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Citation for published version:

Digital Object Identifier (DOI):

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Publisher's PDF, also known as Version of record

Published In:
Molecular and Cellular Biology

Publisher Rights Statement:
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Cells Expressing Murine RAD52 Splice Variants Favor Sister Chromatid Repair†

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Received 11 July 2005/Returned for modification 2 February 2006/Accepted 24 February 2006

The RAD52 gene is essential for homologous recombination in the yeast Saccharomyces cerevisiae. RAD52 is the archetype in an episist group of genes essential for DNA damage repair. By catalyzing the replacement of replication protein A with Rad51 on single-stranded DNA, Rad52 likely promotes strand invasion of a double-stranded DNA molecule by single-stranded DNA. Although the sequence and in vitro functions of mammalian RAD52 are conserved with those of yeast, one difference is the presence of introns and consequent splicing of the mammalian RAD52 pre-mRNA. We identified two novel splice variants from the RAD52 gene that are expressed in adult mouse tissues. Expression of these splice variants in tissue culture cells elevates the frequency of recombination that uses a sister chromatid template. To characterize this dominant phenotype further, the RAD52 gene from the yeast Saccharomyces cerevisiae was truncated to model the mammalian splice variants. The same dominant sister chromatid recombination phenotype seen in mammalian cells was also observed in yeast. Furthermore, repair from a homologous chromatid is reduced in yeast, implying that the choice of alternative repair pathways may be controlled by these variants. In addition, a dominant DNA repair defect induced by one of the variants in yeast is suppressed by overexpression of RAD51, suggesting that the Rad51-Rad52 interaction is impaired.

Homology-directed repair (HDR) allows cells to repair DNA double-strand breaks (DSBs) in an error-free way and is a key mechanism for maintaining genome integrity in all eukaryotes (11, 48, 52). RAD52 is an essential gene for HDR in the yeast Saccharomyces cerevisiae (20, 30, 46). Identified as one member of an episist group of radiation-sensitive yeast mutants, rad52 strains are defective in HDR, the major DSB repair pathway in yeast. RAD52 is largely conserved throughout eukaryotes, and both the human and yeast Rad52 proteins stimulate the replacement of replication protein A with Rad51 on single-stranded DNA (4, 29, 39, 45). Additionally, Rad52 promotes DNA annealing and strand exchange between a DNA duplex and complementary single-stranded DNA (14, 21, 27). Three-dimensional structures of the N terminus of human RAD52 (hRAD52) reveal a multimeric ring containing a positively charged surface groove that has been suggested to bind single-stranded DNA (15, 40). Relative to the N terminus of Rad52, the C terminus is poorly conserved between homologs. The C terminus of Rad52 is essential for its interaction with Rad51, a bacterial RecA homolog (26, 37). Consistent with its ability to bind both itself and other DNA repair proteins, Rad52 forms discrete nuclear foci both spontaneously and in response to DNA damage, and these DNA repair centers are the sites of DSB repair (23–25, 32).

Although yeast RAD52 plays a critical role in HDR, surprisingly, RAD52 knockout mice are resistant to DNA damage (35). However, it is likely that mammalian RAD52 is important for DNA repair, as its activities are conserved with yeast Rad52; in addition, HDR in chicken cells requires RAD52 (5, 42). Furthermore, RAD52 null mice have a defect in gene targeting (35), and loss of RAD52 rescues the tumorigenic phenotype of ATM-deficient mice (50). An alternative explanation for the phenotype of the RAD52 knockout mice is that the functions of yeast Rad52 are redundant in higher eukaryotes. Mammals have a number of other genes, such as XRCC3 and BRCA2, that may encode complementary functions with yeast RAD52 (9, 10, 38).

Another difference between the mammalian and yeast RAD52 genes is the presence of introns. The open reading frames (ORFs) of both human and mouse RAD52 are divided into 12 exons whose positions relative to the RAD52 open reading frame are conserved. Splice variants of hRAD52 have been isolated from cDNA libraries derived from both brain and testes tissues, and the encoded protein variants were found to bind to DNA but not to wild-type hRAD52 in vitro (16).

Here we report the isolation of two new splice variants from adult mouse tissues. We show that these variant transcripts act as dominant alleles in both mammalian cells and the yeast S. cerevisiae. In both mammalian cells and yeast, the variants perturb HDR, specifically by elevating the frequency of sister chromatid repair. In yeast, both variants also decrease the frequency of repair from a homologous chromatid.

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more, for one variant in yeast, the defect in DNA repair is suppressed by overexpression of RAD51. These data show that the splice variants have the potential to alter the ratio of repair from the homologous to the sister chromosome, possibly by impairing the interaction of Rad52 with Rad51.

**MATERIALS AND METHODS**

**RNA isolation and reverse transcription-PCR (RT-PCR).** Whole-tissue samples were homogenized first by passing approximately 15 mg tissue several times through a 27-gauge needle in RNAlater buffer (Ambion) and then through a QIAshredder column (Qiagen). RNA was purified from the resulting suspension without delay using the RNAeasy column purification system (Qiagen). Three to 5 µg of RNA was used for cDNA synthesis using random hexamer primers, thus ensuring that the cDNA represents all the transcripts present in the tissue. Either the Superscript first-strand synthesis system for RT-PCR (Invitrogen) or first-strand cDNA synthesis kit for RT-PCR (Roche) was used for this cDNA synthesis. Approximately 10% of the resulting cDNA was used as a template for PCR using gene specific primers.

As a control for cDNA yield, primers were designed to amplify a 200-bp product spanning exon 8 from a splice variant of the murine glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH). Each RT reaction included a matched control that omits the reverse transcriptase enzyme to confirm that the subsequent PCR products are derived from cDNA and not from DNA contamination. GAPDH primers used for PCR were 5'-TGAAGAAGGCTGATGGTAGAC-3' and 5'-AGTCCGATGATGGCATGGAC-3'. RAD52 exon 1 to 7 primers were 5'-CCATGGTCTTGCGAGCCATTCTC-3', 5'-CCATGGTCTTGCGAGCCATTCTC-3', and 5'-GGAGGAGGCTGTGAAGC-3'. For RAD52 exon 6 to 11 primers were 5'-GGAGGAGGCTGTGAAGC-3' and 5'-GGAAGGCGCTCTGACTGATG-3'. The two variant plasmids, pRAD52(pWJ1456) and pRAD52+intron8(pWJ1458), were created using the Quickchange mutagenesis kit (Stratagene) to alter the RAD52 sequence. Briefly, pRAD52Δexon4 is identical to pRAD52, except for the deletion of 94 bp corresponding to pRAD52 exon 4. pRAD52+intron8 is identical to pRAD52, except for the inclusion of 139 bp of mouse intron 8 (derived from the RT-PCR of mouse tissues described above). pCMV6-wt (Invitrogen), lacking the RAD52 gene, is a control vector. Chinese hamster ovary cells (CHO) cells were used to assay HDR. These cells contain a direct repeat of the GFP gene in which one repeat is full length but is inactivated by the inclusion of an I-SceI endonuclease target site. The second repeat lacks both the 5' and 3' ends of the gene. After cleavage with the I-SceI endonuclease, the resulting DSB can be repaired by gene replacement from the second GFP repeat either from the same or from a sister chromosome, resulting in a functional GFP allele (see Fig. 5A). The efficiency of repair is scored by fluorescence-activated cell sorting (FACS). The plasmid encoding the homing endonuclease I-SceI (pCAGGS-I-SceI) as well as the CHO and CHO-XRCC3-/- cells containing the DR-GFP reporter were kind gifts from Maria Jasin and Jeremy Stark, and their use has been described elsewhere (31). Cells were grown in serum-plus medium (Dulbecco’s modified Eagle medium containing 10% newborn bovine serum, 4.5 µl/g glucose, 4.5 µl/g sodium pyruvate, and 100 U/ml penicillin-streptomycin; all from Invitrogen-GibcoBRL) at 37°C, 6% CO2. Cells were harvested from culture dishes after 2 to 10 min of incubation with trypsin-EDTA (Invitrogen) and then washed once in PBS. Cells (2 x 10^6) were plated on 60-mm tissue culture dishes and grown overnight in serum-plus medium. The following day the cells were transfected in OPTIMEM medium (Invitrogen) with 4 µg/dish plasmid DNA (2 µg of pRAD52 and 2 µg of experimental plasmid as indicated), using 4 µl/dish lipofectin (Invitrogen) following the manufacturer’s instructions (this DNA/lipofectin ratio was determined to be optimal in the absence of an I-SceI target site). CHO cells were transfected with plasmid pIRES-EGFP vector. Transfection was carried out for 5 h, after which the medium was replaced with serum-plus medium. After 48 h, the cells were collected using trypsin-EDTA, washed twice in PBS, and resuspended in 200 to 600 µl PBS. FACS analysis was performed on the cells as previously described (49). Expression levels of I-SceI from the plasmid vector were assumed to be equivalent in each case. The expression of the mammalian variants was assayed by a parallel experiment in which CHO cells were transfected with plasmid pIRES-EGFP as described above but including the internal ribosomal entry site (IRES)-GFP sequence. This GFP sequence produces fluorescence signal in all cells that successfully take up and express the plasmid vector. FACS analysis of the resulting transfections allowed the measurement of the proportion of CHO cells transfected (GFP-). For each of the four vectors (pIRES-EGFP, pRAD52+ IRES-EGFP, pRAD52Δexon4-IRES-EGFP, and pRAD52+intron8-IRES-EGFP) the number of cells transfected was equivalent (Fig. 5B in the supplemental material).

**Mammalian cell synchronization.** To obtain a synchronous culture of mouse 3T3 cells, the cells were grown to near confluence in 150-cm^2 tissue culture flasks and then arrested with 400 ng/ml (1.3 µM) nocodazole for 6 h. The mitotic cells were then collected by mitotic shake-off, which involves mechanical shaking of the cell culture flasks for 5 min. The mitotic cells were collected by centrifugation, washed twice in nocodazole-free media, and then grown in nocodazole-free media. Cells were plated in 60-cm^2 dishes and harvested at various time points using trypsin. One aliquot was fixed in 100% ethanol at -20°C for at least 30 min. These fixed cells were then stained in PBS with 50 µg/ml propidium iodide and 200 µg/ml RNase A (Sigma) at 4°C for at least 30 min prior to FACS analysis. A second aliquot was placed directly into RLT buffer (Qiagen) and stored at -80°C for RNA preparation as described above.

**Yeast strains and methods.** Standard genetic methods were used for the growth and manipulation of yeast (36). The yeast strains used in this study are listed in Table 1. The strains encoding Rad52 tagged with yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) have been reported previously (23). We modified W3749-14C (Rad52-YFP) using our previously published allele replacement method (8). Briefly, PCR primers were designed to generate targeting fragments that eliminate either the 3' or 5' end of the targeted gene. This targeted gene is inactivated by the inclusion of an I-SceI endonuclease target site. The second allele replacement method was performed as described above.

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and quantifying the relative fluorescence using Volocity software (Improvision, Lexington, MA).

The strains encoding the two heteroalleles of \textit{ADE2} were a kind gift from Lorraine Symington, and their use has been described elsewhere (12). The \textit{ade2-} allele contains a fill-in mutation of the 5' Ndel restriction endonuclease target site of the yeast \textit{ADE2} gene, and the \textit{ade2-a} allele contains a fill-in mutation of the 5' AartII site (2). The frequency of heteroallelic recombination was measured for at least nine independent isolates of each diploid strain. Cells were grown overnight in synthetic complete (SC) medium and washed once in H2O before being briefly sonicated. Serial 10-fold dilutions were plated both on SC medium and in duplicate on adenine-deficient medium. Colonies were counted after 48 h, and the method of Lea and Coulson was used to estimate the mean number of recombinants per culture (22).

The strains used for the direct-repeat assay contain two defective \textit{LEU2} alleles separated by a \textit{URA3} marker (pop-out), which was used to measure the frequency of single-strand annealing (SSA) events, which are scored by the loss of a \textit{URA3} marker (pop-out), which is placed between the two repeated \textit{lea2} alleles. As the \textit{lea2} direct repeat is present as a diploid, \textit{LEU2} cells resulting from SSA events will be \textit{ura3} / \textit{URA3}, compared to \textit{URA3} / \textit{URA3} for replacement events. Plating the \textit{LEU2} colonies on medium containing 5-fluoroorotic acid (5-FOA), which selects against \textit{URA3}, allowed us to differentiate the \textit{URA3} genotype. The colonies that were \textit{ura3} / \textit{URA3}, when replica plated to 5-FOA, produced small papillae, indicative of their ability to lose the single \textit{URA3} gene. In contrast, the \textit{URA3} / \textit{URA3} colonies failed to grow at all on 5-FOA. Sporulating a selection of each class of \textit{LEU2} colonies and testing the ability of the haploid spores to grow on uracil-deficient medium confirmed their \textit{URA3} genotypes (data not shown). This allowed us to estimate the proportion of \textit{LEU2} recombinants resulting in pop-out events compared to replacement events (illustrated in Fig. 6A).

**RESULTS**

**Identification of mouse splice variants.** Preliminary data derived from our sequencing of the mouse \textit{RAD52} gene (unpublished observations) and from RT-PCR from an array of different mouse tissues (see Fig. S5 in the supplemental material) suggested that at least two splice variants of mouse \textit{RAD52} are commonly expressed. To investigate these findings further, tissue samples of lung and liver were dissected from two C57BL/6J mice and used for RT-PCR. A series of overlapping PCRs were performed covering the length of the mouse \textit{RAD52} cDNA, as illustrated in Fig. 1A. The murine \textit{RAD52} gene contains 12 exons. Three PCRs amplify cDNA from exons 1 to 7, 6 to 11, and 10 to 12. These PCRs were designed to detect most alterations in splicing that are pre-
dicted from earlier studies (Fig. 5A), such as the removal of exon sequence or the inclusion of intron sequences. Gels were blotted and probed with radiolabeled oligonucleotides to ensure that the RT-PCR products are specific for the RAD52 gene. Using a relatively low number of PCR cycles and subsequent gel blotting also allows a semiquantitative assessment of the template RNA, as the RT-PCRs have not saturated.

We observed two variant products consistent with alternative splicing in addition to the expected RAD52 transcript products corresponding to the annotated RAD52 sequence (Fig. 1B and C). The expected product from exons 1 to 7 is 591 bp; however, a smaller, ~500-bp product was also identified (Fig. 1B). As depicted in Fig. 1C, products amplified from exons 6 to 11 give rise to a larger product of 750 to 800 bp in addition to the expected 631-bp product. No alternatively spliced products were observed from exons 10 to 12 (data not shown). Figure 1D shows the GAPDH control.

Both of these variant products are predicted to encode truncated RAD52 peptides (Fig. 2). DNA sequencing of the nov...
formed on the RNA as described above. Figure 4A shows the FACS profile of the cells following release from nocodazole arrest, and Fig. 4B shows the results of RT-PCR. We could detect no significant difference in the ratio of splice variant to normal transcript for either RAD52Δexon4 or RAD52+intron8 at any stage in the cell cycle. The RT-PCR products appear the same as asynchronously dividing cells (as in Fig. 3).

Splice variants modeled in mammalian cell culture cause dominant recombination effects. To test whether the mammalian splice variants cause functional changes in HDR, an established assay for gene repair in Chinese hamster ovary (CHO) cells was used. These cells contain the HDR reporter system outlined in Materials and Methods (also see Fig. 5A). Briefly, an I-SceI-induced DSB, within a nonfunctional GFP gene, can be repaired by gene conversion from a neighboring sequence to restore a functional GFP open reading frame. CHO cells were transfected with vectors expressing the normal RAD52 cDNA, the two variant cDNAs, RAD52Δexon4 and RAD52+intron8, or the control (summarized in Fig. 5B). Expression of RAD52 has no significant effect on HDR in this assay (Fig. 5C), similar to the expression of RAD52 in a cell line mutated in rad52 (43). Interestingly, expression of either of the splice variant constructs elevates the frequency of repair compared to that of full-length RAD52 (Fig. 5C).

Since there is evidence from chicken cells to suggest that RAD52 acts in parallel with XRCC3 in DNA damage repair (10), the mammalian HDR experiments were repeated with a CHO cell line that lacks XRCC3 (CHO-XRCC3−/−) to see how the splice variants act in this mutant. Consistent with previous observations (31), the overall frequency of HDR in this cell line is more than 10-fold lower than in wild-type cells. However, overexpression of the RAD52 splice variants did not change the levels of HDR (Fig. 5D).

Finally, we attempted to knock down the level of the RAD52+intron8 variant in CHO cells by designing small interfering RNA against the hamster intron 8 sequence. However, transfection of this small interfering RNA had no detectable effect on the level of the intron 8 variant (as judged by RT-PCR) or the frequency of recombination of the GFP targets in the presence of I-SceI (data not shown).

Splice variants modeled in yeast cause dominant recombination effects. The ability to conduct functional recombination studies in mammalian cells is currently hindered by the limited number of functional assays that distinguish between recombination products; for example, if it is difficult to differentiate repair from a homolog as opposed to a sister chromosome. Consequently, we employed the budding yeast Saccharomyces cerevisiae to assay for dominant-negative behavior of the splice variants. To mimic the C-terminal truncations that are predicted to be encoded by the two mouse splice variants, RAD52Δexon4 and RAD52+intron8, the RAD52-YFP (encoding Rad52 fused to yellow fluorescent protein) gene from Saccharomyces cerevisiae (23) was modified at the yeast chromosomal locus. The RAD52Δexon4 and RAD52+intron8 mouse splice variants were recreated in the yeast RAD52-YFP gene as rad52Δ77 and rad52Δ284, respectively (the numbers correspond to the final amino acid in the deleted Rad52 sequences). It is important to note that the mouse sequences were not transferred into yeast; rather, the yeast sequence was used to model the mouse transcripts by creating truncations of the endogenous yeast RAD52 gene. We refer to these two yeast alleles as “splice variant equivalents.”

As the mouse variants showed increased HDR when expressed in mammalian cells (Fig. 5), a similar repair assay was performed in yeast. Recombination between direct repeats was assayed using a reporter system containing two defective alleles of LEU2 (leu2ΔEcoRI and leu2ΔBstEI) (Fig. 6A). Similar to the HDR assay used in CHO cells, the leu2 direct-repeat reporter assays primarily intrachromatid and sister chromatid recombination (17, 41). Commonly, two kinds of LEU2+ results product: replacements, where one of the leu2 alleles is repaired without loss of the intervening URA3 sequence; or pop-out events, where the URA3 is lost by deletion of the sequence between the two LEU2 alleles (Fig. 6A). As described in Materials and Methods, these two events are distinguished by a
simple replica-plating assay. Since the mouse splice variants detected in our screen are expressed together with the normal transcript (Fig. 1B and C, 3, and 4B), we next created diploid yeast strains that contain a full-length \textit{RAD52} allele in addition to a splice variant-equivalent allele to screen for dominant effects on direct-repeat recombination. Both of the \textit{RAD52} splice variant equivalents elevate the frequency of LEU2 recombinants (Fig. 6B). While the effects upon recombination

FIG. 5. (A) Schematic representation of the reporter system developed by Pierce and colleagues (31). puro, puromycin-N-acetyltransferase gene. A double-strand break can be introduced into this reporter using the I-SceI endonuclease, and subsequent HDR from a downstream GFP fragment enables the repair of a functional GFP sequence. These HDR events can be scored by FACS analysis. Four expression vectors were created from a derivative of the pIRES-EGFP vector (Clontech) as described in Materials and Methods (B). The four vectors include the empty vector control pCMV, full-length mouse \textit{RAD52} (pRAD52), or the two splice variants, pRAD52\textsubscript{exon4} and pRAD52\textsubscript{intron8}, as indicated in panel B. (C) The frequency of HDR events in CHO cells (scored as % GFP cells using FACS analysis) is shown. For each transfection the plasmid is indicated below. In addition, each transfection included an I-SceI-expressing plasmid; the mean of four experiments is indicated (±standard deviation). Analysis of variance indicates significant differences between the groups (data not shown). Both of the variant plasmids (pRAD52\textsubscript{exon4} and pRAD52\textsubscript{intron8}) have a higher frequency of HDR events than the wild-type \textit{RAD52} plasmid (one-tailed \textit{t} test, \(P = 4.9 \times 10^{-5}\) and \(P = 7.5 \times 10^{-5}\), respectively). A CHO cell line, deficient in XRCC3, was also tested in the same way (D). The difference between the wild-type \textit{RAD52} plasmid and the pRAD52\textsubscript{exon4} plasmid is not significant (one-tailed \textit{t} test, \(P = 0.094\)). The difference between the wild-type \textit{RAD52} plasmid and the pRAD52+intron8 plasmid is significant (one-tailed \textit{t} test, \(P = 0.044\)).

FIG. 6. (A) Frequency of direct-repeat recombination was assayed as indicated. Two \textit{leu2} alleles are schematically represented (top), with dark triangles indicating the two mutations that inactivate the gene. Recombination between the two repeated sequences can restore a functional \textit{LEU2} gene, and two possible \textit{LEU2} recombinant structures are shown. First, a replacement event restores the wild-type \textit{LEU2} sequence to one of the \textit{leu2} alleles (e.g., the upstream sequence is restored). Second, a pop-out event is shown where single-strand annealing recombines the two repeated \textit{leu2} alleles, leading to loss of the \textit{URA3} gene and both mutations within \textit{LEU2}. Other possible events such as triplications are discussed elsewhere (41). (B) The median \textit{leu2} recombination frequency \(m\), as defined by Lea and Coulson (22), is shown, where the error bars indicate ± standard deviation of \(m\). The wild-type \textit{RAD52} allele is abbreviated wt. Both variant alleles (\textit{rad52}\textsubscript{exon4} and \textit{rad52}\textsubscript{intron8}) have a higher frequency of direct-repeat recombination than the heterozygous wild-type strain (wt/null), (one-tailed \textit{t} tests, \(P = 8.5 \times 10^{-5}\) and \(4.4 \times 10^{-6}\), respectively). The frequency of recombination in the heterozygous wild-type strain (wt/null) versus a wild-type diploid strain (wt/wt) is not significantly different (one-tailed \textit{t} test, \(P = 0.41\)). Similarly, the recombination frequency in the \textit{rad52}\textsubscript{exon4} strain is not significantly different from that of the \textit{rad52}\textsubscript{intron8}/null strain (one-tailed \textit{t} test, \(P = 0.089\)).
TABLE 2. Proportion of replacements versus pop-outs from the direct-repeat recombination assay

<table>
<thead>
<tr>
<th>Straina</th>
<th>RAD52 genotype</th>
<th>n</th>
<th>Proportion of LEU2+ colonies that are ura3−/URA3b</th>
<th>Frequency of replacement events (10−3)c</th>
<th>Frequency of pop-out events (10−6)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>W5977</td>
<td>wt/wt</td>
<td>933</td>
<td>0.17</td>
<td>2.1</td>
<td>0.43</td>
</tr>
<tr>
<td>W5978</td>
<td>wt/rad52::HIS5</td>
<td>532</td>
<td>0.32</td>
<td>2.2</td>
<td>1.0</td>
</tr>
<tr>
<td>W5979</td>
<td>wt/rad52Δ77-YFP</td>
<td>1291</td>
<td>0.30</td>
<td>2.7</td>
<td>1.2</td>
</tr>
<tr>
<td>W5980</td>
<td>wt/rad52Δ284-YFP</td>
<td>1132</td>
<td>0.53</td>
<td>2.3</td>
<td>2.5</td>
</tr>
</tbody>
</table>

a wt, wild-type RAD52 genotype. The full genotypes are listed in Table 1.
b n is the number of LEU2+ colonies screened on 5-FOA-containing medium.
c The estimated frequencies of replacements and pop-outs are calculated using the total recombination frequencies, shown in Fig. 6B, adjusted by the proportion shown in this table.

are modest, they are significantly different from those of a heterozygous strain (one-tailed t tests, \( P = 8.5 \times 10^{-3} \) and \( 4.4 \times 10^{-6} \) for \( \text{rad52}\Delta 77 \) and \( \text{rad52}\Delta 284 \), respectively). These effects are similar to those seen in mammalian cell culture (Fig. 5). Interestingly, in yeast, the \( \text{rad52}\Delta 77 \) allele increases replacement events, while the \( \text{rad52}\Delta 284 \) allele increases the frequency of \( \text{URA3} \) pop-outs (Table 2).

The yeast system has the advantage that the variants are easily assayed for effects on other types of DNA repair. Therefore, we analyzed the splice variant equivalents for their effects on heteroallelic recombination, focus formation, and DNA damage sensitivity. First, spontaneous heteroallelic recombination was assayed in diploid yeast with two defective \( \text{ade2} \) heteroalleles (\( \text{ade2}-n \) and \( \text{ade2}-a \)). Recombination between these alleles, which reside at each end of the \( \text{ADE2} \) gene on homologous chromosomes, restores a functional \( \text{ADE2} \) gene (Fig. 7A). Surprisingly, in contrast to the elevated frequency of direct-repeat recombination seen in both mammalian cells (Fig. 5) and yeast (Fig. 6B), both of the splice variant equivalents lowered the frequency of heteroallelic recombination (Fig. 7B). These disparate phenotypes suggest that the variants may be promoting sister chromatid repair while at the same time suppressing repair from a homologous chromosome.

In yeast, Rad52 and other DNA repair proteins form distinct nuclear foci at the sites of double-strand break repair (23–25). These DNA repair centers appear predominantly in S and G2 phases of the cell cycle. Altered levels of foci within yeast strains are indicative of DNA repair deficiencies (25). Next, the formation of foci in the splice variant equivalent strains was measured using fluorescence microscopy. Both splice variant equivalents fail to form spontaneous foci in a haploid strain (data not shown). Diploid strains containing the splice variant equivalents tagged with YFP and wild-type Rad52 tagged with CFP (cyan fluorescent protein) were assayed for focus formation. Interestingly, in these strains, the \( \text{Rad52}\Delta 284\)-YFP protein forms foci that also contain wild-type \( \text{Rad52}\)-CFP (Fig. 8A), suggesting that \( \text{Rad52}\Delta 284\)-YFP and wild-type \( \text{Rad52} \) are incorporated into the same DNA repair complex. The same phenotype is not observed for \( \text{Rad52}\Delta 77\)-YFP. Additionally, both splice variant equivalents elevate the proportion of S/G2 cells that contain spontaneous wild-type \( \text{Rad52} \) foci, indicative of either increased DNA damage or an altered DNA repair activity within these cells (Fig. 8B).

Unlike in mammalian cells, \( \text{RAD52} \) is essential for HDR in yeast, and furthermore HDR is its major DSB repair pathway. Therefore, DNA damage sensitivity is a standard assay for the function of yeast Rad52. First, we estimated protein levels in diploid strains containing differentially tagged Rad52 peptides (CFP and YFP) to ensure that the splice variant equivalent alleles do not alter the expression of \( \text{RAD52} \) from either the wild-type or mutant allele. Table 3 shows the amount of the YFP and CFP detected in the nucleus of the different strains, and there is no significant difference between the two mutant strains and the wild-type diploid. Next, the repair capacity of the splice variant equivalent strains was assayed by measuring viability with increasing doses of \( \gamma \)-irradiation. Both \( \text{rad52}\Delta 284 \) and \( \text{rad52}\Delta 77 \) are as sensitive to \( \gamma \)-irradiation as \( \text{rad52} \) null strains, either as haploid strains (data not shown) or as heterozygous diploids containing a null allele (Fig. 9A, first four rows). The diploid strains containing both a mutant and wild-type \( \text{RAD52} \) allele were tested for sensitivity to \( \gamma \)-irradiation, and \( \text{rad52}\Delta 284 \) (Fig. 9A, sixth row) is as sensitive as a wild type/null (Fig. 9A, top row). In contrast, \( \text{rad52}\Delta 284 \) confers a dominant-negative phenotype for \( \gamma \)-irradiation sensitivity (Fig. 9A, last row, indicated by the white arrow).

![FIG. 7. (A) Heteroallelic recombination is measured using two \( \text{ade2} \) alleles as shown schematically. The dark triangles indicate the two mutations that inactivate the gene: \( \text{ade2}-n \) is a fill-in mutation of the AatII site in the 5′ end of the gene, and \( \text{ade2}-a \) is a fill-in mutation of the NdeI site in the 3′ end of the gene (12). Recombination between the two homologs restores a functional \( \text{ADE2} \) gene, as indicated. The gene conversion event depicted has lost the 3′ \( \text{ade2}-n \) mutation, leading to restoration of a functional \( \text{ADE2} \) gene. The frequency of heteroallelic recombination is calculated using the methods described by Lea and Coulson (21). (B) The median \( \text{ade2} \) recombination frequency \( m \) as defined by Lea and Coulson; the error bars indicate ± standard deviation of \( m \). The wild-type allele is abbreviated \( \text{wt} \). Both variant alleles (\( \text{rad52}\Delta 284\)-null and \( \text{rad52}\Delta 77\)-null) have a lower frequency of heteroallelic recombination than the heterozygous wild-type strain (\( \text{wt/null} \)) (one-tailed \( t \) tests, \( P < 10^{-4} \)). The frequency of recombination in the diploid wild-type strain (\( \text{wt/wt} \)) does not differ significantly from the heterozygous wild-type strain (\( \text{wt/null} \)) (two-tailed \( t \) test, \( P = 0.87 \)). The \( \text{rad52}\Delta 284\)-null strain has a significantly lower frequency of recombination than the \( \text{rad52}\Delta 284\)-null strain (one-tailed \( t \) test, \( P < 2 \times 10^{-4} \)).]
Rad52 recruits Rad51 to sites of DNA damage and stimulates C-terminal truncations of RAD52 do in

rad52Δ284 is dependent on its disruption of Rad51 function(s).

**DISCUSSION**

Identification of novel RAD52 transcripts from adult mouse tissue. Here we identify two novel RAD52 transcripts from adult mouse tissue which are the major alternatively spliced mRNAs from this gene (Fig. 1). One variant aberrantly splices the RAD52 mRNA from exon 3 directly to exon 5, skipping exon 4 (RAD52Δexon4), while the other variant fails to remove the short intron 8 (RAD52+intron8). These two variant mRNAs are predicted to encode truncations of the RAD52 peptide and are likely translated in vivo, since both are found on polyribosomes in the cytoplasm of mouse cells (Fig. 3).

Furthermore, a RAD52 IMAGE clone sequence was identified that lacks exon 4 (locus id:BC010673, gi:14715025), and sequences including RAD52 intron 8 were identified from the National Center for Biotechnology Information Mouse Uni- gene Clusters Map (data not shown). However, definitive identification of these variants using database screening fails, because many cDNA clone sequences are derived from partially spliced transcripts. In addition, examination of the sequences at the intron-exon boundaries of murine RAD52 does not identify deviations from the canonical splice sites to account for the alternate splicing. Thus, a solely bioinformatics approach is inadequate for identifying these novel splice variants, which requires the rigor of the RT-PCR analysis described here.

Testing the recombination phenotype of variant RAD52 transcripts in cell culture. RAD52Δexon4 and RAD52+intron8 are predicted to encode truncated mouse RAD52 peptides of 80 and 290 amino acids, respectively (Fig. 2). We next tested the effects of the mouse variants on HDR in mammalian cells using direct repeats of GFP (Fig. 5A). Expression of the murine RAD52 variants increase the frequency of gene replacement compared to wild-type mouse RAD52 (Fig. 5), establishing that these variants are capable of altering the DNA repair phenotype of mammalian cells. Although the increase in HDR is small, it is consistent with the relatively modest effect on this type of assay seen when complementing rad52 null cells (43). Several years ago, Kito et al. speculated that human RAD52 splice variants similar in size to the mouse RAD52Δexon4 transcript might confer a dominant-negative phenotype (16). Here we demonstrate that C-terminal truncations of RAD52 do in

- **TABLE 3. Quantification of Rad52 protein levels**

<table>
<thead>
<tr>
<th>Strain</th>
<th>RAD52 genotype</th>
<th>n</th>
<th>Quantification of CFP signal mean fluorescence</th>
<th>Quantification of YFP signal mean fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>W5937</td>
<td>RAD52-CFP/RAD52-YFP</td>
<td>54</td>
<td>477.5 (-/±71)</td>
<td>257.2 (-/±12.5)</td>
</tr>
<tr>
<td>W5938</td>
<td>RAD52-CFP/rad52-YFP</td>
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<td>504.6 (-/±40.7)</td>
<td>239.04 (-/±2.9)</td>
</tr>
<tr>
<td>W5939</td>
<td>RAD52-CFP/rad52Δ284-YFP</td>
<td>62</td>
<td>454.4 (-/±39.9)</td>
<td>264 (-/±12.5)</td>
</tr>
</tbody>
</table>

*The full genotypes are listed in Table 1. n indicates the number of cells measured for fluorescence. The amount of CFP is greater than YFP in all cases, as CFP gives a brighter fluorescence signal. It should be noted that nuclear fluorescence was measured in all cases, as Rad52 is localized within the nucleus. However, Rad52Δ284-YFP is localized throughout the cell.

*The mean fluorescence intensity (+standard deviation of the mean) is given in relative units.*
fact confer a dominant phenotype in mammalian cells by increasing recombination.

**Testing the recombination phenotype of variant RAD52 transcripts in yeast.** The recombination assays available in mammalian cells have a number of limitations. We therefore modeled the splice variants in yeast to allow a further assessment of their role in DNA repair and recombination. A key advantage of modeling the mouse splice variants in yeast is that a range of well-characterized assays are available against which other defined alleles of ScRad52 have previously been tested. Furthermore, the HDR assays available in yeast are more diverse and informative than the current mammalian assays.

The two mouse variants, \( \text{RAD52}\text{/H9004 exon4} \) and \( \text{RAD52}\text{/H11001 intron8} \), were modeled in the Sc\( \text{Rad52} \) gene (\( \text{rad52}\text{/H9004} \text{77} \) and \( \text{rad52}\text{/H9004} \text{284} \), respectively). These alleles are referred to as “splice variant equivalents,” since they truncate the yeast Rad52 protein at po-

![Image of yeast cultures with different genotypes and treatments](image-url)
sitions homologous to the mouse variants. The mammalian assay suggests that expression of the mouse variants elevates repair from the same or a sister chromosome (Fig. 5), therefore an equivalent yeast assay was performed. Direct-repeat recombination in yeast (Fig. 6) shows results similar to those seen in mammalian cells (Fig. 5), suggesting that the yeast splice variant equivalents do indeed model their mammalian counterparts. The splice variant equivalents show additional functional differences, which were revealed by further analysis of the LEU2 recombination products from the leu2 direct-repeat assay. For rad52Δ277, the increased recombination is due to a modest increase in replacement events, whereas for rad52Δ284 the increase is due to pop-out events (Table 2). In contrast to the increase in leu2 direct-repeat recombination, the splice variant equivalents dominantly decrease the frequency of recombination between ade2 heteroalleles (Fig. 7). These opposing recombination phenotypes suggest that the variants may bias the HDR pathway away from a homologous template toward a sister or intrachromatid template.

To probe further the dominant effects of the two splice variant equivalents, DNA damage sensitivity was tested. While mammalian rad52 mutant cells exhibit limited DNA damage sensitivity, yeast rad52 strains are exquisitely sensitive to DNA damage. Thus, in yeast, sensitivity to DNA damage provides a simple assay for RAD52 function. As haploid cells, both rad52Δ277 and rad52Δ284 are sensitive to γ-irradiation (data not shown). These results were not surprising, since previous studies showed that truncations removing more than ~200 C-terminal amino acids of ScRad52 render strains as sensitive as a rad52 null strain to methyl methanesulfonate (MMS), a DNA-damaging agent (1, 6). In addition, a deletion of the last 175 codons of ScRAD52 confers a defective DSB repair phenotype (51).

Since the mammalian variants are coexpressed with full-length transcripts (Fig. 1B, C, 3, and 4B), diploid strains containing a splice variant equivalent allele and a wild-type ScRAD52 allele were tested for γ-ray sensitivity. The rad52Δ284 allele is dominant negative, while the rad52Δ277 allele is not (Fig. 9A). Previous work has shown that overexpression of two ScRad52 C-terminal truncations confers a dominant-negative phenotype for sensitivity to MMS (26), suggesting that N-terminal Rad52 peptides are capable of disrupting the DNA repair ability of the full-length protein. Interestingly, structural analysis of both the full-length human RAD52 protein and an N-terminal fragment reveals a multimeric complex with RAD52 monomers arranged in a ring configuration (15, 40, 44). Therefore, it is likely that the RAD52+intron8 variant is incorporated into the ring and disrupts its function. This view is supported by our finding that Rad52Δ284 and wild-type Rad52 colocalize in foci (Fig. 8A). In addition, increased levels of wild-type Rad52 foci caused by the splice variant equivalents may indicate increased DNA damage. However, it is unclear how increased damage could cause a specific preference for repair from the same or a sister chromosome. Indeed, DNA damage elevates repair from both sister and homologous chromosomes (13). In addition, elevated Rad52 foci levels in yeast mutants typically indicate delayed or impaired repair processes.

Disruption of the Rad52-Rad51 interaction and its effects on recombination. C-terminal truncations of Rad52 are known to disrupt its interaction with Rad51, thus impairing the formation of Rad51 filaments (19, 26, 37, 39). Such Rad52 C-terminal truncation mutants are complemented by overexpression of RAD51 (1, 26). Furthermore, disruption of the Rad52-Rad51 interaction in both yeast and mammalian cells channels recombination intermediates to Rad51-independent repair pathways, such as single-strand annealing (SSA) or break-induced replication (33, 43, 51). The dominant recombination phenotype exhibited by the splice variants may be due to disruption of this Rad52-Rad51 interaction. Indeed, we find that the dominant-negative γ-ray sensitive phenotype of rad52Δ284 is partially rescued by overexpression of the RAD51 gene (Fig. 9B). In addition, the rad52Δ284 allele increases pop-out recombination between leu2 direct repeats that likely occurs by SSA. Together, these data support the notion that Rad51-dependent pathways have been disrupted.

On the surface, expression of all the variants results in increased direct-repeat recombination in both mammalian cells and yeast. However, further analysis of the recombinants in yeast reveals some differences. For example, the shorter rad52Δ277 variant increases the frequency of gene conversion events in contrast to rad52Δ284, which increases pop-out events. In addition, Rad52Δ277 does not colocalize with wild-type Rad52 in DNA repair foci or cause a dominant-negative γ-ray sensitivity phenotype, like Rad52Δ284 does. However, despite these differences, both yeast variants increase wild-type Rad52 focus levels and increase direct-repeat recombination. Thus, we conclude that these variants may disrupt HDR in a similar way and represent a new phenotype for rad52 N-terminal mutants (28).

RAD52 variants favor sister chromatid repair. We show that the RAD52 splice variants dominantly increase the frequency of direct-repeat recombination from the same or a sister chromosome in both mammalian cells and yeast. In yeast, where we are able to easily measure the frequency of recombination between heteroalleles on homologous chromosomes, the splice variant equivalents lower the frequency of recombination. Rad51-independent repair pathways are also required for interchromosomal but not intrachromosomal repair in yeast (2). Therefore, it is possible that expression of the RAD52 splice variants in mammalian cells inhibits RAD51-dependent interchromosomal repair of spontaneous lesions, consequently forcing them to be repaired from the sister chromatid. Perhaps expression of RAD52+intron8 inhibits the activity of RAD51, impairing the homology search ability of the repair complex. This, in turn, may favor the repair of DSBs using the sister chromatid template, which is tethered by cohesin, rather than searching for a homolog (18, 34). Similarly, deletion of p53 also favors intrachromosomal or sister chromatid repair (53), perhaps mediated through the inhibition of RAD51. Recently, differential expression of splice variants of p53 was found in human breast tumors compared to normal breast tissue (7). Similarly, the differential expression of the RAD52 splice variants in the various cell types, shown in Fig. SA of the supplemental material, may control selective preferences for recombination in different adult tissues.

ACKNOWLEDGMENTS

We thank Scott Hughes for help with preparing mouse samples, Maureen Ward and Arthur Bank for mouse 3T3 cells and tissue culture reagents, and Lorene Rozier for help with the mitotic shake-off technique. Further, we are indebted to Maria Jasin, Jeremy Stark, and...
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