Genome instability in rad54 mutants of Saccharomyces cerevisiae

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ABSTRACT

The RAD54 gene of Saccharomyces cerevisiae encodes a conserved dsDNA-dependent ATPase of the Swi2/Snf2 family with a specialized function during recombinational DNA repair. Here we analyzed the consequences of the loss of Rad54 function in vegetative (mitotic) cells. Mutants in RAD54 exhibited drastically reduced rates of spontaneous intragenic recombination but were proficient for spontaneous intergenic recombinant formation. The intergenic recombinants likely arose by a RAD54-independent pathway of break-induced replication. Significantly increased rates of spontaneous chromosome loss for diploid rad54/rad54 cells were identified in several independent assays. Interestingly, the increase in chromosome loss appeared to depend on the presence of a homolog. In addition, the rate of complex genetic events involving chromosome loss were drastically increased in diploid rad54/rad54 cells. Together, these data suggest a role for Rad54 protein in the repair of spontaneous damage, where in the absence of Rad54 protein, homologous recombination is initiated but not properly terminated, leading to misrepair and chromosome loss.

INTRODUCTION

In Saccharomyces cerevisiae double-stranded DNA breaks (DSBs) are primarily repaired by homologous recombination (HR). In vegetative (mitotic) wild-type cells, the primary HR pathway appears to be gene conversion without associated crossing-over, whereas other mechanisms such as break-induced replication (BIR) and single-strand annealing (SSA) may become more prominent with special DNA substrates (e.g. direct and inverted repeats) or in certain mutants (1–4). DSB repair by a conversion-type mechanism initiates with processing of the DSB to produce 3' ssDNA tails that are presumably covered with the eukaryotic ssDNA-binding protein RPA. The mediator proteins, Rad52 and the Rad55/ Rad57 complex, catalyze the exchange of the RPA filament for the Rad51:ssDNA filament, which is active in homology search and DNA strand exchange (5). Strand invasion and heteroduplex DNA (hDNA) formation by the Rad51:ssDNA filament are enhanced by its interaction with the Rad54 protein (6–9). Rad54 protein also augments hDNA extension in Rad51 protein-mediated DNA strand exchange (10). Thus, biochemical experiments using purified proteins suggest a role of Rad54 protein in HR after the initial DSB processing and assembly of the Rad51 nucleoprotein filament. This notion is supported by genetic and cytological data that place RAD54 downstream of RAD51, RAD52, RAD55 and RAD57 in the recombination pathway (4,11–14).

Rad54 protein is a member of the Swi2/Snf2 family of DNA-dependent ATPases that modulate protein:dsDNA interactions in various molecular processes (15). Inactivation of RAD54, as well as of RAD51 and RAD52, leads to extreme sensitivity to DSBs. Genetically, RAD52 is required for almost all forms of HR, whereas the requirement for RAD51 and RAD54 is not as strict. Most studies with rad54 cells have been performed using repeat recombination substrates. An increase or decrease of direct repeat recombination, depending on the particular assay used (16–20), and a consistent decrease in two inverted repeat systems (3,11) were described. The limited data with natural chromosomes suggested a decrease in spontaneous intragenic recombination in two studies using the identical assay system (19,21) but an increase in intergenic recombination and chromosome loss (22). During meiotic recombination, the effect of a rad54 mutation is far less pronounced due to the function of the TID1 gene which encodes a protein highly related to Rad54 (19,21,23–25). The effects of a tid1 mutation during vegetative growth are somewhat strain-dependent and less severe compared with the DNA damage sensitivity of a rad54 mutant (19,23). It has been proposed that Rad54 protein functions primarily in recombination between sister chromatids, whereas Tid1 protein mediates primarily recombination between homologs (19,21,23).

Given the critical function of Rad54 protein during DSB repair by HR, we were interested to study the effect of rad54 null mutations on recombination and genome stability involving natural chromosomes during vegetative growth of S.cerevisiae cells in the absence of external genotoxic stress.
DSBs and other types of DNA damage (e.g. ssDNA gaps) that are repaired by HR may occur during growth even in the absence of external genotoxic stress (4). We found that Rad54 protein is important for proper recombination between homologous chromosomes. Chromosome loss and complex genetic events that included chromosome loss were greatly elevated in diploid rad54/rad54 cells. Strikingly, chromosome loss was only found elevated in diploid cells containing two copies of the test chromosome. This suggests that genomic instability in rad54 cells is caused not by the absence of HR but by misrepair leading to elevated mutation and chromosome loss rates.

**MATERIALS AND METHODS**

**Strains, plasmids and media**
The *S. cerevisiae* strains used in this study are listed in Table 1. Deletion/substitution alleles were constructed by the one-step gene disruption method (26). To disrupt the RAD54 gene, plasmids pSM31 (rad54–:LEU2) (kindly supplied by Dr. D. Schuld) and pWDH252 (rad54–:URA3) were digested with *Stu*I/*Bgl*II and *Xba*I/*Eco*RI, respectively, to transform strains selecting for the respective marker gene. The marker genes replaced a 1.9 kb internal *Bam*HI fragment, eliminating approximately 2/3 of the RAD54 coding region. The *rad54–::Kan^R* mutation substituting the RAD54 coding region between the first 10 and last 27 amino acids with the kanamycin-resistance gene was created by PCR product-mediated transformation (27). In the *his1–*::*Kan^R* allele, the entire *HIS1* open-reading frame was replaced by the kanamycin-resistance gene (27). All deletion/disruption alleles were verified by appropriate Southern blot analysis. Plasmid YCp50-RAD54 (pWDH205) was constructed by inserting the *Pst*I–*Sal*I RAD54 fragment of YEp13-RAD54-216A (28) into *Sal*I–*Eco*RI cleaved YCp50 (29) after converting all ends with Klenow DNA polymerase to blunt ends. Standard media and growth conditions were used (30). L-Canavanine sulfate (60 mg/l) was added to synthetic media lacking arginine cooled to 60°C.

**Mitotic recombination, chromosome loss and mutation rates**
Rates of spontaneous mitotic recombination were determined in fluctuation tests by the median method (31). Nine independent single colonies were analyzed in each experiment. All diploid strains were freshly constructed before each experiment. To determine mitotic intragenic recombination rates, single colonies were picked and inoculated in 10 ml of YPD medium and incubated at 30°C for 20 h. Cells (5 × 10^6^) were collected by centrifugation, resuspended in 1.6 ml of H_2O, and appropriate dilutions were plated on selective plates to determine the number of recombinants and on full medium to determine the total live cell count. Plates were scored after incubation for 4 days at 30°C. Mitotic intergenic recombination and chromosome loss rates for chromosome V markers were determined by measuring the rates of canavanine-resistant colonies in a fluctuation test after incubation for 4 days at 30°C. Genotypes of colonies were inferred from phenotypic analysis on the appropriate media to follow all chromosome V markers. Due to the extremely low rate, the p(0) method (31) was used to determine the wild type rate in Figure 5.

**Rates of chromosome fragment loss**
The rates were determined by fluctuation tests using the method of the median (31). Single colonies were grown on SD-uracil to select for the presence of the chromosome fragment (CF) and inoculated in 1 ml of YPD. After growth for 20 h at 30°C, ~200 cells were spread per YPD plate. Plates were scored after 4 days of incubation at 30°C and 1 day at 4°C. Nine independent colonies were used per data point. To determine the rate for the loss of a single CF in a diploid cell, white colonies grown on YPD plates were taken, as they contain two copies of the CF (32).

**Rates of YAC loss**
The rates of yeast artificial chromosome (YAC) instability were determined by the method of the median (31). Briefly, single yeast colonies harboring the YACs were resuspended in water and appropriate dilutions were plated onto YPD. After 16–24 h of growth at 30°C, five to nine colonies were resuspended in water, and an appropriate dilution was plated on YPD for total cell counts, while the remaining suspension or dilution was plated on selective complete media containing 1 mg of 5-fluoro-orotic acid per milliliter. To ensure reproducibility, a minimum of three repetitions of the fluctuation assay were performed per strain, and at least three independently isolated clones were tested.

**Targeted integration assay**
PCR product-mediated transformation was performed as described (27) using the transformation protocol of Schiestl *et al.* (33). Transformants were analyzed by Southern blotting using the kanamycin-resistance gene as a probe and several restriction enzymes that cleave outside the fragment used for transformation. PCR primer P1 (5′-biotin-gATTCgATA-CTAACgCCgC3-′) was biotinylated to allow direct sequencing of the genomic amplification products with primer P2 (5′-CTACCgTCAgAAACTgCCTgTAC-3′) as described (34).

**UV survival**
UV survival curves were performed at fluencies of 0–150 J/m^2^ with cells spread on YPD plates. Plates were incubated in the dark at 30°C and scored after 4 days.

**Statistical analysis**
The ranking method described by Wierdl *et al.* (35) was used to determine the statistical significance of differences in the rate data.

**RESULTS**

**RAD54 is important for spontaneous intragenic recombination between homologs**

RAD54 is an important member of the recombinational DNA repair pathway in *S. cerevisiae*. Mutants in this gene show an almost complete deficiency in DSB repair. We wanted to ascertain the function of RAD54 in spontaneous intragenic mitotic recombination between homologs using heteroallelic...
URA3 chromosome loss in Spontaneous intergenic mitotic recombination and mutants are severely defective in gene conversion between rad54 between homologs. Since intragenic recombination is largely wild-type strains (Fig. 2B). The upper chromosome was determined the rates for mitotic intergenic recombination chromosome V [see Fig. 2A; derived from (36)], we sion, and crossing-over (4). Using a marker system on various mechanisms, including BIR, long tract gene conver-

Table 1. Saccharomyces cerevisiae strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>FF18728</td>
<td>MATa lys9 met2 leu2-3,112</td>
</tr>
<tr>
<td>FF1873</td>
<td>MATa leu2-3,112 trpl-289 ura3-52 his7-2 lys1-1</td>
</tr>
<tr>
<td>FF18734</td>
<td>MATa leu2-3,112 trpl-289 ura3-52 his7-2 lys1-1</td>
</tr>
<tr>
<td>FF18970</td>
<td>MATa leu2-3,112 trpl-289 ura3-52 his7-2 lys1-1 rad54-Δ::LEU2</td>
</tr>
<tr>
<td>FF18974</td>
<td>MATa leu2-3,112 trpl-289 ura3-52 his7-2 lys1-1 rad54-Δ::LEU2</td>
</tr>
<tr>
<td>FF18984</td>
<td>MATa leu2-3,112 ura3-52 his7-1 lys2-1</td>
</tr>
<tr>
<td>JH320</td>
<td>MATa ura3-52 lys2-801 ade2-101 trpl-Δ cyh2Δ +CF [CEN6 URA3 SUP11 CYH2Δ]</td>
</tr>
<tr>
<td>REE209</td>
<td>MATa ura3-13 ade2 ade5 can1 leu1-12 trp5-d his7-2 tyr1-1 lys2-1 met13-1</td>
</tr>
<tr>
<td>REE218</td>
<td>MATa ura3-1 ade2 cyh2 leu1-1 trp5-c his7-1 tyr1-2 lys2-2 met13-c</td>
</tr>
<tr>
<td>WDHY545</td>
<td>MATa leu2-3,112 ura3-52 his7-1 lys2-1 rad54-Δ::LEU2</td>
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<tr>
<td>WDHY707</td>
<td>MATa lys9 met2 leu2-3,112 rad54-Δ::LEU2</td>
</tr>
<tr>
<td>WDHY768</td>
<td>MATa ura3-13 ade2 ade5 can1 leu1-12 trp5-d his7-2 lys1-1 met13-1 rad54-Δ::URA3</td>
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<tr>
<td>WDHY769</td>
<td>MATa ura3-1 ade2 cyh2 leu1-1 trp5-c his7-1 tyr1-2 lys2-2 met13-c rad54-Δ::URA3</td>
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<tr>
<td>WDHY909</td>
<td>MATa ura3-52 lys2-801 ade2-101och trpl-Δ cyh2Δ [CF (CEN6) URA3 SUP11 CYH2Δ]</td>
</tr>
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<td>WDHY910</td>
<td>MATa ura3-52 lys2-801 ade2-101och trpl-Δ cyh2Δ [CF (CEN6) URA3 SUP11 CYH2Δ]</td>
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<td>WDHY980</td>
<td>MATa leu2-3,112 trpl-289 ura3-52 his7-2 lys1-1 trpl-Δ::Kan8</td>
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<tr>
<td>WDHY982</td>
<td>MATa leu2-3,112 trpl-289 ura3-52 his7-2 lys1-1 trpl-Δ::Kan8 rad54-Δ::LEU2</td>
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<td>WDHY1074</td>
<td>MATa can1R ura3-52 his1-Δ::Kan8 iso ade5 lys1 leu2-13 trpl-289</td>
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<tr>
<td>WDHY1138</td>
<td>MATa can1R ura3-52 his1-Δ::Kan8 iso ade5 lys1 leu2-13 trpl-289 rad54-Δ::LEU2</td>
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<tr>
<td>WDHY1216</td>
<td>MATa ura3-52 lys2-801 ade2-101 trpl-1 cyh2Δ +CF [CEN6 URA3 SUP11 CYH2Δ] rad54-Δ::Kan8</td>
</tr>
<tr>
<td>WDHY1246</td>
<td>MATaMATat CAN1/CAN1 ura3/URA3 his1-Δ::Kan8 HIS1 ilv1/ILV1 leu2/leu2 lys1/LYS1 lys9/LYS9 trpl-1 TRP1 met2/MET2 ade5/ADE5</td>
</tr>
<tr>
<td>WDHY1251</td>
<td>MATaMATat CAN1/CAN1 ura3/URA3 his1-Δ::Kan8 HIS1 ilv1/ILV1 leu2/leu2 lys1/LYS1 lys9/LYS9 trpl-1 TRP1 met2/MET2 ade5/ADE5 rad54-Δ::LEU2</td>
</tr>
<tr>
<td>WDHY1657</td>
<td>MATaMATat leu2/leu2 ade2/ade2 lys2/lys2 trpl/trpl his4/HIS4 ura3/ura3 can1/CAN1 [YAC 18ED5::URA3::ADE2, YAC WDX4932::LEU2::TRP1]</td>
</tr>
<tr>
<td>WDHY1700</td>
<td>MATaMATat leu2/leu2 ade2/ade2 lys2/lys2 trpl/trpl his4/HIS4 ura3/ura3 can1/CAN1 [YAC 18ED5::URA3::ADE2, YAC 18ED5::LEU2::TRP1]</td>
</tr>
<tr>
<td>WDHY1792</td>
<td>MATaMATat leu2/leu2 ade2/ade2 lys2/lys2 trpl/trpl his4/HIS4 ura3/ura3 can1/CAN1 rad54-Δ::Kan8 rad54-Δ::Kan8 [YAC 18ED5::URA3::ADE2, YAC 18ED5::LEU2::TRP1]</td>
</tr>
<tr>
<td>WDHY1805</td>
<td>MATaMATat Leu2/Leu2 ade2/ade2 lys2/lys2 trpl/trpl his4/HIS4 ura3/ura3 can1/CAN1 rad54-Δ::Kan8 rad54-Δ::Kan8 [YAC 18ED5::URA3::ADE2, YAC WDX4932::LEU2::TRP1]</td>
</tr>
</tbody>
</table>

All FF strains were kindly supplied by Dr F. Fabre. The REE strains were kindly provided by Dr R. Easton Esposito. JH320 was a kind gift of Dr H. Hegemann. All WDHY strains were constructed during this study.

mutations in five different genes. Rate determinations demonstrated a significant decrease in prototrophic recombinant formation in diploid rad54/rad54 strains compared with their isogenic wild-type strains (Fig. 1). Depending on the strain background, the rate decrease was 14-fold or 6-fold for the his7-1/his7-2 heteroalleles. The rate decreases for the TYR1, URA3, TRP5 and LEU1 genes ranged from 5- to 33-fold (Fig. 1). This is consistent with previous reports of a 10- to 12-fold reduction in prototroph formation between HIS4 heteroalleles (19,21). From these data, we conclude that RAD54 plays an important role in mitotic intragenic recombination between homologs. Since intragenic recombination is largely achieved by gene conversion (4), we conclude that rad54 mutants are severely defective in gene conversion between homologs.

Spontaneous intergenic mitotic recombination and chromosome loss in rad54/rad54 cells

Intergenic recombinants in vegetative cells can arise by various mechanisms, including BIR, long tract gene conversion, and crossing-over (4). Using a marker system on chromosome V [see Fig. 2A; derived from (36)], we determined the rates for mitotic intergenic recombination and chromosome loss in diploid rad54/rad54 and isogenic wild-type strains (Fig. 2B). The upper chromosome was marked by the recessive canavanine-resistance marker. CAN1 codes for the arginine permease of S. cerevisiae, and CAN1 mutants cannot take up arginine or its toxic analog canavanine, leading to canavanine resistance. In addition, this chromosome carried three additional recessive markers (ura3, his1, ilv1) on both sides of the centromere and a dominant kamycin-resistance marker (Kan8) replacing the HIS1 gene on the right arm of chromosome V. The lower chromosome was wild-type for all yeast markers but lacked the kamycin-resistance gene. After selecting for canavanine-resistant colonies, we determined the rates of all phenotypic classes for the chromosome V markers. The overall rate of canavanine-resistant colonies was elevated in rad54/rad54 cells 13-fold. Class 1 (Can8 Ura+ His+ Kan8 Ilv+) can arise from conversion of the CAN1S marker to can1R in G1 or G2, from cross-over between the CAN1 and ura3 genes in G2, or from BIR of CAN1S to can1R in G1 or G2. It was the majority class in wild-type cells, representing 62.2% of all events. This class experienced a slight rate reduction (1.5-fold) in rad54/rad54 cells. This may represent the net result of several independent changes depending on the effect of RAD54 on conversion, BIR, and cross-over. Classes 2, 4 and 5 cannot be explained by a single event and due to the low rates of occurrence are not discussed here. Class 3 (Can8 Ura+ His+ Kan8 Ilv+), which accounts for 29.4% of the wild-type
events, can arise from conversion of the \textit{CAN1}^S and \textit{URA3} markers to \textit{can}^R and \textit{ura}^3 in G1 or G2, by cross-over between \textit{ura}^3 and the centromere in G2, or by BIR of the \textit{CAN1}^S and \textit{URA3} markers in G1 or G2. This class experienced a 2.4-fold rate increase in the rad54 strain. Class 6 (CanR Ura± His± KanR rad54) respectively. Shown are means from five independent determinations. The black and white bars labeled HIS7^* show the data for a one set of strains: wild-type diploid (FF18734xFI18984, WT) and rad54/ rad54 diploid (FF18974xWDHY543, rad54), respectively. Shown are means from three independent determinations. Error bars represent one standard deviation. The extent of the rate differences between the mutant and the wild type is given as fold reduction.

To test if the elevated rate of chromosome V loss in \textit{S.cerevisiae} cells exhibits a defect in spontaneous, intragenic mitotic recombination. Intragenic mitotic recombination rates at the indicated gene loci were determined in two different isogenic wild-type and rad54/rad54 strain pairs by fluctuation tests as described in Materials and Methods. The black and white bars labeled HIS7^* show the data for one set of strains: wild-type diploid (FF18734xFI18984, WT) and rad54/ rad54 diploid (FF18974xWDHY543, rad54), respectively. Shown are means from five independent determinations. The black and white bars labeled HIS7, TYR1, URA3, TRP5 and LEU1 show the data for a second set of strains: wild-type diploid (REE209xREE218, WT) and rad54/rad diploid (WDHY768xWDHY769, rad54), respectively. Shown are means from three independent determinations. Error bars represent one standard deviation. The extent of the rate differences between the mutant and the wild type is given as fold reduction.

Figure 1. Diploid \textit{S.cerevisiae} rad54 cells exhibit a defect in spontaneous, intragenic mitotic recombination. Intragenic mitotic recombination rates at the indicated gene loci were determined in two different isogenic wild-type and rad54/rad54 strain pairs by fluctuation tests as described in Materials and Methods. The black and white bars labeled HIS7^* show the data for one set of strains: wild-type diploid (FF18734xFI18984, WT) and rad54/ rad54 diploid (FF18974xWDHY543, rad54), respectively. Shown are means from five independent determinations. The black and white bars labeled HIS7, TYR1, URA3, TRP5 and LEU1 show the data for a second set of strains: wild-type diploid (REE209xREE218, WT) and rad54/rad diploid (WDHY768xWDHY769, rad54), respectively. Shown are means from three independent determinations. Error bars represent one standard deviation. The extent of the rate differences between the mutant and the wild type is given as fold reduction.

Chromosome loss in \textit{rad54} cells depends on the presence of a homolog

Mutants that increase chromosome loss identify genes which function in different cellular processes, including recombination, DNA replication, and chromosome segregation. To learn more about the role of \textit{RAD54} in chromosome stability, we analyzed chromosome loss in haploid cells. Since all \textit{S.cerevisiae} chromosomes carry essential genes, the loss of a non-essential CF was determined (32). The left arm of the CF was 125 kb in size and is derived from the left arm of chromosome III of \textit{S.cerevisiae}. The right arm of the CF carried \textit{URA3} and the \textit{SUP11}, encoding an ochre-suppressor tRNA, separated by \textit{CEN6} from the left arm. Chromosome loss rates were measured by a visual assay that utilizes the red pigment phenotype of \textit{S.cerevisiae} colonies carrying the ade2-1 ochre mutation. The haploid indicator strain with the CF containing the \textit{SUP11} suppressor formed white colonies because the \textit{ade2-1} mutation was suppressed by the suppressor tRNA, whereas CF loss leads to red colonies. Rate determinations in isogenic haploid wild-type and rad54 strains containing the CF did not reveal a statistically significant difference in the loss of the CF (Table 2). Thus, we conclude that loss of a single chromosome in haploid cells is not increased in \textit{rad54} cells.

Since we identified greatly enhanced chromosome loss in diploid \textit{rad54}/\textit{rad54} cells using a test system with native homologous chromosomes (Fig. 2) but no increase in haploid cells using the CF system, we determined the rate of CF loss in diploid cells. Diploid strains carrying two copies of the CF were isolated as white colonies in which the \textit{ade2-1} mutations were fully suppressed. Loss of a single CF is signaled by the appearance of pink colonies, loss of both CFs leads to red colonies (32). The rates for the loss of one CF in isogenic

Analysis of intergenic recombinants

While intragenic recombination is largely accomplished by a conversion-type mechanism, the intergenic recombinants analyzed in the chromosome V system (Fig. 2B) could arise from either conversion, BIR, or crossing-over. As \textit{rad54}/\textit{rad54} mutants are highly defective in intragenic recombination, we concluded that they drastically reduce conversion. To distinguish whether the intergenic recombinants found in classes 1 and 3 of Figure 2B were generated by BIR or crossing-over in G2, we devised the scheme shown in Figure 3A. Using non-selectively grown cells, we identified colonies containing canavanine-resistant cells by replica plating. The original colony was identified, re-streaked on fresh medium, and single colonies were phenotyped. The rationale to distinguish BIR/conversion from cross-over is shown in Figure 3B. A BIR/conversion event leads to two daughter cells, of which one is resistant (homozygous \textit{can}^1\textit{S}/\textit{can}^1\textit{S}) and the other is heterozygous (\textit{can}^1\textit{S}/\textit{can}^1\textit{S}). The heterozygous daughter cells will give rise to a colony that will form canavanine-resistant papillae (paps), which are easily scored. In contrast, a cross-over event leads to two products, of which one is resistant (homozygous \textit{can}^1\textit{S}/\textit{can}^1\textit{S}) and the other is sensitive (\textit{can}^1\textit{S}/\textit{can}^1\textit{S}). In analyzing 94 events, we did not detect canavanine-sensitive cells, the evidence for the reciprocal cross-over product, in wild-type (0/94). Only four in 77 \textit{rad54}/\textit{rad54} events showed evidence for the reciprocal cross-over product. Thus, we conclude that either cross-over is not the major mechanism to generate mitotic intergenic recombinants or that the cross-over process in mitotic cells is not reciprocal, leading to loss of the unselected chromosome.
diploid wild-type and rad54/rad54 cells containing two CFs were determined to be 13.2 \pm 3 \times 10^{-3} and 33.1 \pm 3 \times 10^{-3}, respectively (Table 2). This represents a 2.5-fold increase in rad54/rad54 cells, which was statistically significant. It is interesting to note that the CF was 2.4-fold less stable in diploid compared to haploid cells even in wild-type cells. We conclude that in diploid rad54/rad54 cells containing two CFs, the CF loss rates increased. Since there was no increase in haploid cells containing one CF, this result suggests that the presence of the homolog contributed to the elevated CF loss rate. In wild-type diploid cells loss of both CFs was never observed (rate estimated to be <2 \times 10^{-4}), whereas in rad54/rad54 cells the rate of losing both CFs was \sim 1 \times 10^{-3}.

The magnitude of the increase in CF loss in diploid cells was less than expected from the data with the native chromosomes III and V (Fig. 2). Several reasons may have contributed to this. For both haploid and diploid wild-type cells, the CF loss rate is already significantly higher than that of natural chromosomes. For comparison, chromosome V is lost at a rate of 3 \times 10^{-6} in wild-type cells (Fig. 2), an over 4000-fold difference relative to the diploid CF loss rate. This may be due to the size difference or to the lack of stabilizing DNA sequences found in natural chromosomes but lacking in the CF. Another contributing factor to the lower stability of the CF might be its significant sequence homology with chromosome III from which it was derived. To overcome this limitation and to test the idea that the presence of a homolog destabilizes chromosomes in rad54/rad54 cells, we devised the system shown in Figure 4A. We made use of YACs that contained inserts of homologous or non-homologous human chromosomes.

### Table 2. Loss of one non-essential CF is increased in diploid but not in haploid rad54 cells

<table>
<thead>
<tr>
<th>Strains</th>
<th>Rate/1000 cell divisions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1n + 1 CF</td>
<td>Wild type: 5.55 (^c)</td>
</tr>
<tr>
<td>2n + 2 CF</td>
<td>Wild type: 13.2 (^d)</td>
</tr>
</tbody>
</table>

\(^a\)Haploid strains were WDHY909 (wild type) and WDHY910 (rad54), diploid strains were WDHY909xJH320 (wild type) and WDHY910x-WDHY1216 (rad54/rad54).

\(^b\)Given are means from two (haploid strains) or three (diploid strain) independent rate determinations.

\(^c\)Difference between wild type and rad54 is statistically not significant (P > 0.05).

\(^d\)Difference between wild type and rad54 is statistically significant (P < 0.01).
The homologous YAC pair 18ED5-URA and 18ED5-LEU differs only in their content of marker genes, whereas 18ED5-URA and WXD4932-LEU represent a heterologous YAC pair. The loss rates of the heterologous and homologous YAC pairs were determined in isogenic wild-type and rad54/rad54 diploid strains by establishing the rate of Ura- cells and determining the proportion of the events where also the second marker gene, ADE2, was lost. In wild-type diploid cells, the loss rates of the homologous and heterologous YAC pairs were very similar, and the difference was not statistically significant (Fig. 4B). In contrast, in rad54/rad54 cells the homologous pair exhibited a significantly higher loss rate of YAC 18ED5-URA than the heterologous pair (P < 0.025). The inherent instability of the WXD4932-URA YAC precluded a meaningful analysis of the reciprocal heterologous and homologous YAC pairs (data not shown). From the CF and YAC loss data we conclude that chromosome loss in rad54 cells is specifically increased by the presence of two homologous chromosomes.

Complex genetic events are highly elevated in rad54/ rad54 cells

The analysis of spontaneous chromosome loss using selected (Fig. 2) and unselected events (data not shown) suggested the possibility that complex genetic events may be more frequent in rad54/rad54 cells. While the previous experiments were not designed to test this question directly, we modified our chromosome V marker system to determine rates of more complex genetic events. As a baseline reference, we first determined the mutation rate in the CAN1 gene (Table 3). rad54 cells showed a statistically significant 2.3-fold increase over the isogenic wild-type. This increase was complemented to wild-type rates, when the wild-type RAD54 gene was re-introduced on a centromeric plasmid (Table 3). This is consistent with published data of a 6-fold increase in the CAN1 mutation rate in a different strain background (18).

To measure the rate of complex genetic events, we determined the rate of canavanine-resistant cells in isogenic diploid wild-type and rad54/rad54 strains homozygous for the CAN13 gene (Fig. 5A). Generation of canavanine-resistant derivatives necessitates at least two genetic events which were inferred from genetic analysis. The rate of canavanine-resistant derivatives was extremely low in wild-type cells (3.1 \times 10^{-10}), as expected (Fig. 5B). In rad54/rad54 cells this rate was elevated 84-fold. Six genotypic classes could be distinguished in the ensuing genetic analysis (Fig. 5B). Class 1 of Figure 5, which represented the great majority (95.8%) of the events in wild-type cells, has likely arisen by a mutation in one CAN13 gene followed by conversion or BIR of the remaining CAN13 gene, or by cross-over between CAN1 and ura3. The rate for this class is elevated 3.7-fold in rad54/rad54 cells, but this class represents only 10.9% of all mutant events. The only two other classes in wild-type show one homolog with a mutation at CAN13 and loss of the upper (class 5 of Fig. 5) or lower (class 6 of Fig. 5) chromosome. Both were found at a very low rate (7 × 10^{-12}) represented by single cases constituting together only 4.2% of the wild-type events. Both class 5 and 6 events (Fig. 5B) were found to exhibit a tremendous rate increase in rad54/rad54 cells of 2206- and 1229-fold, respectively. In addition, three additional classes were identified (classes 2-4 of Fig. 5). They represent together 3.6% of the mutant events and were not found in wild-type cells. These classes can arise by a variety of different pathways that were not further explored due to their low frequency. From this data we conclude that complex genetic events are highly elevated in rad54/rad54 diploid cells.

Gene targeting is reduced, but not absent, in rad54 cells

Gene targeting of linear fragments into the chromosome is mediated by HR. Since Rad54 protein plays an important role in HR, we determined the ability of rad54 cells to integrate a linear fragment that had 332 and 260 bp of homology at its ends (Fig. 6A). We chose the YNL311 gene, whose deletion in a systematic analysis of S. cerevisiae open-reading frames did not demonstrate any appreciable phenotype (M. Sigrist and W.-D. Heyer, unpublished results). As a control for transformation efficiency, the competent cells were also transformed with the centromeric plasmid YCp50. The transformation efficiency of wild-type and rad54 cells was found to be very similar within a 2-fold or smaller difference (Fig. 6B). To determine the efficiency of homologous integration of the linear fragment independent of the transformation efficiency, we calculated the ratio of the number of kanamycin-resistant transformants obtained with the linear fragment per microgram DNA over the number of plasmid transformants per microgram DNA. Haploid rad54 cells

<table>
<thead>
<tr>
<th>Strain</th>
<th>can8 rate × 10^-7</th>
<th>Fold increase</th>
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</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>5.9 ± 0.9</td>
<td>1×</td>
</tr>
<tr>
<td>rad54</td>
<td>13.3 ± 2.9</td>
<td>2.3</td>
</tr>
<tr>
<td>rad54 + YCp50-RAD54</td>
<td>3.7 ± 0.9</td>
<td>0.6</td>
</tr>
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Data shown are means from five independent fluctuation tests with one standard deviation.

*Strains were FF18733 (wild type) and FF18973 (rad54).*

Figure 4. Homolog-dependent increase of chromosome loss in rad54/rad54 cells. (A) Diagram of the two pairs of YACs used in this experiment. 18ED5-URA and 18ED5-LEU contained the same 190 kb human DNA insert in vector backbones differing in their marker genes as indicated. WXD4932 contains a 100 kb human DNA insert that is different from the 18ED5 YACs. (B) Chromosome loss rates of YAC 18 ED5-URA in wild-type cells containing the homologous (WDHY1700) or heterologous (WDHY1657) YAC pair and rad54/rad54 strains containing the homologous (WDHY1792) or heterologous YAC pair (WDHY1805). The rates for chromosome loss were determined as described in Materials and Methods. Means and one standard deviation of at least three independent experiments are shown.
showed a highly significant 17-fold decrease in the gene-targeting efficiency in comparison with an isogenic wild-type strain. In diploid rad54/rad54 cells the reduction was 5-fold (Fig. 6B).

To determine the accuracy of the integration events, Southern blot and PCR analysis of fragment transformants were performed. In 43 wild-type and 30 rad54 transformants, all integration events were found to be at the chromosomal YNL311c locus and appeared to be correct (data not shown). To provide higher resolution, the right junction of the integration (Fig. 6A) was amplified by PCR and a 351 bp fragment covering the junction was entirely sequenced in 12 diploid wild-type and 13 rad54/rad54 integrants. No changes in the DNA sequence were identified (data not shown). From these data we conclude that gene targeting is reduced, but not eliminated, in rad54 cells and that the targeting events are not detectably mutagenic.

The UV sensitivity of a histone H2A/B mutant is not epistatic with rad54 and does not suppress the UV sensitivity, mitotic intragenic recombination defect, and enhanced mutation rate of rad54.

Rad54 protein is a member of the Swi2/Snf2 family of ATPases, of which many members are active in chromatin remodeling. It has been speculated that Rad54 might function to remodel chromatin during recombinational repair (37). The transcriptional activation defect of snf2 cells can be suppressed by a deletion of TRT1 locus, one of two loci encoding histone H2A and histone H2B (38). trt1 mutant cells are viable but exhibit gross, genome-wide changes in chromatin structure (39), which apparently render transcriptional activation independent of chromatin remodeling by Snf2/Swi2. Thus, we hypothesized that a deletion of TRT1 might also suppress the phenotypes of a rad54 mutation. The deletion of TRT1 caused a mild UV-sensitive phenotype, which was less pronounced than that caused by rad54 (Fig. 7). These findings permitted epistasis analysis. In isogenic haploid and diploid strains, trt1 did not suppress the UV sensitivity caused by rad54. On the contrary, trt1 rad54 double mutants were more sensitive than either single mutant to an extent that suggests that both mutations caused additive sensitivity to UV. These data were confirmed by single-dose experiments comparing stationary phase cells that have a G1 DNA content and logarithmically grown cells that represent a mixture of G1, S and G2 cells (Fig. 7C). In addition to UV sensitivity, MMS sensitivity, spontaneous mutation rates, and spontaneous mitotic intragenic recombination rates were measured in isogenic wild-type, rad54, trt1 and rad54 trt1 strains. Deletion of TRT1 did not cause a phenotype in any of these assays and did not suppress the defects caused by the rad54 mutation.
deficient in generating prototrophs during spontaneous mitotic intragenic recombination (Fig. 1). The multiple heteroallele system used for most of the intragenic recombination analysis with rad54/rad54 strains had been previously used with rad52-1/rad52-1 diploids (40). rad52-1 is a missense mutation changing amino acid 90 from alanine to valine (41) and displays a null phenotype in most assays, including mitotic recombination. RADS2 is essential for all forms of HR in S. cerevisiae, reducing it to ~1% of wild-type levels when this gene is mutated (4). The rad52 analysis reported frequency data which allows comparison with our frequency data underlying the rate data of Figure 1. Although frequency data are subject to experimental variation, we observed that for three heteroallele pairs the reported wild-type data (40) and our wild-type data are near identical, allowing direct comparison. In these heteroallele pairs, rad52-1 caused a 6-fold reduction in prototroph formation between his7-1 and his7-2, a 32-fold reduction between tyr1-1 and tyr1-2, and a 9-fold reduction between ura3-1 and ura3-13. For the same markers, rad54/rad54 diploids showed a reduction of 8-, 26- and 21-fold, respectively. It appears that within likely experimental error, the reduction of the frequency of spontaneous mitotic intragenic recombination in rad52-1/rad52-1 diploids and rad54/rad54 diploid is equivalent. These data demonstrate the importance of Rad54 protein for the interaction between homologous chromosomes. It appears from studies of meiotic recombination (19,21,23) that during meiosis the function of the related Tid1 protein overlaps with that of Rad54. It is likely that this reflects the distinct and overlapping meiotic roles of Rad51 and Dmc1, the respective interaction partners of Rad54 and Tid1 (13,42–44). While Tid1 may not play a role in sister chromatid recombination (21,23), Rad54 is important for recombination between sister chromatids and homologs. This conclusion is consistent with the genetic requirement for RAD54 in the repair of an HO-induced DSB by conversion (45). Incidentally, DSB-induced gene conversion, a recombination event involving a broken and an unbroken homologous chromosome, is independent of TID1 (45).

The defect in prototroph formation during intragenic recombination in rad54 cells could result from the absence of recombination or from mis-recombination, producing recombinant chromosomes that contain mutations in the marker gene and hence cannot be selected for. We tried to distinguish between both possibilities by analyzing a gene-targeting experiment where the accuracy of the recombination event is not strictly selected for. Gene targeting was significantly reduced, but not absent, in rad54 cells, a result that is consistent with observations made in chicken and mouse RAD54-deficient cells (46,47). Physical analysis of integration junctions failed to detect any recombination-associated mutations, suggesting that at least the selectable targeted integration events are not mutagenic in rad54 cells. However, the reduced frequency of targeted integration events could mean that the mutagenic events have been lost, possibly by associated chromosome loss.

**DISCUSSION**

Rad54 is essential for accurate mitotic gene conversion between homologous chromosomes

Using marker systems in five different genes and two strain backgrounds, we showed that rad54/rad54 mutants are highly deficient in generating prototrophs during spontaneous mitotic intragenic recombination (Fig. 1). The multiple heteroallele system used for most of the intragenic recombination analysis with rad54/rad54 strains had been previously used with rad52-1/rad52-1 diploids (40). rad52-1 is a missense mutation changing amino acid 90 from alanine to valine (41) and displays a null phenotype in most assays, including mitotic recombination. RADS2 is essential for all forms of HR in S. cerevisiae, reducing it to ~1% of wild-type levels when this gene is mutated (4). The rad52 analysis reported frequency data which allows comparison with our frequency data underlying the rate data of Figure 1. Although frequency data are subject to experimental variation, we observed that for three heteroallele pairs the reported wild-type data (40) and our wild-type data are near identical, allowing direct comparison. In these heteroallele pairs, rad52-1 caused a 6-fold reduction in prototroph formation between his7-1 and his7-2, a 32-fold reduction between tyr1-1 and tyr1-2, and a 9-fold reduction between ura3-1 and ura3-13. For the same markers, rad54/rad54 diploids showed a reduction of 8-, 26- and 21-fold, respectively. It appears that within likely experimental error, the reduction of the frequency of spontaneous mitotic intragenic recombination in rad52-1/rad52-1 diploids and rad54/rad54 diploid is equivalent. These data demonstrate the importance of Rad54 protein for the interaction between homologous chromosomes. It appears from studies of meiotic recombination (19,21,23) that during meiosis the function of the related Tid1 protein overlaps with that of Rad54. It is likely that this reflects the distinct and overlapping meiotic roles of Rad51 and Dmc1, the respective interaction partners of Rad54 and Tid1 (13,42–44). While Tid1 may not play a role in sister chromatid recombination (21,23), Rad54 is important for recombination between sister chromatids and homologs. This conclusion is consistent with the genetic requirement for RAD54 in the repair of an HO-induced DSB by conversion (45). Incidentally, DSB-induced gene conversion, a recombination event involving a broken and an unbroken homologous chromosome, is independent of TID1 (45).

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**rad54/rad54 cells are proficient in BIR to generate intergenic recombinants**

The analysis of a marker system on chromosome V showed that rad54/rad54 cells were proficient in generating intergenic recombinants. The most abundant recombinant classes
(classes 1 and 3 in Fig. 2) could result from gene conversion, BIR, or crossing-over. Since rad54/rad54 cells are highly deficient in intragenic recombination which is primarily the result of conversion events, we consider it unlikely that gene conversion is contributing much to these classes in wild-type or rad54/rad54 cells. Hence, we tried to distinguish between BIR and crossing-over as the mechanism to generate the observed recombinants. From further genetic analysis it appeared that these recombinants were almost exclusively the result of BIR. This is consistent with previous observations that crossing-over is quite rare in vegetatively growing cells (4). An HO-induced DSB can be repaired by BIR in a RAD54-independent fashion (45), and it appears from our data that also spontaneous DNA damage can result in RAD54-independent BIR. It is unlikely that this spontaneous damage, which triggered the recombination events scored here, is a DSB, because rad54 mutants are extremely sensitive to DSB damage with most of the cells not surviving a single DSB (48). It was recently realized that direct repeat recombination can be the result of a combination of BIR and SSA (2). The proficiency of rad54/rad54 cells in BIR and SSA is consistent with the observed disparate effects of RAD54 mutants in direct repeat recombination assays (16–20).

**Genomic instability in rad54/rad54 cells**

Cells lacking Rad54 exhibit significantly increased levels of genome-wide chromosome loss (22; this study). Importantly, we showed that the increased chromosome loss phenotype is unlikely to be caused by a defect in chromosome segregation. Defects in chromosome segregation (e.g. mutations of the centromere or the spindle apparatus) lead to increased chromosome loss of non-essential test chromosomes in haploid cells (32). rad54 mutants, however, did not lead to increased chromosome loss in haploid cells. Instead, it appeared that chromosome loss was specifically increased in diploid cells carrying two homologous, but not two heterologous, chromosomes (Table 2 and Fig. 4). This suggests that attempted recombination between homologous chromosomes results in chromosome loss. Biochemical, genetic and cytological data support the notion that Rad54 protein acts after the initial formation of the Rad51-ssDNA filament (see Introduction), which could lead to recombination intermediates that cannot be properly processed in the absence of Rad54.

Mutation rates to canavanine-resistant cells in haploid rad54 cells are only 2–6-fold elevated over wild type (18) (Table 3). In determining the rate of events leading to canavanine-resistance in homozygous CAN1+/CAN1+ diploid cells, we found an over 80-fold increase in rad54/rad54 cells (Fig. 5). The generation of canavanine-resistant offspring required at least two events. By far the majority of these events in rad54/rad54 mutants, but not in wild-type cells, were a mutation in one CAN1 gene and loss of the other chromosome. The rates of these two classes (classes 5 and 6 in Fig. 5) were elevated over 1700-fold compared with wild type. Since the rates of the component events (mutation of CAN1, Table 3; chromosome V loss, Fig. 2) have been determined independently, it is possible to evaluate the independence of both mechanisms. The rates of CAN1 mutation + chromosome loss was $1.4 \times 10^{-11}$ in wild type and $~2400 \times 10^{-11}$ in rad54/rad54 cells (classes 5 and 6 in Fig. 5). In wild type, the component rates were $5.9 \times 10^{-7}$ (haploid mutation rate, Table 3) and $3 \times 10^{-6}$ (loss rate of one chromosome in CAN1+/can1R diploid, Fig. 2) resulting in an expected rate of $1.8 \times 10^{-12}$, if both events were independent. The observed rate of $1.4 \times 10^{-11}$ was ~8-fold higher, indicating that the two events might not be entirely independent in wild-type cells. In rad54/rad54 cells, the component rates ($13.3 \times 10^{-7} \times 401 \times 10^{-6}$) predicted a rate of $53 \times 10^{-11}$, but the observed rate was $2400 \times 10^{-11}$, an over 45-fold difference, suggesting that both mutation and chromosome loss are highly coincident.

This calculation makes no assumption about the possible mechanisms and order of events leading to canavanine-resistant clones with loss of one chromosome V. If one were to assume that the mutation happens first and the chromosome loss second, one might argue to multiply the haploid mutation rate by a factor of two, since there are two CAN1 genes. In this sequence of events, mutation would result in a CAN1+/can1R diploid, which after loss of the CAN1 chromosome, leads to the observed class (can1R with loss of the other chromosome). Thus, only one half of the possible chromosome V loss events leads to the selected class, leading to consider another factor of two in the calculation. However, one could equally assume that the chromosome loss events happens first and the mutation second, since chromosome loss is considerably more frequent than the haploid mutation event in both wild-type and rad54 cells. In this case, the loss would produce an aneuploid cell with a single chromosome V where the CAN1 gene would then mutate to can1R. Here one could argue to introduce a factor of two because either of the two chromosomes could be lost, whereas in the determination of the component rate one particular chromosome must be lost to produce a selectable event. For the mutation rate, the haploid rate would apply under the assumption that the mutation rate is not influenced by overall ploidy and mating-type status. In the absence of specific insight into the mechanisms leading to the can1R + chromosome loss events, we favor not introducing any factors. This operation may lead to an overestimate of the absolute extent by how much the two events are coincident, but the relative difference between wild-type and rad54/rad54 cells remains constant.

Keeping the above mentioned caveat in mind, the calculation suggests that in wild-type cells both events, mutation and chromosome loss, may not be entirely independent showing an 8-fold coincidence, whereas in rad54/rad54 cells both events are highly coincident (45-fold). We suggest that attempted recombinational repair of spontaneous DNA damage in rad54/rad54 cells leads to associated chromosome loss and mutations.

Previous studies in diploid *S. cerevisiae* cells have identified an association between spontaneous mutation and homoygosis, leading to diploid cells with homozygous mutations (49,50). These authors suggested that the mutations arose from recombinogenic lesions causing the coincidence between spontaneous mutagenesis and homoygosis. This observation fits our data in wild type, where many more canavanine-resistant cells retaining both chromosomes (rate $2.97 \times 10^{-10}$) Fig. 5, class 1) were found than predicted from the haploid mutation rate ($5.9 \times 10^{-7} \times 5.9 \times 10^{-2} = 3.5 \times 10^{-14}$, Table 3). We surmise that the associated recombination in rad54/rad54 cells leads to the observed chromosome loss, explaining the high coincidence between both events in the mutant.
RAD52 mutants, the rare intragenic recombination events were found to be highly associated with loss of one chromosome, an association seen in wild-type cells only infrequently (51).

Together, these data suggest that HR is an important factor in genomic stability, particularly in diploid cells. It will be interesting to expand the present studies on genomic instability, which are being performed in haploid cells (52), to diploid cells in order to understand the interplay between DNA replication, HR, DNA checkpoints and the chromosome segregation apparatus to maintain genomic stability.

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