 37.5% of ductal carcinoma in situ of the breast displayed LOH (RADFORD et al. 1995).

Identifying the factors that stimulate interhomolog recombination may provide insight into the molecular mechanisms that promote LOH and cell transformation. Defects in DNA replication proteins like Dna2, Pol δ, and Rad27, stimulate mitotic recombination (AGUILERA et al. 2000; LOPES et al. 2002; MICHEL et al. 2004; SYMINGTON 1998), perhaps by interfering with DNA replication fork progression. Alternatively, a DNA replication fork may encounter a lesion that blocks leading or lagging strand synthesis (HOLMES and HABER 1999; LOPES et al. 2006b). Lesions can be bypassed using error-prone mechanisms, or through template switching to the sister-chromatid. If the lesion persists, leaving a daughter-strand nick or gap (LOPES et al. 2006b), subsequent DNA replication may generate a collapsed fork that resembles a single-ended DSB (CORTES-LEDESMA and AGUILERA 2006; SALEH-GOHARI et al. 2005). Whereas mutagenic bypass and template switching may aid in preventing fork collapse and DSB formation, the requirement for HR to rescue collapsed forks is clearly suggested
by the observation that certain DNA replication mutations are lethal in combination with mutations in the HR machinery (Debrauwere et al. 2001; Galli et al. 2003; Michel et al. 2004; Symington 1998; Tishkoff et al. 1997).

In yeast, the RAD27 gene codes for a 5’-3’ flap exo- and endonuclease that processes Okazaki fragments during lagging strand DNA synthesis and is the homolog to the human FEN-1 protein (Harrington and Lieber 1994; Lieber 1997; Reagan et al. 1995; Sommers et al. 1995). In the rad27-null mutant, all consequences of defective lagging strand synthesis are observed. For example, rad27-null mutant cells display increased levels of single-stranded DNA (Parenteau and Wellinger 1999; Vallen and Cross 1995), mutagenesis (Tishkoff et al. 1997), microsatellite instability (Refsland and Livingston 2005; Schweitzer and Livingston 1998; Xie et al. 2001), minisatellite instability (Kokoska et al. 1998; Lopes et al. 2006a), telomeric repeat instability (Parenteau and Wellinger 1999), and recombination (Negritto et al. 2001; Symington 1998; Tishkoff et al. 1997). Combining the rad27Δ-null allele with null mutations in a large number of HR genes leads to synthetic lethality, strongly suggesting the need for HR to rescue DNA replication defects in these cells (Debrauwere et al. 2001; Symington 1998; Tishkoff et al. 1997).

Previous studies in yeast showed that certain alleles of the RAD3, SSL1, and SSL2 genes, which encode subunits of the transcription factor IIH (TFIH) and nucleotide excision repair (NER) complexes, display elevated levels of mitotic recombination (Bailis and Maines 1996; Bailis et al. 1995; Garfinkel and Bailis 2002; Golin and Esposito 1977; Malone and Hoekstra 1984; Montelone and Liang-Chong 1993; Montelone et al. 1992; Montelone et al. 1988). This suggests a role for these DNA

Recently, the Ssl1 subunit has also been shown to possess E3 ubiquitin ligase activity (\textit{Takagi et al.} 2005). Since Ssl1 and Rad3 interact physically and genetically (\textit{Bardwell et al.} 1994; \textit{Maines et al.} 1998) it is possible that this function may be integral to the roles already described for these proteins.

Mutant alleles of the \textit{RAD3}, \textit{SSL1}, and \textit{SSL2} genes confer several, genetically separable phenotypes, indicative of their roles in multiple cellular functions. Consistent with these observations, human homologs of these genes have been linked to the diseases xeroderma pigmentosum, Cockayne’s syndrome, and trichothiodystrophy, which are characterized by transcriptional and DNA repair defects, and increased tumor formation (\textit{Garfinkel and Bailis} 2002). While mutations in the yeast \textit{RAD3} and \textit{SSL1} genes create separable transcription, NER and recombination phenotypes (\textit{Feaver et al.} 1993; \textit{Maines et al.} 1998; \textit{Montelone and Malone} 1994; \textit{Montelone et al.} 1988; \textit{Song et al.} 1990; \textit{Wang et al.} 1995), it remains unclear how these changes are related.
One \textit{RAD3} mutant allele, \textit{rad3-102}, was identified on the basis of its ability to confer elevated levels of mitotic recombination and mutagenesis (MALONE and HOEKSTRA 1984). The \textit{rad3-102} allele contains a point mutation that alters amino acid residue 661 in the seventh, conserved domain of the Rad3 helicase (MONTELONE and MALONE 1994). Further, like the \textit{rad27}-null allele, \textit{rad3-102} confers synthetic lethality when combined with mutations in the HR genes \textit{RAD52} and \textit{RAD50} (MALONE and HOEKSTRA 1984). This may be independent from the transcription initiation and NER function of Rad3 as the \textit{rad3-102} mutation does not confer any apparent transcription defect, and only a minor NER defect (HOEKSTRA and MALONE 1987; MALONE \textit{et al.} 1988). In this paper, we examine the epistatic interactions between \textit{rad3-102} and a null allele of \textit{RAD27}, in order to determine if their elevated rates of mutation and recombination are mechanistically related.
Yeast strains and plasmids: All strains used in this study are isogenic with W303-1A (THOMAS and ROTHSTEIN 1989) and are shown in Table 1. All yeast strain construction used conventional methods (BURKE et al. 2000). The construction of the rad27::LEU2 allele was described previously (FRANK et al. 1998) and the rad3-102 allele was incorporated into yeast from the plasmid pBM3-102 that was a generous gift of Beth Montelone and Robert Malone (MONTELONE and MALONE 1994; MONTELONE et al. 1988). All strains derived from those containing the rad27::LEU2 mutation were checked for differences in growth to assure absence of suppressors. The rad27-null mutation confers a slow germination phenotype that is easily distinguishable from wild type strains. ABX761, used for the unequal sister-chromatid recombination (USCR) assay, was constructed by transforming XbaI-digested PNN287, the generous gift of Michael Fasullo (FASULLO and DAVIS 1987), into ABX731-8C (MATa, his3-∆200) by electroporation. Insertion of the plasmid at the targeted site was verified by Southern blot analysis (M. S. NAVARRO and A. M. BAILIS, unpublished results). Yeast strains containing the hom3-10, lys2-Bgl, and CAN1 alleles were derived from strain RKY2672 from Richard Kolodner (TISHKOFF et al. 1997) and backcrossed into our background at least four times. The RAD52-YFP allele was the generous gift of Michael Lisby and Rodney Rothstein (LISBY et al. 2001). Assays were conducted from haploids taken immediately from dissection plates, or diploids constructed from newly dissected spore colonies.
The impact of the rad5-G535R allele on growth, mutation, recombination, and chromosome loss was shown to be minimal and did not change the effects exerted by the rad3-102 or rad27-null alleles singly, or in combination (M. S. NAVARRO and A. M. BAILIS, unpublished results).

Centromere-containing plasmids pRS416 (CHRISTIANSEN et al. 1991), which contains the URA3 gene, and pJM3, which contains the URA3 and MATa genes were generously provided by Phil Hieter and Jim Haber.

**Determination of mutation rates:** The lys2-Bgl, hom3-10, and CAN1 mutation rates for ABX471-2C (wild type), ABX474-4B (rad27), ABX460-6A (rad3-102), and ABX481-1C (rad27Δ rad3-102) were determined as described previously (TISHKOFF et al. 1997). Strains were plated on YPD (2% peptone, 1% yeast extract, 2% dextrose) for single colonies at 30°. For each genotype, seven individual colonies from at least five strains were excised from plates and individually suspended in sterile water. Appropriate dilutions were plated onto YPD to determine the total number of viable cells, onto synthetic medium lacking lysine to determine the number of lysine prototrophic (Lys⁺) cells per colony, onto synthetic medium lacking threonine to determine the number of threonine prototrophic (Thr⁺) cells per colony, and onto synthetic medium without arginine plus 60µg/ml of canavanine to determine the number of canavanine resistant (Can⁺) cells per colony. Individual rates were calculated using the method of the median (LEA and COULSON 1949) and are expressed as the number of mutation events/cell/generation. 95% confidence intervals were determined by a previously
described method (SPELL and JINKS-ROBERTSON 2004). A minimum of 30 cultures were tested per strain.

**Determination of unequal sister-chromatid recombination rates:** The rates of unequal sister-chromatid recombination were determined as previously described (FASULLO and DAVIS 1987). At least five, freshly dissected ABX761 segregants (of each genotype) containing the USCR construct and the \( \text{his}^3-\Delta200 \) allele were streaked out for single colonies on YPD. After three days of growth at 30\(^\circ\), at least three single colonies were excised from each plate and suspended in sterile water. Appropriate dilutions were plated onto YPD to determine the total number of viable cells and synthetic complete medium lacking histidine to determine the number of His\(^+\) cells per colony. After growth at 30\(^\circ\), for 3-4 days, the number of His\(^+\) events was determined. Unequal sister-chromatid recombination rates and 95% confidence intervals were calculated from a minimum of 15 trials as described above.

**Determination of direct repeat recombination rates:** The direct repeat assay was performed as described previously (MAINES et al. 1998). Two truncated \( \text{HIS3} \) sequences, sharing 415bp of homology, flank a \( \text{URA3} \) marker at the \( \text{HIS3} \) locus on chromosome XV. Recombination between the duplicate sequences results in deletion of the \( \text{URA3} \) marker and generation of a wild type \( \text{HIS3} \) allele. At least 15, freshly dissected segregants containing the direct repeat were grown to saturation in synthetic medium lacking uracil at 30\(^\circ\). Appropriate dilutions of cells were plated onto YPD medium to determine the number of viable cells and onto synthetic complete medium lacking histidine to determine the number of His\(^+\) recombinants. Plates were incubated at 30\(^\circ\) for four days.
Deletion rates and 95% confidence intervals were calculated from a minimum of 15 trials as described above.

**Determination of chromosome V loss and interhomolog recombination rates:** The rates of interhomolog recombination (IHR) and chromosome V loss were determined as described previously (KLEIN 2001). Freshly dissected segregants containing either **CAN1** and **HOM3**, or **can1-100** and **hom3-10** alleles were crossed to generate **CAN1/can1-100, HOM3/hom3-10** diploid strains. At least five independent diploids of each genotype were prepared. Seven fresh colonies of each diploid were dispersed in sterile water. Appropriate dilutions of cells were plated onto YPD medium to determine the total number of viable cells, and onto synthetic medium lacking arginine and containing 60µg/ml of canavanine to determine the number of Canr colonies. The total number of Canr colonies was determined after growth at 30°C for four days. Canr colonies were replica plated onto synthetic medium lacking threonine. After two days of growth at 30°C, the fractions of colonies that were Canr Thr+, and Canr Thr− were determined. Cells that were Canr Thr− were scored as chromosome loss events and cells that were Canr Thr+ were scored as interhomolog recombination events. Canr Thr+ colonies may also represent mutation events, however it was found that Canr Thr+ colonies result predominantly from mitotic recombination (GOLIN and ESPOSITO 1977). Rates and 95% confidence intervals were determined from a minimum of 35 trials as described above.

In order to distinguish break-induced replication events from gene conversion events among interhomolog recombination events, we inserted the **URA3** marker within the telomere proximal hxt13 locus on chromosome V as previously described (CHEN and
KOLODNER 1999). For the assay, the $hxt13::URA3$ allele is on the same copy of chromosome V copy that contains the wild type $HOM3$ and $CAN1$ alleles. Appropriate dilutions of diploid cells were plated onto YPD medium and onto synthetic medium lacking arginine and containing canavanine to determine the number of Can$^r$ colonies. The total number of Can$^r$ colonies was determined after growth at 30° for four days. Can$^r$ colonies were replica plated onto synthetic medium lacking threonine and onto synthetic medium lacking uracil. After two days of growth at 30°, the fractions of colonies that were Can$^r$ Thr$^+$ Ura$^+$ were scored as gene conversion events, cells that were Can$^r$ Thr$^+$ Ura$^-$ were scored as break-induced replication events, and cells that were Can$^r$ Thr$^-$ Ura$^-$ were scored as chromosome loss events. It is important to note that we were unable to distinguish between BIR and crossover events with this assay.

**Fluorescence microscopy:** All experiments were performed according to previously described methods (LISBY et al. 2001). In brief, 5ml of cells were grown in complete synthetic medium to an OD$_{600}$ of 0.2 at room temperature. Growth at room temperature is required to allow the chromophore to form efficiently (LIM et al. 1995). One ml of cells was then washed and resuspended in 200-300 µl of complete synthetic medium. A small aliquot of cells was placed on a glass slide, and sealed with VALAP solution (combination of equal volumes of petroleum jelly, lanolin, and paraffin). Cells were visualized using an Olympus AX70 automated upright microscope (Olympus, Melville, NY) containing a mercury illumination source and U-MWIBA filter cube (excitation 460-490nm) for visualizing Rad52-YFP. Ten live cell images were taken at 0.1µm intervals along a z-axis, using a Spot RT slider high-resolution B/W camera and a
Plan/Apochromat 60x, 1.4 numerical aperture lens and prepared using the Image Pro Plus software (Media Cybernetics, Silver Spring, MD) and Image J software (National Institutes of Health, Bethesda, MD). Brightfield images were used to count total cell number and define cell-cycle phase, and each z-stack fluorescence image was inspected for the presence of a Rad52-YFP focus.

**Determination of doubling time:** YPD liquid (5ml) was inoculated with a single colony and grown overnight at 30°C. Aliquots from each wild type, rad3-102, rad27-null, and rad3-102 rad27-null culture were used to inoculate 5ml of YPD to an approximate cell density of 1 x 10⁷ cells/mL and grown at 30°C. Culture density was measured each hour by monitoring turbidity using a Klett-Summerson colorimeter fitted with a red filter. Doubling times were calculated using a common algorithm (Singleton 1995). Growth assays with strains containing either pJM3 or pRS416 were done using synthetic complete medium lacking uracil to maintain selection for the plasmids.
RESULTS

Mutagenic response to DNA replication defects is unaltered in the rad3-102 rad27-null double mutant.

The rad27-null mutant confers a defect in lagging strand synthesis that was previously shown to increase mutagenesis in several mutation assays (TISHKOFF et al. 1997). Since the rad3-102 mutant also displays an increased mutation rate (MALONE and HOEKSTRA 1984; MONTELONE and LIANG-CHONG 1993; MONTELONE and KOELLIKER 1995; MONTELONE et al. 1992), we investigated the epistatic relationship between the rad27-null and rad3-102 mutations with respect to mutagenesis. The CAN1 forward mutation assay and the hom3-10 and lys2-Bgl reversion assays previously utilized to characterize the rad27-null mutant were used (TISHKOFF et al. 1997). The CAN1 forward mutation rate assay selects for cells made resistant to canavanine (Canr) by mutagenesis of the arginine permease gene (GRENSON et al. 1966). The reversion assays select for revertants of either a 4-base insertion in the LYS2 gene (lys2-Bgl), or a +1 T insertion within a run of six T’s in the HOM3 gene (hom3-10). Consistent with previous results (TISHKOFF et al. 1997), we observed significant increases in mutation rate with all three assays in the rad27-null mutant cells (Table 2); a 16-fold increase in the rate of CAN1 mutation, an 8-fold increase in the rate of hom3-10 reversion, and a 17-fold increase in the rate of lys2-Bgl reversion. rad3-102 cells displayed significantly increased rates with only the hom3-10 (7-fold increased) and lys2-Bgl (8-fold increased) reversion assays. Interestingly rad3-102 did not alter the mutator effect of the rad27-null mutation, even in the hom3-10 reversion assay where its stimulation is similar to that seen with the rad27-
null mutation. These results suggest that the rad3-102 and rad27-null mutations affect the same mutagenic mechanism. To further confirm this conclusion, we determined the spectrum of representative lys2-Bgl reversion mutations from wild type, rad3-102, rad27-null, and rad3-102 rad27-null double mutants. Sequencing of nucleotides 315-540 of the LYS2 gene from at least 20 independent Lys+ revertants revealed that the mutation spectrum of the rad3-102 rad27-null double mutant was similar to that of the rad27-null single mutant, particularly with regard to the deletion/insertion mutations that are characteristic of rad27-null mutant cells (44% and 36%, respectively). No deletion/insertion mutations appeared in wild type or rad3-102 Lys+ revertants (L. B1 and A. M. BAILIS, unpublished results).

Unequal sister-chromatid and direct-repeat recombination are unaffected by the rad3-102 allele in the rad3-102 rad27-null double mutants.

Template switching with the sister-chromatid is another response to replication lesions (DONG and FASULLO, 2003). Consequently, we investigated the epistatic relationship between the rad3-102 and rad27-null mutations with respect to USCR using an assay developed by Fasullo and Davis (FASULLO and DAVIS 1987). The assay monitors the frequency of recombination between 3’ and 5’ truncated copies of the HIS3 gene that share 300bp of homologous sequence and are arranged, tail to head at the TRP1 locus on chromosome IV. This arrangement restricts the production of a functional HIS3 gene until S-phase. The rate of USCR in the rad27-null single mutant was 48-fold over the wild type rate and the rad3-102 mutant increased recombination less than four-fold.
over wild type (Table 3). USCR in the rad3-102 rad27-null double mutant was not significantly different from the rad27-null single mutant.

Unlike USCR, deletion formation by recombination between non-tandem, direct repeats on a chromosome can occur before or after the initiation of replication. We determined the rates of recombination between 3’ and 5’ truncated copies of the HIS3 gene that share 415bp of homology and flank a URA3 selectable marker in haploid and diploid cells (MAINES et al. 1998). The rates of direct-repeat recombination in haploid cells were increased seven-fold in the rad27-null mutant and only two-fold in the rad3-102 mutant (Table 3). Like USCR, the direct-repeat recombination rate in double mutant haploids was not significantly different from the rate in rad27-null single mutants, although the possible presence of additive effects of combining the rad3-102 and rad27-null alleles can not be dismissed. Very similar effects were observed in diploids, suggesting that the presence of a homolog does not affect the mechanism of recombination. These results indicate that the rad27-null and rad3-102 mutations may affect both recombinagenic responses to replication lesions by similar mechanisms.

Together, the rad3-102 and rad27-null mutations confer synergistic increases in chromosome loss and recombination between homologs.

Chromosome loss and recombination between homologs have previously been shown to be stimulated in DNA replication mutant cells (HABER 1999), presumably in response to lesions generated during DNA replication. In particular, daughter-strand nicks or gaps that are not repaired by error-prone bypass or template switching with the sister-chromatid may persist to form collapsed replication forks that can stimulate
chromosome loss and recombination with the homolog in diploid cells (DAIGAKU et al. 2006). Since these may be important consequences of DNA replication lesion formation, we examined the epistatic interactions between rad3-102 and the rad27-null allele with respect to chromosome loss and interhomolog recombination. Using a previously described assay for measuring the loss of one copy of chromosome V and recombination between chromosome V homologs (KLEIN 2001), we observed wild type levels of chromosome loss and a seven-fold increase in interhomolog recombination in rad3-102/rad3-102 homozygotes, and a 29-fold increase in chromosome loss and 71-fold increase in interhomolog recombination in the rad27-null/rad27-null homozygotes (Table 3). However, in contrast to the previous assays, the combination of the rad3-102 and rad27-null alleles led to synergistically increased rates of chromosome loss and interhomolog recombination; a 172-fold increase in chromosome loss and a 1,400-fold increase in interhomolog recombination in the rad3-102/rad3-102 rad27-null/rad27-null double homozygotes. These results suggest that DNA replication fork collapse is greatly stimulated in rad3-102 rad27-null mutants.

The rad3-102 and rad27-null mutations together increase the percentage of G2/M phase cells with Rad52-YFP foci.

Previously, Lisby and Rothstein tagged the yeast Rad52 homologous recombination protein with the yellow-fluorescent protein, YFP (ORMÖ et al. 1996), and observed that it forms nuclear foci in response to DNA damage induced by ionizing radiation, DSB formation by the HO-endonuclease, and collapsed replication forks (LISBY et al. 2001; LISBY et al. 2003; LISBY et al. 2004). If, as suggested by the
chromosome loss and interhomolog recombination data, the *rad3-102* and *rad27*-null alleles together lead to synergistic increases in replication fork collapse, more *rad3-102* *rad27*-null double mutant cells might be expected to display foci than *rad3-102* and *rad27*-null single mutant cells. As observed previously, we found that most wild type cells exhibited diffuse, nuclear fluorescence and that only 6.6% of S- and 4.1% of G2/M-phase cells contained one or more foci in an asynchronous population (Table 4) (Lisby et al. 2001). Consistent with the levels of recombination observed in *rad3-102* and *rad27*-null mutant cells, 23% of S- and 14% G2/M-phase *rad3-102* mutant cells displayed a focus, while 23% of S- and 47% of G2/M-phase *rad27*-null mutant cells displayed foci. As predicted, the *rad3-102* *rad27*-null double mutant exhibited a greater percentage of cells with foci than either of the single mutants, with 47% of S-phase and 68% of G2/M phase cells containing foci.

A profound growth defect in the *rad3-102* *rad27*-null double mutant haploid is suppressed in the homozygous double mutant diploid.

The *rad3-102* and *rad27*-null single mutant haploid strains exhibit mild growth defects at 37° (Sommers et al. 1995; Symington 1998) (Table 5). However, combining the *rad3-102* and *rad27*-null alleles synergistically increases doubling time in haploids. Strikingly, the doubling time of the *rad3-102/rad3-102 rad27-null/rad27-null* double homozygote is very similar to that in the *rad27-null/rad27-null* single homozygote (Table 5). This result may indicate an effect of diploidy, *MAT* heterozygosity, or both. *MAT* heterozygosity has been demonstrated to increase recombination in diploid cells as well as resistance to DNA damage in both haploid and diploid cells (Fasullo and Dave
1994; FASULLO et al. 1999; FRIIS and ROMAN 1968; HEUDE and FABRE 1993). In order to distinguish if MAT heterozygosity is solely responsible for rescuing the growth defect of the double mutant cells, we transformed both wild type and rad3-102 rad27-null MATα haploids with a plasmid that contains the MATα sequence and repeated the growth assays. As a control, we also transformed wild type and double mutant MATα haploids with pRS416 that lacks MAT sequences. MAT heterozygosity did not significantly alter the doubling time of rad3-102 rad27-null double mutant haploids. This suggests that the presence of a homolog is required to rescue the growth defect imposed by the combination of the rad3-102 and rad27-null alleles, perhaps by permitting chromosome loss, or enabling interhomolog recombination to rescue collapsed forks that otherwise inhibit growth.
DISCUSSION

The TFIIH and NER helicase Rad3 has been variously implicated in spontaneous mutagenesis and recombination, as well as in the processing of recombination intermediates (Bailis and Maines 1996; Bailis et al. 1995; Malone and Hoekstra 1984; Montelone and Liang-Chong 1993; Montelone and Koelliker 1995; Montelone and Malone 1994; Montelone et al. 1992; Montelone et al. 1988). This manuscript addresses the effect of a known hypermutagenic and hyperrecombinagenic allele, rad3-102, in the context of the defined DNA replication defect conferred by the rad27-null mutation in order to better understand how Rad3 functions in the development of mutations, genome rearrangements and LOH. We revealed that two mechanisms that promote LOH, chromosome loss and interhomolog recombination, were synergistically stimulated in the rad3-102 rad27-null double mutant. However, mutation rate, unequal sister-chromatid and direct-repeat recombination, mechanisms that do not rely on the presence of a homologous chromosome, were not stimulated any further in the double mutant than in the rad27-null single mutant. These results suggest that rad3-102 confers a preference for mechanisms that utilize a homolog in the rescue of replication lesions generated in the absence of Rad27. In support of this hypothesis, differential growth of rad3-102 rad27-null haploids and homozygous diploids suggests these lesions, which we suggest may be collapsed forks, are either efficiently repaired by interhomolog recombination or result in chromosome loss in diploids.

The Rad27 nuclease functions primarily in the removal of the 5’- RNA/DNA flap generated during lagging-strand synthesis (Harrington and Lieber 1994; Lieber 1997;
In its absence, processing by other nucleases may create nicks or gaps in the daughter-strand, which have been observed in rad27-null mutants (Parenteau and Wellinger 1999; Vallen and Cross 1995). These daughter-strand nicks and gaps may be utilized for mutagenic bypass or template switching and give rise to mutations or sister-chromatid exchange events (Fasullo and Davis 1987; Holmes and Haber 1999; Lopes et al. 2006b; Michel et al. 2004; Saleh-Gohari et al. 2005), as observed in our rad27-null mutant (Table 2 and Table 3). Persistence of a flap would lead to replication fork collapse upon confrontation with the replicative polymerase during the next cell cycle. Chromosome breaks have been observed in rad27-null mutant cells (Callahan et al. 2003; Vallen and Cross 1995), which could elicit checkpoint signals that lead to arrest in G2, slow growth, and enhanced chromosome loss and interhomolog recombination (Table 3, Table 5, and L. Bi and A. M. Bailis, unpublished results). These observations are most consistent with the rad27-null mutation primarily affecting the creation of DNA damage. Previous studies have implicated Rad27 in the processing of recombination intermediates (Kikuchi et al. 2005; Negritto et al. 2001; Wu et al. 1999; Zheng et al. 2005). The results reported here do not support such a conclusion, perhaps, because the effects of increased chromosome breakage on our assays eclipse the effects on the processing of intermediates in rad27-null mutant cells. Substantial increases in all of the consequences of replication lesion formation in the rad27-null mutant cells argues that blocking Okazaki fragment maturation leads to dramatic increases in genome instability.

The role that the Rad3 helicase plays in the maintenance of genome stability is unclear. While the participation of Rad3 in NER and transcription is not inconsistent with
the effects of \textit{rad3} mutations on mutagenesis and recombination, \textit{rad3-102}, the hypermutable and hyperrecombinogenic mutant described here, displays minimal or no defects in nucleotide excision repair or transcription (HOEKSTRA and MALONE 1987; MONTELONE \textit{et al.} 1988). Certain mutations in \textit{SSL1}, which encodes another core subunit of TFIIH and the NER repairosome, stimulate short-sequence recombination and attenuate the processing of DSBs and Ty1 cDNA (MAINES \textit{et al.} 1998; LEE \textit{et al.} 1998). Recently, Ssl1 has also been shown to have ubiquitin ligase activity that may influence its role in genome stability. Since \textit{RAD3} and \textit{SSL1} have been shown to interact genetically (BARDWELL \textit{et al.} 1994; MAINES \textit{et al.} 1998), and Rad3 and Ssl1 to interact physically (BARDWELL \textit{et al.} 1994; MAINES \textit{et al.} 1998), it is possible that \textit{rad3-102} influences genome stability through an interaction between Rad3 and Ssl1, but this has yet to be explored.

It was previously suggested that the hyperrecombinogenic effect of \textit{rad3-102} resulted from the mutant protein generating lesions in the DNA that ultimately became DSBs (MONTELONE \textit{et al.} 1988). This hypothesis was especially attractive since \textit{rad3-102}, like \textit{rad27}\(\Delta\), is synthetically lethal in mutant \textit{rad52} and \textit{rad50} backgrounds (MALONE and HOEKSTRA 1984). While the hypermutagenic and hyperrecombinogenic nature of the \textit{rad3-102} mutant is reminiscent of the \textit{rad27}-null mutant, we observed several, essential differences. In general, the hypermutagenic and hyperrecombinogenic characteristics of the \textit{rad3-102} mutant were significantly less severe than those of the \textit{rad27}-null mutant. These are reflected by the minimal effects of the \textit{rad3-102} allele on cell-cycle profile, Rad52-YFP focus formation, and growth, which are inconsistent with substantial increases in DNA replication lesions (Table 4, Table 5, and L. Bì and A. M.
Therefore, it seems likely that the rad3-102 allele exerts its effect subsequent to the formation of DNA replication lesions. In further support of this hypothesis, we have previously shown that another allele of RAD3, rad3-G595R, has a substantial effect on recombination that has been initiated by an HO-endonuclease catalyzed DSB, perhaps by changing their exonucleolytic processing (BAILIS et al. 1995). Consequently, we suggest that the rad3-102 allele may exacerbate the effect of the rad27-null allele on loss of heterozygosity primarily by altering the cellular response to DNA replication lesions.

The data presented here are consistent with a model for the interaction between the rad27-null and rad3-102 mutant alleles where the loss of Rad27 leads to the accumulation of DNA replication lesions and rad3-102 alters their processing. Loss of Rad27 results in the inefficient cleavage of RNA primer sequences from the 5’ ends of Okazaki fragments, such that at least one RNA residue remains, blocking their ligation to adjacent fragments. Other nucleases remove these 5’ ends, creating daughter-strand nicks and gaps that accumulate in rad27-null mutant cells (MERRILL and HOLM 1998; PARENTEAU and WELLINGER 1999). The 3’ ends of these gaps may be recognized by polymerases with an increased tendency for mis-insertion that synthesize across the gap in an error-prone manner, contributing to the robust mutation rate in the rad27-null mutants (Table 2). Alternatively, the gaps may be repaired by template switching with the sister-chromatid (ZHENG and FASULLO 2003) that can also account for the duplications that accumulate in rad27-null mutants (TISHKOFF et al. 1997), as well as the increases in USCR and direct-repeat recombination (Table 3).
We suggest that rad3-102 may attenuate the removal of the residual RNA primer sequences from Okazaki fragments by blocking nucleases that can compensate for the loss of Rad27 (Symington 1998). This may occur because the NER repairosome, of which Rad3 is a component, may recognize and bind to the primer sequences as it does other polymerase blocking lesions (Johansson et al. 2004), limiting nuclease access in rad3-102 mutant cells. Under these circumstances, unprocessed and unligatable Okazaki fragments would be expected to accumulate. An increase in unprocessed Okazaki fragments may lead to widespread DSB formation by DNA replication fork collapse in the subsequent S-phase as the discontinuities would lie on the template for leading strand synthesis (Figure 1). Alternatively, the nicks may stimulate DSB formation prior to the following S-phase (Tishkoff et al. 1997). The DSBs may be repaired by interhomolog recombination, or give rise to chromosome loss that can be tolerated in diploids but may be fatal in haploids. Evidence for this in rad3-102 rad27-null double mutant haploids exists in the significant increase in the percentage of cells with Rad52-YFP foci (Table 4) and the synthetic growth defect in haploid cells that lack an efficient means of rescuing the collapsed forks (Table 5). However, in rad3-102 rad27-null double mutant diploids, the presence of a homolog appears to rescue the synthetic growth defect (Table 5), perhaps through the repair of DSBs by interhomolog recombination, which is increased 1400-fold, or through supporting chromosome loss, which is stimulated 172-fold (Table 3). Interestingly, the presence of a homolog failed to stimulate direct-repeat recombination in the rad3-102 rad27-null double mutant diploids beyond that observed in the rad27-null single mutant haploids, and these rates were not significantly different from those observed in rad27-null and rad3-102 rad27-null haploids (Table 3). This is
consistent with fork collapse leading to the formation of single-ended DSBs that are thought to be ideal substrates for interhomolog recombination by break-induced replication (BIR) (MCEACHERN and HABER 2006), but not, perhaps, direct-repeat recombination, which is thought to occur by single-strand annealing (DAVIS and SYMINGTON 2004; DONG and FASULLO 2003; IVANOV et al. 1996; LIN et al. 1990).

The interhomolog recombination observed in wild type diploids is largely RAD51-independent (KLEIN 2001; Table 6) suggesting that the rad3-102 allele may stimulate a RAD51-independent form of BIR. In support of this notion, rad3-102 is not synthetically lethal in combination with the rad51-null allele (M. S. NAVARRO and A. M. BAILIS, unpublished results). Interestingly, when we modified the interhomolog recombination assay such that gene conversion could be distinguished from BIR, we observed that a six- to seven-fold increase in gene conversion and BIR in a rad3-102 diploid was suppressed in a rad3-102 rad51-null diploid (Table 6). This may suggest that the large stimulation in interhomolog recombination observed in the rad3-102 rad27-null diploids occurs by a Rad51-dependent mechanism. However, this hypothesis cannot be directly addressed due to the inviability of rad27-null rad51-null double mutant cells (DEBRAUWERE et al. 2001; SYMINGTON 1998; TISHKOFF et al. 1997).

The high degree of conservation of the DNA replication and repair apparatus throughout eukaryotic phylogeny supports the speculation that similar genetic or pharmacological disruptions of Okazaki fragment maturation and processing in human cells could lead to massive increases in LOH, and the initiation of carcinogenesis. In fact, such collisions between pharmacology and genotype may help to explain differential responses to chemotherapeutic drugs, some of which disrupt DNA synthesis in a manner
that may elicit unforeseen DNA repair responses. We are further pursuing the role that
Rad3, and, by extension, its human homolog Xpd, may play in the response to replication
lesions at the DNA level in the hope of better understanding the link between DNA
replication and LOH through homologous recombination.
ACKNOWLEDGMENTS

This work was supported by U.S. Public Health Service grant GM57484 to A.M.B, and F31-GM067568 to M.S.N., as well as funds from the Beckman Research Institute of the City of Hope and the City of Hope National Medical Center.

We thank J. Haber, M. Lisby, R. Rothstein, J. McDonald, M. Fasullo, B. Montelone, R. Malone, B. Montelone, and R. Kolodner for strains and plasmids. We also thank M. C. Negritto, L. Hoopes, P. Frankel and members of the Bailis laboratory for stimulating discussions. We would also like to thank several anonymous reviewers for suggesting improvements to the manuscript.
LITERATURE CITED


Mutations in yeast replication proteins that increase CAG/CTG expansions also increase repeat fragility. Mol. Cell. Biol. 23: 7849-7860.


CHEN, S., A. D. DAVIES, D. SAGA, and H. D. ULRICH, 2005 The RING finger ATPase Rad5p of Saccharomyces cerevisiae contributes to DNA double-strand break repair in a ubiquitin-independent manner.


DAIGAKU, Y., S. MASHIKO, K. MISHIBA, S. YAMAMURA, A. Ui et al., 2006 Loss of heterozygosity in yeast can occur by ultraviolet irradiation during the S phase of the cell cycle. Mutat. Res. 600: 177-183.


requirement for homologous recombination in the absence of Rad27 activity.


DONG, Z., and M. FASULLO, 2003 Multiple recombination pathways for sister chromatid exchange in *Saccharomyces cerevisiae*: role of *RAD1* and the *RAD52* epistasis group genes. Nucleic Acids Res. 31: 2576-2585.


FASULLO, M., T. BENNET, and P. DAVE, 1999 Expression of *Saccharomyces cerevisiae MATα* and *MAT* alpha enhances the HO-endonuclease stimulation of chromosomal rearrangements directed by *his3* recombinational substrates. Mutat. Res. 433: 33-44.


FRANK, G., J. QIU, M. SOMSOUK, Y. WENG, L. SOMSOUK et al., 1998 Partial functional
deficiency of E160D flap endonuclease-1 mutant in vitro and in vivo is due to

FRIEDL, A. A., B. LIEFSHITZ, R. STEINLAUF, and M. KUPIEC, 2001 Deletion of the SRS2
gene suppresses elevated recombination and DNA damage sensitivity in rad5 and

FRIIS, J., and H. ROMAN, 1968 The effect of the mating-type alleles on intragenic

GALLI, A., T. CERVELLI, and R. H. SCHIESTL, 2003 Characterization of the
hyperrecombination phenotype of the pol3-t mutation of Saccharomyces

GAME, J., and R. K. MORTIMER, 1974 A genetic study of X-ray sensitive mutants in

GARFINKEL, D. J., and A. M. BAILIS, 2002 Nucleotide excision reapir, genome stability,
60.

GOLIN, J. E., and M. ESPOSITO, 1977 Evidence for joint genic control of spontaneous

GRAY, J. W., and C. COLLINS, 2000 Genome changes and gene expressions in human


KLEIN, H. L., 2001 Spontaneous chromosome loss in *Saccharomyces cerevisiae* is suppressed by DNA damage checkpoint functions. Genetics 159: 1501-1509.


LOPES, J., C. RIBEYRE, and A. NICOLAS, 2006a Complex minisatellite rearrangements generated in the total or partial absence of Rad27/hFEN1 activity occur in a single generation and are Rad51 and Rad52 dependent. Mol. Cell. Biol. 26: 6675-6689.


MALONE, R. E., and M.F. HOEKSTRA, 1984 Relationships between a hyper-rec mutation 

MALONE, R. E., B. A. MONTELONE, C. EDWARDS, K. CARNEY, and M. F. HOEKSTRA,
1988 A reexamination of the role of the RAD52 gene in spontaneous mitotic 

MCEACHERN, M. J., and J. E. HABER, 2006 Break-induced replication and 

MERRILL, B. J., and C. HOLM, 1998 The RAD52 recombinational repair pathway is 
essential in pol30 (PCNA) mutants that accumulate small single-stranded DNA 

MICHEL, B., G. GROMPONE, M. FLORES, and V. BIDNENKO, 2004 Multiple pathways 

MITELMAN, F., B. JOHANSSON, and F. MERTENS, 1994 Catalog of Chromosomal 


MONTELONE, B. A., and R. E. MALONE, 1994 Analysis of the rad3-101 and rad3-102 
mutations of Saccharomyces cerevisiae: Implications for structure/function of 


REFSLAND, E. W., and D. M. LIVINGSTON, 2005 Interactions among DNA ligase 1, the flap endonuclease and proliferating cell nuclear antigen in the expansion and contraction of CAG repeat tracts in yeast. Genetics 171: 923-934.


### Table 1. Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABX471-2C</td>
<td>$MAT \alpha_{hom3-10} \text{ lys}2\text{-}Bgl \text{ CAN1}$</td>
<td>This study</td>
</tr>
<tr>
<td>ABX474-4B</td>
<td>$MAT \alpha_{hom3-10} \text{ lys}2\text{-}Bgl \text{ CAN rad27::LEU2}$</td>
<td>This study</td>
</tr>
<tr>
<td>ABX460-6A</td>
<td>$MAT \alpha_{hom3-10} \text{ lys}2\text{-}Bgl, \text{ CAN rad3-102}$</td>
<td>This study</td>
</tr>
<tr>
<td>ABX481-1C</td>
<td>$MAT \alpha_{hom3-10} \text{ lys}2\text{-}Bgl \text{ CAN rad27::LEU2 rad3-102}$</td>
<td>This study</td>
</tr>
<tr>
<td>ABX761</td>
<td>$MAT \alpha/\alpha_{CAN1/can1-100 \text{ his}3\text{-}\Delta200/\text{HIS3 trp1-1/trp1-1:his3-\Delta3':his3-\Delta5':URA3 rad3-102/RAD3 rad27::LEU2/RAD27}$</td>
<td>This study</td>
</tr>
<tr>
<td>ABX1465</td>
<td>$MAT \alpha/\alpha_{his3::URA3::his3/\text{HIS3 rad3-102/RAD3}}$</td>
<td>This study</td>
</tr>
<tr>
<td>ABX1368</td>
<td>$MAT \alpha/\alpha_{his3\Delta-200/his3::URA3::his3 \text{ TRP1/trp1-1 rad27::LEU2/RAD27}}$</td>
<td>This study</td>
</tr>
<tr>
<td>ABX1308</td>
<td>$MAT \alpha/\alpha_{CAN1/can1-100 \text{ his}3\Delta-200/his3::URA3::his3 rad27::LEU2/RAD27 rad3-102/RAD3}$</td>
<td>This study</td>
</tr>
<tr>
<td>ABX633</td>
<td>$MAT \alpha_{can1-100/CAN1 \text{ hom3-10/HOM3 HIS3/his3-11, 15, trp1-1/TRP1, URA3/ura3-1 LYS2/lys2\Delta-Bgl}}$</td>
<td>This study</td>
</tr>
<tr>
<td>ABX647</td>
<td>$MAT \alpha_{can1-100/CAN1 \text{ HOM3/hom3-10 HIS3/his3::ura3::LEU2 TRP1/trp1-1 rad3-102/rad3-102}}$</td>
<td>This study</td>
</tr>
<tr>
<td>Strain</td>
<td>Description</td>
<td>Source</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------------------------------------</td>
<td>--------------</td>
</tr>
<tr>
<td>ABX658</td>
<td>MAT α/α. can1-100/CAN1 HOM3/hom3-10 TRP1/trp1-1</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>his3::URA3::his3/HIS3 rad27::LEU2/rad27::LEU2</td>
<td></td>
</tr>
<tr>
<td>ABX693</td>
<td>MAT α/α. can1-100/CAN1 HOM3/hom3-10 ura3-1/URA3 LYS2/lys2ΔBgl rad3-102/rad3-102 rad27::LEU2/rad27::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>ABX861</td>
<td>MAT α/α. ADE2/ade2-1 his3::URA3::his3/HIS3 rad3-102/RAD3 rad27::LEU2/RAD27 RAD52/RAD52-YFP</td>
<td>This study</td>
</tr>
<tr>
<td>W961-5A</td>
<td>MAT α HIS3</td>
<td>John McDonald</td>
</tr>
<tr>
<td>ABX362-14C</td>
<td>MAT α rad3-102</td>
<td>This study</td>
</tr>
<tr>
<td>ABX217-3C</td>
<td>MAT α HIS3 rad27::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>ABX397-3A</td>
<td>MAT α rad3-102 rad27::leu2::hisG</td>
<td>This study</td>
</tr>
<tr>
<td>ABX447</td>
<td>MAT α/α. leu2-3, 112 RAD27/rad27::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>ABX430</td>
<td>MAT α/α. rad3-102/rad3-G595R</td>
<td>This study</td>
</tr>
<tr>
<td>ABX1869</td>
<td>MAT α/α. ADE2/ade2-1, CAN1/can1-100, his3-11, 17/HIS3, TRP1/trp1-1, leu2-3, 112 rad27::LEU2/RAD27 RAD3/rad3-102</td>
<td>This study</td>
</tr>
<tr>
<td>ABT576</td>
<td>MAT α, LEU2, pRS416 (URA3)</td>
<td>This study</td>
</tr>
<tr>
<td>ABT577</td>
<td>MAT α, LEU2, pJM3 (MATα, URA3)</td>
<td>This study</td>
</tr>
<tr>
<td>ABT581</td>
<td>MAT α, HIS3, rad27::LEU2, rad3-102, pRS416 (URA3)</td>
<td>This study</td>
</tr>
<tr>
<td>ABT582</td>
<td>MAT α, HIS3, TRP1, rad27::LEU2, rad3-102, pJM3 (MATα, URA3)</td>
<td>This study</td>
</tr>
<tr>
<td>Strain</td>
<td>Genotype Description</td>
<td>Reference</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------------------------------------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>ABX1358</td>
<td>MATa/α his3::URA3::his3/his3-Δ200, TRP1/trp1-1</td>
<td>This study</td>
</tr>
<tr>
<td>ABX1369</td>
<td>MATa/α his3::URA3::his3/his3-Δ200, TRP1/trp1-1, rad3-102/rad3-102</td>
<td>This study</td>
</tr>
<tr>
<td>ABX1362</td>
<td>MATa/α his3::URA3::his3/his3-Δ200, TRP1/trp1-1, rad27::LEU2/rad27::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>ABX2010</td>
<td>MATa/α his3::URA3::his3/his3-Δ200, TRP1/trp1-1, rad27::LEU2/rad27::LEU2, rad3-102/rad3-102</td>
<td>This study</td>
</tr>
<tr>
<td>ABX1498</td>
<td>MATa/α can1-100/CAN1, hom3-10/HOM3, ura3::KANMX/ura3::KANMX, HXT13/hxt13::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>ABX1204</td>
<td>MATa/α can1-100/CAN1, hom3-10/HOM3, ura3::KANMX/ura3::KANMX, HXT13/hxt13::URA3, rad3-102/rad3-102</td>
<td>This study</td>
</tr>
<tr>
<td>ABX1175</td>
<td>MATa/α can1-100/CAN1, hom3-10/HOM3, ura3::KANMX/ura3::KANMX, HXT13/hxt13::URA3, rad51::LEU2/rad51::LEU2</td>
<td>This study</td>
</tr>
<tr>
<td>ABX1611</td>
<td>MATa/α can1-100/CAN1, hom3-10/HOM3, HIS3/HIS3, ura3::KANMX/ura3::KANMX, HXT13/hxt13::URA3, rad51::LEU2/rad51::LEU2, rad3-102/rad3-102</td>
<td>This study</td>
</tr>
</tbody>
</table>
All strains were isogenic to W303-1A (MAT a ade2-1 can1-100 his3-11,17 leu2-3,112 trp1-1 ura3-1 rad5-G535R) (THOMAS and ROTHSTEIN 1989). Only deviations from this genotype are listed.
**Table 2. Mutation rate analysis in wild type and mutant strains.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Can^r (x10^{-7})</th>
<th>Hom^+ (x10^{-7})</th>
<th>Lys^+ (x10^{-7})</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABX471-2C</td>
<td>wild type</td>
<td>4.0 (3.8-4.4)</td>
<td>0.26 (0.21-0.29)</td>
<td>0.32 (0.27-0.42)</td>
</tr>
<tr>
<td>ABX460-6A</td>
<td>rad3-102</td>
<td>5.7 (4.8-7.5)</td>
<td>1.9 (1.6-2.6)</td>
<td>2.6 (1.9-3.2)</td>
</tr>
<tr>
<td>ABX474-4B</td>
<td>rad27</td>
<td>64 (54-88)</td>
<td>2.2 (1.3-3.0)</td>
<td>5.3 (3.6-7.6)</td>
</tr>
<tr>
<td>ABX481-1C</td>
<td>rad3-102 rad27</td>
<td>69 (47-85)</td>
<td>1.7 (0.85-2.0)</td>
<td>4.0 (2.9-4.8)</td>
</tr>
</tbody>
</table>

*a All rates were determined from a minimum of 30 trials as described in the Materials and Methods. 95% confidence intervals are indicated in parentheses.
Table 3. Recombination and chromosome loss rates in wild type and mutant strains.

<table>
<thead>
<tr>
<th></th>
<th>Haploid</th>
<th>Diploid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relevant genotype</td>
<td>USCR(^a)  (\times 10^{-6})</td>
<td>DRR(^a) (\times 10^{-5})</td>
</tr>
<tr>
<td>wild type</td>
<td>1 (0.8-1.2)</td>
<td>6.6 (5.5-7.8)</td>
</tr>
<tr>
<td>rad3-102</td>
<td>3.3 (1.3-5.6)</td>
<td>12 (11-13)</td>
</tr>
<tr>
<td>rad27</td>
<td>46 (26-61)</td>
<td>44 (32-59)</td>
</tr>
<tr>
<td>rad3-102 rad27</td>
<td>79 (54-107)</td>
<td>84 (32-220)</td>
</tr>
</tbody>
</table>

\(^a\) USCR and DRR rates were determined from a minimum of 15 trials as described in the Materials and Methods section using haploids dissected from ABX761, ABX1308, ABX1368, and ABX1465. 95% confidence intervals are indicated in parentheses.

\(^b\) IHR and CL rates were determined from a minimum of 35 trials as described in the Materials and Methods section using diploids ABX633, ABX647, ABX658, and ABX693.

\(^c\) DRR rates were determined from a minimum of seven trials as described in the Materials and Methods using diploids ABX1358, ABX1369, ABX1362, and ABX2010.
Table 4. Rad52-YFP focus formation in wild type and mutant cells.

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>S-phase</th>
<th>G2/M-phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>6.6 ± 5.3</td>
<td>4.1 ± 1.8</td>
</tr>
<tr>
<td>rad3-102</td>
<td>23 ± 3.8</td>
<td>14 ± 1.5</td>
</tr>
<tr>
<td>rad27</td>
<td>23 ± 7.6</td>
<td>47 ± 10</td>
</tr>
<tr>
<td>rad3-102 rad27</td>
<td>47 ± 8.0</td>
<td>68 ± 2.9</td>
</tr>
</tbody>
</table>

*Percentage of cells containing a focus was measured by dividing the number of cells in S- or G2/M phase containing one or more foci by the total number of S- or G2/M cells and is reported as the median percentage ± two standard errors of at least five trials. Each trial consisted of at least 50 cells.*
Table 5. Doubling times of wild type and mutant strains.$^a$

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>Haploid DT ± 2SE (min)</th>
<th>Diploid DT ± 2SE (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>94±3.9</td>
<td>95±2.0</td>
</tr>
<tr>
<td>wild type + pRS416</td>
<td>102±2.7</td>
<td>ND</td>
</tr>
<tr>
<td>wild type + pJM3</td>
<td>96±3.4</td>
<td>ND</td>
</tr>
<tr>
<td>rad3-102</td>
<td>105±3.9</td>
<td>98±3.6</td>
</tr>
<tr>
<td>rad27</td>
<td>114±8.0</td>
<td>130±4.7</td>
</tr>
<tr>
<td>rad3-102 rad27</td>
<td>175±11</td>
<td>130±10</td>
</tr>
<tr>
<td>rad3-102 rad27 + pRS416</td>
<td>192±11</td>
<td>ND</td>
</tr>
<tr>
<td>rad3-102 rad27 + pJM3</td>
<td>189±11</td>
<td>ND</td>
</tr>
</tbody>
</table>

$^a$ Doubling times were determined at 30$^\circ$ as described in the Materials and Methods and are reported as the median doubling time ± two standard errors in minutes, from at least five independent trials.
Table 6. BIR and GC rates in wild type and mutant diploids.

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>BIR Rate&lt;sup&gt;a&lt;/sup&gt; (x10&lt;sup&gt;-5&lt;/sup&gt;)</th>
<th>GC Rate&lt;sup&gt;a&lt;/sup&gt; (x10&lt;sup&gt;-5&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>1.3 (0.93-1.8)</td>
<td>0.22 (0.17-0.30)</td>
</tr>
<tr>
<td>rad3-102</td>
<td>8.5 (7.3-11)</td>
<td>1.3 (0.97-1.6)</td>
</tr>
<tr>
<td>rad51</td>
<td>3.5 (2.9-5.0)</td>
<td>0.37 (0.24-0.75)</td>
</tr>
<tr>
<td>rad3-102 rad51</td>
<td>4.6 (3.4-6.5)</td>
<td>0.47 (0.24-0.89)</td>
</tr>
</tbody>
</table>

<sup>a</sup> BIR and GC rates were determined from a minimum of seven trials in the diploids ABX1498, ABX1204, ABX1175, and ABX1611. 95% confidence intervals are indicated in parentheses.
Figure 1. Model of the consequences of DNA replication fork failure in \textit{rad27}-null mutant cells. (A) During DNA replication, the 5’ RNA/DNA flaps (gray line) of Okazaki fragments generated on the lagging-strand are inefficiently processed in the \textit{rad27}-null mutant. (B) The 5’ RNA/DNA flap may be displaced by mutagenic polymerases and later cleaved by other endo-/exonucleases. (C) Alternatively, the 3’ end of the next Okazaki fragment might interact with the sister-chromatid to facilitate synthesis past the unligateable flap that is later displaced by the newly synthesized strand and degraded by exonucleases. (D) Unligated ends may persist until the next round of replication where they will serve as the leading strand template and consequently force the fork to collapse, creating single-ended DSBs that may not be optimal substrates for template switching or direct-repeat recombination. (E) This often results in chromosome loss and death in haploids, whereas, (F) in diploid cells, recombination with the homolog enables restart of the replication fork. Defective exonucleolytic digestion of residual flaps in \textit{rad3-102 rad27}-null double mutants increases the incidence of unligated ends and results in synergistic increases in chromosome loss and interhomolog recombination in diploids, but kills haploids.
Figure 1.

A. 

B. 

C. 

D. 

E. 

F.

- Mutagenic bypass
- Mutations

- Sister-chromatid interaction
- USCR

- Replication fork stalling
- Fork collapse

- DSB formation
- Chromosome loss, death in haploids

- DSB formation
- Interhomolog recombination, replication