RPA antagonizes microhomology-mediated repair of DNA double-strand breaks

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Microhomology-mediated end joining (MMEJ) is a Ku- and ligase IV–independent mechanism for the repair of DNA double-strand breaks that contributes to chromosome rearrangements. Here we used a chromosomal end-joining assay to determine the genetic requirements for MMEJ in Saccharomyces cerevisiae. We found that end resection influences the ability to expose microhomologies; however, it is not rate limiting for MMEJ in wild-type cells. The frequency of MMEJ increased by up to 350-fold in rfa1 hypomorphic mutants, suggesting that replication protein A (RPA) bound to the single-stranded DNA (ssDNA) overhangs formed by resection prevents spontaneous annealing between microhomologies. In vitro, the mutant RPA complexes were unable to fully extend ssDNA and were compromised in their ability to prevent spontaneous annealing. We propose that the helix-destabilizing activity of RPA channels ssDNA intermediates from mutagenic MMEJ to error-free homologous recombination, thus preserving genome integrity.

Chromosomal double-strand breaks (DSBs) are cytotoxic lesions that occur spontaneously during normal cellular processes or as a result of the treatment of cells with DNA-damaging agents. Failure to repair DSBs, or inappropriate repair, can lead to chromosome loss, deletions, duplications or translocations. Two mechanistically distinct pathways have evolved to repair DSBs: homologous recombination (HR) and non-homologous end joining (NHEJ). HR relies on an intact homologous duplex to serve as a template for repair, whereas NHEJ involves the direct ligation of DSB ends. Canonical NHEJ is defined as being dependent on Ku and ligase IV and can occur with high fidelity or be associated with small deletions or insertions at the junctions. NHEJ junctions exhibit either no homology or short (1–4 bp) microhomologies (MHs). In the absence of Ku or ligase IV, end joining occurs at a reduced frequency and is characterized by larger deletions with longer MHs at the junctions.

There may be several distinct alternative end-joining pathways in mammals, but in budding yeast, Ku-independent ligation occurs by MMEJ exclusively. Mechanistically, MMEJ is similar to single-strand annealing (SSA), a pathway that can be used to repair DSBs formed between long direct repeats. Both processes initiate by nucleolytic degradation of the 5′ strands of DSBs to yield 3′ ssDNA tails, a process referred to as 5′-3′ resection. Resection is required to expose homologies internal to the DNA ends that are subsequently annealed, resulting in loss of one of the repeats and the sequence between them.

Studies in budding yeast have shown that the conserved Mre11–Rad50–Xrs2 (MRX) complex (Mre11–Rad50–Nbs1 in mammals), together with Sae2, initiates end resection, and extensive processing of the 5′ strands requires the 5′-3′ exonuclease, Exo1, or the combined activities of the Sgs1 helicase and Dna2 endonuclease. MRX and Sae2 can act directly to initiate resection by endonucleolytic cleavage of the 5′ strand, resulting in limited end processing, or MRX can act indirectly by recruiting Sgs1, Dna2 and Exo1 (refs. 7–9). Although the activities of Sae2 and the Mre11 nuclease are essential to remove covalent adducts from ends, they are dispensable for resection of endonuclease-induced DSBs and function only to accelerate resection initiation. In contrast, Schizosaccharomyces pombe or mammalian cells depleted of the Sae2 ortholog, Ctp1 or CtIP (also called Rbbp8), respectively, greatly reduce resection initiated from an endonuclease-induced DSB, which is similar to the effect of loss of the MRN complex. Consistent with the requirement for end resection to reveal MHs internal to DSB ends, knockdown of CHN (Rbbp8) using short interfering RNA (siRNA) reduces the frequency of MMEJ. Furthermore, siRNA knockdown of CHN reduces the frequency of DSB-induced translocations in mouse cells, and the translocation breakpoints are associated with shorter deletions and reduced MH usage. In mouse and yeast cells, elimination of Mre11 reduces both NHEJ and MMEJ, but the junctions recovered after Mre11 depletion in mouse cells do not show a change in the length of MHs.

Annealing between exposed homologies is critical for MMEJ and SSA. Rad52 catalyzes annealing of complementary ssDNA in vitro and is required for SSA in vivo; however, the role of Rad52 in MMEJ is under debate. MMEJ between chromosomal MHs of ≤14 bp was reported to be Rad52 independent, whereas annealing of complementary ssDNA overhangs of >8 nt in a plasmid end-joining assay was shown to be partially Rad52 dependent. A recent study in yeast showed that the efficiency of MMEJ is extremely sensitive to the...
length of MHs, and the sequence composition and distance between the DSB and MH also influence repair efficiency\textsuperscript{24,25}. These findings suggest that annealing between MHs is spontaneous and driven by the thermal stability of the annealed sequence.

RPA is a heterotrimeric ssDNA binding protein (encoded by RFA1, RFA2 and RFA3 in \textit{S. cerevisiae}) that is required for multiple transactions involving ssDNA\textsuperscript{26}. RPA prevents spontaneous annealing between complementary ssDNA \textit{in vitro}; however, this inhibitory effect can be overcome by Rad52 (ref. 27). A hypomorphic mutation in \textit{rfai} resulting in a D228Y substitution in the protein (\textit{rfai}-D228Y) was identified as a suppressor of the rad52A SSA defect\textsuperscript{28,29}. Genetic screens identified several other \textit{RFA1} alleles (\textit{rfai}-t11, \textit{rfai}-t133 and \textit{rfai}-t48) that impart sensitivity to DNA-damaging agents and reduce mitotic and meiotic recombination\textsuperscript{30,31}. Of the \textit{rfai} alleles tested, none reduced end resection, but the \textit{rfai}-t11 mutant was defective for SSA.

A recent study showed that complete depletion of RPA from yeast cells prevents long-range resection by Exo1 and Sgs1–Dna2, and short (5–9 bp) inverted repeats within the partly resected ends anneal to form hairpin structures, suggesting that one important function of RPA is to prevent spontaneous annealing between MHs \textit{in vivo}\textsuperscript{12}.

Here we used a chromosomal end-joining assay to examine the requirement for end resection and strand annealing during MMEJ. We found that resection influences the ability to expose MHs but is not rate limiting, especially when the MHs are close to the break ends. Furthermore, our studies reveal a role for RPA in preventing MMEJ, suggesting that annealing between MHs is spontaneous in budding yeast and that this critical step is inhibited by RPA bound to ssDNA.

RESULTS

Resection initiation prevents NHEJ repair of DSBs

We developed a chromosomal end-joining assay to elucidate the role of resection initiation in directing repair by MMEJ or NHEJ in yeast. We inserted two inverted 18-bp I-SceI endonuclease cleavage sites separated by a 4-bp linker and flanked by 12-bp direct repeats corresponding to the ADE2 coding sequence within the \textit{ADE2} open reading frame (ORF), thereby inactivating the gene. We used inverted I-SceI cut sites to minimize multiple cycles of repair by accurate ligation and re-cleavage by I-SceI\textsuperscript{3}. The 12-bp repeats were designed to restore the \textit{ADE2} coding region when repair occurs by MMEJ (Fig. 1a). Yeast \textit{ade2} mutants accumulate a red pigment resulting in the formation of red colonies, whereas cells with wild-type \textit{ADE2} form white colonies; thus, repair by MMEJ is scored directly by the plating efficiency of cells on medium containing galactose compared with medium containing glucose.

The survival frequency of wild-type cells was 0.0036, and a majority (~98%) of survivors were \textit{ADE2}\textsuperscript{+} (Fig. 1b and Table 1). DNA sequencing revealed that 95% of the \textit{ADE2}\textsuperscript{+} survivors repaired the DSB by NHEJ, and most used the 2-bp MH within the 3′ ATAA overhangs produced by I-SceI cleavage (Supplementary Fig. 1). The remaining \textit{ADE2}\textsuperscript{+} events resulted from MMEJ between imperfect 16-bp repeats (two mismatches within 18 bp) located 5 kb apart (Supplementary Table 1). We also sequenced the \textit{ADE2}\textsuperscript{+} survivors and found that 31 out of 37 used the 12-bp MH, but 6 events were due to NHEJ using the 2-bp MH within the 3′ overhangs associated with a frame shift, or deletion of 4 bp, to restore the \textit{ADE2} reading frame (Supplementary Fig. 1). The \textit{ADE2}\textsuperscript{+} NHEJ events represent <1% of the total NHEJ events and are combined with the \textit{ADE2}\textsuperscript{−} NHEJ class in Table 1.

Survival of yku70A and \textit{dnl4A} mutants was reduced by >24-fold as compared to wild-type cells. The \textit{ADE2}\textsuperscript{−} and \textit{ADE2}\textsuperscript{+} events due to

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
Gene & \textit{ADE2}\textsuperscript{−} NHEJ & \textit{ADE2}\textsuperscript{+} MMEJ \\
\hline
\textit{ade2} & 31/37 & 6/37 \\
\textit{ade2} & 31/37 & 6/37 \\
\textit{ade2} & 31/37 & 6/37 \\
\textit{ade2} & 31/37 & 6/37 \\
\textit{ade2} & 31/37 & 6/37 \\
\textit{ade2} & 31/37 & 6/37 \\
\hline
\end{tabular}
\caption{Survival frequencies of \textit{ADE2}\textsuperscript{−} and \textit{ADE2}\textsuperscript{+} events.}
\end{table}
joining by the 2-bp MH within the I-SceI-generated overhanges were eliminated in the yku70Δ and dnl4Δ mutants, which is consistent with their formation by NHEJ. Although 53.0% and 39.1% of survivors in the yku70Δ and dnl4Δ mutants, respectively, were Ade+ as a result of MMEJ at the 12-bp MH, the absolute frequency of these events was the same as that in wild-type cells (Fig. 1b). The majority of Ade− survivors recovered from the yku70Δ and dnl4Δ mutants were formed by MMEJ using the 16-bp MH, but we identified other MHS at the junctions at a lower frequency (Supplementary Table 1). Consistent with previous studies, we found the frequency of survivors to be greatly reduced in the mre11Δ mutant because of loss of NHEJ; however, the frequency of Ade+ MMEJ was higher than in wild-type cells (P = 0.0001) (Fig. 1b).

Survival of the sae2Δ mutant was increased by 7.4-fold relative to wild-type cells because of an increased frequency of NHEJ (Fig. 1c).

The absolute frequency of MMEJ using the 12-bp MH was similar to that in wild-type cells, but we detected no Ade− MMEJ events. Only 5 of 100 Ade− survivors tested from the wild-type cells were the result of MMEJ, so the failure to detect one event among 120 analyzed from the sae2Δ mutant is not unexpected given the large increase in NHEJ frequency. Similarly, survival and NHEJ increased by 4.7-fold in the mre11Δ-H125N mutant (defective for Mre11 nuclease activity) with no alteration in the frequency of MMEJ. End resection of endonuclease-induced DSBs is delayed in the mre11Δ, mre11Δ-H125N and sae2Δ mutants but still occurs as a result of the activities of Exo1 and Sgs1-Dna2. Because the triple mutants are inviable, we could not assess the contributions of Sgs1–Dna2 and Exo1 to MMEJ in the absence of Mre11 or Sae2.

To further assess the role of Sae2 in MMEJ, we used a plasmid-based end-joining assay. Intramolecular joining between the ends of a PCR-generated DNA fragment containing TRP1 and the ARS416 replication origin creates an autonomous replicon that is detected by formation of Trp+ transformants (Supplementary Fig. 2a).

We designed primers to create a substrate for blunt-end joining (0 MH) or with an embedded direct repeat of 8, 12 or 16 bp to promote joining by MMEJ. We found that blunt-end joining is infrequent, but incorporation of repeats within the primers resulted in a length-dependent increase in transformation frequency. The frequency of Trp+ transformants from the 0-MH substrate was increased by five-fold (P = 0.01) in the sae2Δ mutant compared with wild-type cells (Supplementary Fig. 2), but we observed no significant change in the frequency of MMEJ. Sequence analysis of junctions derived from the 12-MH substrate in the sae2Δ mutant revealed that 23 out of 27 junctions were formed by MMEJ, whereas all such junctions were due to MMEJ in wild-type cells, indicating a subtle defect in MMEJ in the absence of Sae2 (Supplementary Fig. 2d).

Together these results show that Sae2 prevents NHEJ but is not essential for MMEJ in yeast.

### Extensive resection is not required for proximal MMEJ

Exo1 and Sgs1–Dna2 act in parallel to degrade the 5′ ends of DSBs, generating extensive tracts of ssDNA. In the absence of Exo1 and Sgs1–Dna2, resection by MRX-Sae2 removes nucleotides from the 5′ ends in increments of ~100 nt, but the 3′ ssDNA tails rarely exceed 700 nt. Thus, we predicted that the limited MRX-Sae2–dependent resection in the exo1Δ sgs1Δ mutant would be sufficient for MMEJ at the 12-bp repeats but not for the Ade− MMEJ events. There was a small but significant increase in both NHEJ and Ade− MMEJ events in the exo1Δ mutant (P < 0.05), but there was no alteration in the frequency or spectrum of events in the absence of Sgs1 (Fig. 1d).

The frequency of Ade− MMEJ increased by 24-fold in the exo1Δ sgs1Δ mutant, indicating that loss of extensive resection promotes MMEJ close to the DSB, and, as expected, we recovered no Ade− MMEJ events (Fig. 1d). Because Ade− survivors are primarily due to NHEJ, we created a yku70Δ derivative of the exo1Δ sgs1Δ mutant to ensure no contribution from NHEJ and found that all of the survivors from the triple mutant were Ade+ as a result of MMEJ using the 12-bp direct repeats (Table 1).

### RPA suppresses MMEJ

As resection is not limiting for end joining by the 12-bp MH but the frequency of chromosomal MMEJ is very low, we considered the possibility that annealing is the limiting process. RPA removes secondary structures from ssDNA, suggesting that it may prevent annealing between MHs. Furthermore, Smith and Rothstein identified a hypomorphic allele of RFA1 in a screen for suppressors of the SSA defect in the rad52Δ mutant, indicating that Rad52-catalyzed ssDNA annealing is no longer needed when RPA is defective. Thus, we predicted that the frequency of MMEJ would be elevated in the rfa1Δ-D228Y mutant. Indeed, the frequency of Ade− and Ade+ MMEJ events increased by 124-fold and 14-fold, respectively, in the rfa1Δ-D228Y background, resulting in higher cell survival in response to the DSB (Fig. 2a and Table 1). The frequency of Ade− NHEJ remained similar to that in wild-type cells, and we recovered no Ade− NHEJ events.

The defect of the rfa1Δ-D228Y mutant could be due to reduced abundance of the RPA-D228Y complex or to an alteration in DNA
binding by the mutant complex. Smith and Rothstein showed that overexpression of rfa1-D228Y could partially suppress the UV sensitivity and hyper-recombination phenotype of the rfa1-D228Y mutant. We verified that the steady-state level of rfa1-D228Y is reduced relative to Rfa1 (Fig. 2b and Supplementary Fig. 3) but found that overexpression of the rfa1-D228Y allele in the rfa1-D228Y background did not reduce MMEJ, whereas overexpression of RFA1 resulted in full suppression (Fig. 2c).

Because rfa1-D228Y was identified in a screen for mutations that suppressed the rad52a direct repeat recombination defect, the increase in MMEJ could be due to a specific property of this allele rather than a general perturbation of RPA binding to ssDNA. The rfa1-t48 (L221P), rfa1-t33 (S373P) and rfa1-t11 (K45E) mutations were isolated by screening for RFA1 alleles that confer a temperature-sensitive, UV-sensitive or methyl methanesulfonate–sensitive phenotype. Similarly to rfa1-D228Y, rfa1-t48 and rfa1-t33 contain point mutations in the DNA binding domains, whereas rfa1-t11 has a mutation in the DNA polymerase-α interaction domain. The rfa1-t48 and rfa1-t11 mutants exhibit recombination defects, and rfa1-t11 is also defective for SSA. The SSA defect of the rfa1-t48 mutant is not due to decreased end resection, and in vitro studies demonstrated that Rad51 displaces RPA from ssDNA more slowly than wild-type RPA. The rfa1-t33 and rfa1-t48 mutations increased the frequency of Ade+ MMEJ by 85- and 350-fold, respectively, as compared to wild-type cells, whereas MMEJ was unaffected by the rfa1-t11 mutation (Fig. 2a). The steady-state protein level of the mutant rfa1 proteins was 61–80% of wild-type level and did not correlate with MMEJ frequency (Fig. 2b). As the Ade+ MMEJ events require resection of 5 kb, and this class of events was also increased in the rfa1-D228Y, rfa1-t48 and rfa1-t11 mutants, it seems unlikely that the increased use of MHs in the rfa1 mutants is due to an extensive resection defect. However, to address this concern, we measured end resection from an HO endonuclease–induced DSB in the rfa1-D228Y, rfa1-t33 and rfa1-t48 mutants by Southern blot hybridization and found the mutants to be resection proficient (Supplementary Fig. 4).

These data suggest that the interaction between RPA and ssDNA is a critical determinant for repair by MMEJ.

In addition to MMEJ mediated by the 12- and 16-bp MHs, we identified other MHs used for repair in the rfa1 hyper-MMEJ mutants (Supplementary Table 1). In RFA1 cells, the only MMEJ events detected were mediated by the 12- and 16-bp MHs; however, we recovered junctions with shorter MHs or MHs with more interruptions and mismatches from the rfa1-D228Y, rfa1-t33 and rfa1-t48 mutants. We also observed use of other MHs in the yku70Δ and dnl4Δ mutants, but at a ten-fold reduced frequency as compared to the rfa1 mutants. Therefore, mutations that impair Rfa1 DNA binding allow for more promiscuous annealing.

Although end resection is not limiting for MMEJ in wild-type cells, we considered the possibility that Sae2 would be required if the constraint on MH annealing was reduced. Surprisingly, we were unable to generate an rfa1-D228Y sae2Δ double mutant (Fig. 2d). The rfa1-D228Y rad51Δ and rfa1-D228Y rad52Δ mutants were viable, indicating that the rfa1-D228Y sae2Δ lethality is not due to an increased need for HR in the rfa1-D228Y background. It is possible that promiscuous annealing in the rfa1-D228Y mutant leads to the formation of secondary structures within ssDNA that require Sae2 for resolution. The rfa1-t33 mutation confers a temperature-sensitive growth defect, and we were able to generate an rfa1-t33 sae2Δ double mutant by germinating the spore clones at 23 °C. The Ade+ and Ade+ MMEJ frequencies were both significantly lower for the rfa1-t33 sae2Δ double mutant than for the rfa1-t33 single mutant (P < 0.001), indicating that resection becomes limiting when the barrier to annealing is lifted (Fig. 2e).

The rfa1-D228Y mutant was reported to be defective for interchromosomal recombination, raising the possibility that the increased frequency of MMEJ is due to reduced competition with HR. To investigate whether rfa1-D228Y cells are defective for DSB-induced gene conversion, we used the well-characterized mating type–switching system (Supplementary Fig. 4). The repair efficiency of the rfa1-D228Y mutant was slightly reduced (71% of the level observed...
**Figure 3** RPA mutants are defective for ssDNA binding and disruption of secondary structure. (a) PAGE to separate substrates (ssDNA) and products (dsDNA) of the strand annealing reaction. Each gel shows time points (0–8 min) from the annealing reaction in the absence of protein or in the presence of 30 nM RPA, RPA<sup>133</sup> or RPA<sup>448</sup>. A representative gel from three independent trials is shown. (b) Quantification of the annealed dsDNA product. The fraction of annealing was calculated as the radioactivity in the dsDNA band divided by the sum of the two bands. Values are a mean of three trials and errors bars indicate the s.d. (c) Schematic of an ssDNA curtain assay showing ssDNA before and after RPA-eGFP binding (RPA-eGFP/ssDNA). (d) Kymographs showing ssDNA binding by RPA-eGFP, RPA<sup>133</sup>eGFP or RPA<sup>448</sup>eGFP (100 pM each), as indicated, followed by exchange with RPA-mCherry (100 pM). (e) Normalized signal intensity for both RPA-eGFP and RPA-mCherry over time. (f) Comparison of the dissociation kinetics of RPA-eGFP, RPA<sup>133</sup>eGFP as RPA<sup>448</sup>eGFP after the injection of RPA-mCherry (not shown). The solid black lines are single exponential fits approximating the dissociation of the eGFP-tagged RPAs during exchange with RPA-mCherry, yielding rates of ~0.006 s<sup>−1</sup> for both RPA mutants and ~0.001 s<sup>−1</sup> for RPA-eGFP.

For wild-type cells, indicating that the rfa1-D228Y mutant is largely proficient for Rad51 loading and gene conversion repair. Thus, we attribute the increased MMEJ frequency of the rfa1-D228Y mutant to increased spontaneous annealing rather than defective HR.

**RPA mutant complexes are defective for DNA binding in vitro**

The increased MMEJ observed for the rfa1 mutants suggests that the mutant complexes are compromised in their ability to prevent spontaneous annealing between short homologies. To test this hypothesis, we purified the RPA<sup>133</sup> and RPA<sup>448</sup> mutant complexes after expression in *Escherichia coli*<sup>39</sup> and then tested them for their ability to inhibit the annealing of complementary oligonucleotides in *vitro*. We were unable to purify RPA<sup>D228Y</sup> because of instability of the complex. We quantified strand annealing by formation of a duplex product after incubating a [<sup>32</sup>P]-labeled 48-mer oligonucleotide with an unlabeled complementary oligonucleotide in the absence or presence of RPA. After 8 min at 30 °C, 78% of the ssDNA oligonucleotide spontaneously annealed to form double-stranded DNA (dsDNA) product in the absence of added protein (Fig. 3a). Consistent with a previous study<sup>37</sup>, less than 10% of the oligonucleotide annealed to its complement in the presence of wild-type RPA, whereas ~30% was annealed when incubated with RPA<sup>133</sup> or RPA<sup>448</sup> (Fig. 3b).

To characterize the interaction of the mutant RPA complexes with ssDNA further, we used a DNA curtain assay and total internal reflection fluorescence microscopy to directly visualize the binding of fluorescently tagged RPA to long ssDNA molecules in real time (Fig. 3c)<sup>39,40</sup>. We generated ssDNA by rolling-circle replication and anchored it to a lipid bilayer on the surface of a microfluidic sample chamber. The ssDNA was not fluorescent and remained highly compacted because of the formation of extensive secondary structure. When enhanced GFP (eGFP)-tagged wild-type RPA (RPA-eGFP) is injected, it can bind to the tethered ssDNA, allowing it to be visualized and also causing an increase in the observed contour length because of the removal of secondary structure (Fig. 3d, top)<sup>39</sup>. RPA remains bound to the ssDNA with a half-life exceeding 2 h when free RPA is not present in solution. However, ssDNA-bound RPA can also undergo much more rapid concentration-dependent turnover when free RPA is present in solution through a mechanism that is consistent with free RPA causing macroscopic dissociation of a microscopically dissociated RPA-ssDNA intermediate; this rapid protein exchange can be visualized as a change in fluorescence color of the ssDNA when switching between RPA-eGFP and RPA-mCherry (Fig. 3d,e)<sup>40,41</sup>. The exchange of RPA-eGFP for RPA-mCherry does not coincide with a change in the observed extension of the ssDNA. RPA<sup>133</sup>eGFP and RPA<sup>448</sup>eGFP both bound to the ssDNA; however, neither was able to extend the ssDNA to the same extent as RPA-eGFP (Fig. 3d, bottom). This defect in removal of the ssDNA secondary
structure was revealed by allowing the binding reactions to continue for 20 min with the mutant RPAs and then chasing with the same concentration of RPA-mCherry, which resulted in both rapid exchange of the mutant RPA for RPA-mCherry and a corresponding increase in the extension of the ssDNA substrates. Exchange of RPA<sup>133</sup>-eGFP and RPA<sup>148</sup>-eGFP for RPA-mCherry resulted in ~33% and ~84% increases in the observed ssDNA extension, respectively. The effect was most pronounced with RPA<sup>148</sup>-eGFP, indicating that this mutant exhibits the most substantial defect in secondary structure removal. In addition, quantification of the loss of eGFP signal after injection of RPA-mCherry revealed that both RPA mutants dissociated from the ssDNA approximately six-fold more rapidly than wild-type RPA-eGFP (Fig. 3f). Taken together, the in vitro studies support the hypothesis that RPA<sup>133</sup> and RPA<sup>148</sup> allow more spontaneous annealing between MHs in vivo as a consequence of defective ssDNA binding and removal of the secondary structure.

HR competes with MMEJ repair

After resection initiation, the 3′ ssDNA coated by RPA is exchanged for Rad51 through the Rad52 mediator to initiate pairing and strand invasion with homologous duplex DNA<sup>5</sup>. If MMEJ results from delayed or failed initiation of HR, we would predict the frequency of MMEJ to increase in rad51Δ and rad52Δ mutants. Consistent with this hypothesis, the rad51Δ, rad52Δ and rad51Δ rad52Δ mutants exhibited a three- to six-fold increase in Ade<sup>+</sup> MMEJ (P = 0.0001) (Table 1 and Fig. 4a). Although a recent report showed that MMEJ between repeats of >14 bp is partially RAD52 dependent<sup>24</sup>, we did not observe a decrease in the frequency of Ade<sup>−</sup> MMEJ in the rad52Δ and rad51Δ rad52Δ mutants.

Eliminating HR increases MMEJ by only three- to six-fold, whereas the rfa1-D228Y mutant exhibits a 124-fold increase in the frequency of Ade<sup>−</sup> MMEJ, indicating that the increase caused by dysfunctional RPA is not due simply to defective HR. If the slight HR defect of the rfa1-D228Y mutant contributed to the increased frequency of MMEJ, we would predict the rfa1-D228Y mutation to be epistatic to rad51Δ and rad52Δ. The frequencies of MMEJ increased by 250- and 272-fold in the rad51Δ rfa1-D228Y and rad52Δ rfa1-D228Y double mutants, respectively, and were significantly higher than in the rfa1-D228Y single mutant (P < 0.005), which is consistent with independent inhibition of MMEJ by RPA and Rad51 (Fig. 4b).

DISCUSSION

Chromosomal DSBs can be repaired by several distinct mechanisms with different mutagenic potentials. Interest in MMEJ has grown with the discovery that breakpoints of chromosome rearrangements frequently show MHs, implicating MMEJ as the underlying mechanism<sup>42–44</sup>. Here we used a chromosomal assay to monitor repair by NHEJ or MMEJ in budding yeast and found the frequency of MMEJ to be very low (0.006%), even though a perfect 12-bp MH flanks the I-SceI cut site. Repair of a chromosomal DSB by homologous recombination or SSA between long (>1 kb) repeats occurs with close to 100% efficiency in <i>S. cerevisiae</i><sup>45</sup>, indicating that MMEJ is rarely used to repair DSBs. By contrast, the frequency of MMEJ in mammalian cells using a substrate similar to the one we describe here, but with only 8- to 9-bp repeats flanking the I-SceI cut site, occurs at a much higher frequency (0.5–1%) than we observed in yeast<sup>41,42</sup>. In a direct comparison of MMEJ and HR using a reporter that can detect both classes of events, the frequency of HR was only five- to ten-fold higher than that of MMEJ, indicating that MMEJ has a substantial role in DSB repair in mammalian cells<sup>46</sup>. The large difference in the frequency of MMEJ could be due to an active mechanism to prevent MMEJ in yeast or the presence of dedicated MMEJ synopsis and/or annealing proteins in mammalian cells. PARP-1 has been shown to synapse DNA ends <i>in vitro</i> and is required for end joining in the absence of Ku<sup>46–48</sup>. DNA ligase III (Lig3) functions with PARP-1 to catalyze alternative NHEJ <i>in vitro</i> and promotes chromosome translocations in mouse cells<sup>46,49–51</sup>. PARP-1 and DNA ligase III are both absent from yeast, and this absence could explain the low frequency of MMEJ observed. In addition, we propose that a critical step during MMEJ is annealing between MHs, which is normally restricted by RPA.

In contrast to a previous report in budding yeast<sup>49</sup>, we found no MMEJ defect in the <i>sae2Δ</i> mutant. The system used by Lee and Lee detects MMEJ at naturally occurring MHs near the native HO cut site by sequencing the junctions present in colonies surviving HO induction<sup>19</sup>. In agreement with our findings, Lee and Lee reported a ten-fold increase in the frequency of NHEJ in the <i>sae2Δ</i> mutant, but of the sequenced events, none used MHs of >5 nt<sup>19</sup>. A later study using the same strain reported the same frequency of MMEJ in wild-type and <i>sae2Δ</i> strains<sup>52</sup>. In the plasmid end-joining assay, we observed similar frequencies of MMEJ in <i>sae2Δ</i> and wild-type cells, but we recovered some aberrant products resulting from blunt-end joining or partial loss of the MH from the <i>sae2Δ</i> mutant, whereas all of the products from wild-type cells used the MH. Thus, a subtle
MMEJ defect may be apparent in sae2Δ mutants depending on the assay used.

The most striking phenotype of the sae2Δ mutant is the increase in canonical NHEJ, a phenotype that is also observed after CtIP depletion from irradiated G2 cells. Ku persists at DNA ends for longer in the absence of Ctip1 or Sac2, suggesting that delayed resection initiation and the presence of Ku allow more time for NHEJ. An increased use of NHEJ to repair breaks in cis could potentially contribute to the decreased frequency of translocations reported for mouse cells depleted of CtIP. However, even in Ku-deficient cells, CtIP depletion results in reduced usage of MHs at the junctions, consistent with the important role for CtIP in resection initiation and MMEJ in mammalian cells.

As we anticipated, we found that extensive resection is not required for MMEJ using the MH close to the break site but is needed for use of the distal MH. The frequency of Ade− MMEJ increased by 24-fold in the exo1Δ sgs1Δ mutant, suggesting that loss of extensive resection stabilizes the partially resected 3′ overhangs to provide increased opportunity for MMEJ to occur between the proximal MHs. Ade+ MMEJ events were increased in the mre11Δ mutant, and the frequency of Ade− MMEJ was decreased relative to wild-type cells, consistent with the requirement for the MRX complex to recruit the extensive resection nuclease. If extensive resection and MMEJ through more distal MHs, resulting in the loss of essential genes, contributed to the low viability of wild-type cells after DSB induction, we might have expected an even higher frequency of survival for the exo1Δ sgs1Δ mutant. However, cell survival of the exo1Δ sgs1Δ mutant was only 0.44%, which is similar to that of wild-type cells. Previous studies have shown that the DNA damage checkpoint is impaired in exo1Δ sgs1Δ cells, and consequently, cell division might occur with an unrepaired DSB, leading to mis-segregation of the acentric fragment. De novo telomere addition at DSB ends is increased in the exo1Δ sgs1Δ mutant and could also contribute to cell death.

A systematic study of MMEJ in S. cerevisiae revealed the end-joining frequency to be sensitive to the length of the MH. Similarly, our results suggest that a critical step during MMEJ is the ability of MHs to spontaneously anneal. RPA has been shown to inhibit annealing between oligonucleotides in vitro and prevent the formation of DNA secondary structures in vivo. By using hypomorphic alleles of RFA1 to perturb the interaction between RPA and ssDNA, we show that the frequency of MMEJ can be increased by up to 350-fold, and purified RPA is less effective in the removal of secondary structure from ssDNA and in preventing spontaneous annealing in vitro. Furthermore, the rfa1 mutants exhibiting the greatest increase in MMEJ show a greater diversity in the sequences used for repair, with use of shorter MHs and MHs with more mismatches than those observed in wild-type cells. Complete depletion of RPA does not prevent the initiation of end resection, but the ssDNA tails formed undergo intramolecular pairing between short inverted repeats to form hairpin-capped ends. If such structures formed in the rfa1 hypomorphic mutants, they would not give rise to viable products. The only rfa1 allele tested that did not confer increased MMEJ frequency was rfa1-t11. In vitro, RPA forms stable complexes with ssDNA and would be expected to inhibit strand annealing. A previous study showed that rfa1 hypomorphic alleles confer greatly elevated rates of spontaneous gross chromosomal rearrangements (GCRs) as compared to wild-type cells. Moreover, most GCRs were due to chromosome truncation and de novo telomere addition in wild-type cells, whereas translocations and inversions mediated by MHs were more frequently observed in the rfa1-t133 mutant. The GCR product spectrum was not determined for the rfa1-t11 and rfa1-t48 mutants. The rfa1-t33 and rfa1-t48 mutants both show elevated rates of spontaneous GCRs and increased frequencies of DSB-induced MMEJ. Although the >100-fold increase in the GCR rate is most likely due to the generation of more initiating lesions when RPA is impaired, our results suggest that the more promiscuous use of MMEJ contributes to the genome instability of rfa1 hypomorphic mutants. The rfa1-t48 allele, which confers a 350-fold increase in Ade− MMEJ, causes embryonic lethality in mice when homozygous and increased chromosome instability and cancer predisposition when heterozygous. The breakpoints of chromosome rearrangements formed in the Rfa1+t48 mouse have not been analyzed, and it would be of interest to determine whether there is increased usage of MHs.

In summary, we demonstrate that in wild-type cells, MMEJ is limited by RPA bound to ssDNA. The role of resection, which was previously thought to drive MMEJ, is necessary to reveal MHs but does not guarantee annealing and completion of repair. Our studies demonstrate important roles for Sac2 and RPA in repair pathway choice. The initiation of end resection by Sac2 prevents repair by NHEJ by creating 3′ ssDNA overhangs that are poor substrates for Ku binding but are of sufficient length for RPA to bind and promote more extensive resection by Sgs1–Dna2 or Exo1. Although ssDNA is essential for homology-directed repair, resection has the potential to reveal MHs internal to the break site that can be used to align ends for mutagenic MMEJ repair. We suggest that RPA has an important role at this step to remove the secondary structure from ssDNA, facilitating Rad51 nucleoprotein filament assembly, and at the same time prevents spontaneous annealing that can give rise to MMEJ. The role of RPA in binding ssDNA is highly conserved and is likely to have a role in preventing MMEJ and the accompanying translocations in mammalians.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

S.K.D., B.G., E.C.G. and L.S.S. designed experiments and wrote the paper. The experiments shown in Figures 1 and 2 and Supplementary Figures 1–4 were carried out by S.K.D., those shown in Figure 3 were carried out by S.K.D. and B.G., and those shown in Figure 4 were carried out by S.K.D. and M.J.d.A.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Yeast strains. The S. cerevisiae strains used in this study are listed in Supplementary Table 2. All strains are in the W303 background and were generated by PCR fragment–mediated gene targeting or by crossing the appropriate haploid strains. To generate the MMEJ chromosomal system, a PCR-generated ade2 fragment with two copies of the 18-bp L-Sce1 cut site in an inverse orientation flanked by a 12-bp MH (ade2-ISR1-12MH) was used to transform a strain with Ade2 replaced with Kluyveromyces lactis URA3 and selection for 5-fluoroorotic acid (5-FOA) resistance. Replacement of URA3 by ade2-ISR1-12MH was confirmed by DNA sequencing. The ade2-ISR1-12MH PCR fragment was synthesized by two-step PCR: the 5′ and 3′ fragments were amplified individually and then joined by overlap PCR. The strains used for the plasmid ligation assay were made by PCR-mediated gene replacement of the TRPI locus of LSY1099 with kanMX6, removing sequences from 400 bp 5′ to the ORF, the complete coding region and the associated ARS416 within the 5′ noncoding region.

The rfa1-t11, rfa1-t13 and rfa1-t148 mutations were introduced into LSY0678 or LSY0679 by transformation with Nhel-linearized pRD3514, pRD3517 and pRS485/pRD4128, respectively58,59. Integration was selected for by the plasmid-borne URA3 marker, followed by selection on medium containing 5-FOA for clones that had lost URA3. Transformants that had replaced the RFA1 allele with the desired mutant allele were identified by gamma-radiation sensitivity (rfa1-t11) or methyl methanesulfonate sensitivity (rfa1-t13 and rfa1-t148). Positive clones were further confirmed by sequencing at the rfa1 locus. A cloned NAT resistance marker (natMX4) was inserted 254 bp downstream of the RFA1 stop codon by transformation. Integration was selected by nourseothricin resistance and confirmed by PCR using primers internal to the natMX4 cassette and RFA1. rfa1::natMX4 strains were then crossed to the ade2-ISR1-12MH strain. To ensure that the natMX4 insertion does not interfere with RFA1 function, the untagged rfa1 alleles were also generated with the ade2-ISR1-12MH reporter, and the resulting strains were shown to have a similar phenotype to the tagged versions (S.K.D., unpublished data). RFA1 and rfa1-D228Y expressed from 2µ vectors (pWJ583 and pWJ585, respectively) were used for overexpression experiments.

Chromosomal MMEJ assay. Cells were grown to the mid-log phase in YPL (1% yeast extract, 2% peptone and 3% lactic acid, pH 5.5), and serial dilutions were then plated on YPL with 1% glucose or YPL with 1% galactose. Plates were incubated at 30 °C for 3–5 d. The frequency of survival was determined by the number of CFUs on galactose-containing plates divided by the number of CFUs on glucose-containing plates62. Phusion polymerase (New England BioLabs) was used to avoid 3′ overextensions in the PCR fragment or with no homology at the ends62. Purification of wild-type and mutant RPA. Purification of RPA-eGFP, RPAt33-eGFP and RPAt48-eGFP was expressed continuously through the time course. The MAT 2.6-kb probe was used (coordinates 201176–201580 on chromosome 3) to detect the fragment 2.6 kb distal to the HO cut site. A POX1 probe (coordinates 108631–109001 on chromosome 12) was used for normalization of band intensities using ImageJ (NIH). DSBR end resection for each time point was estimated as a ratio of the signal intensity corresponding to that before induction and represents the mean of three independent experiments.

Western blot analysis. Whole-cell extracts prepared by trichloroacetic acid precipitation were analyzed by SDS-PAGE and western blotting with antibodies to S. cerevisiae Rfa1 (Agrisera, AS07-214, 1:5,000) and antibodies to α-tubulin (Sigma-Aldrich, T9026, 1:1,500) as a loading control.

Purification of wild-type and mutant RPA. The rfa1-t13, rfa1-t148 and rfa1-D228Y mutations were generated using the QS site-directed mutagenesis kit (New England BioLabs) with RPA-eGFP (p11d-tscRPA_30gph6) and unlabelled RPA (p11d-tscRPA_30mxeHis6) plasmids as templates. The mutations were verified by DNA sequencing. The RPA mutants, both the eGFP tagged and untagged versions, were expressed in BL21DE3 cells overnight at 16 °C. Pellets from 3 l of cells were resuspended in Ni-lysate buffer (50 mM NaKPO4, pH 8.0, 150 mM NaCl and 10 mM imidazole) and frozen at ~80 °C. Purification of RPA-eGFP, RPA-t13, RPA-t148 and RPA-D228Y was performed as described19,20. Briefly, after sonication and centrifugation, the clarified lysate was applied to a 10 ml nickel–nitrotroic acid (Ni-NTA) column and washed with 40 ml of Ni-Wash buffer (50 mM NaKPO4, pH 8.0, 150 mM NaCl and 20 mM imidazole). The protein was eluted in approximately 25 ml of Ni-Elution buffer (50 mM NaKPO4, pH 8.0, 150 mM NaCl and 200 mM imidazole) and dialyzed overnight against 1 l buffer (40 mM NaCl, 20 mM Tris–HCl, pH 7.4, 1 mM DTT and 0.5 mM EDTA). The dialyzed protein was applied to a MonoQ column (5/50 GL, GE Healthcare) and developed with a gradient from 4% to 30% buffer B (1 M NaCl, 20 mM Tris–HCl, pH 7.4, 1 mM DTT and 0.5 mM EDTA) over 100 column volumes. MonoQ fractions containing RPA were pooled, and the MonoQ chromatography step was repeated to increase protein purity. The pooled fractions corresponding to the RPA-eGFP heterotrimeric complex were pooled, concentrated, dialyzed into storage buffer (100 mM NaCl, 20 mM Tris–HCl, pH 7.4, 1 mM DTT and 50% glycerol) and aliquoted for storage at ~80 °C.

Purification of unlabelled RPA, RPA-t13 and RPA-D228Y was identical to that of the RPA-eGFP tagged versions until the after Ni-NTA elution. The Ni-Elution buffer was applied directly to a 10 ml chitin column and washed with four column volumes of chitin wash buffer (20 mM Tris–HCl, pH 8.0, 250 mM NaCl and 1 mM EDTA). The column was then exchanged into chitin wash buffer containing 50 mM DTT and incubated overnight at 4 °C to allow for intein-mediated cleavage. The cleaved protein was collected as flow through and concentrated to approximately 5 ml. The concentrated protein was applied to a Superdex 200 16/60 column (GE Healthcare) and run with 40 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA and 1 mM DTT. Fractions corresponding to the pure heterotrimeric complex were pooled, concentrated, dialyzed into storage buffer and aliquoted for storage at ~80 °C. Concentrations of unlabelled RPA and selecting for Trp+ transformants. The frequency of end joining was determined by the number of Trp+ transformants derived from the linear fragment relative to pL111. The junctions formed by end joining were amplified by PCR from at least 16 independent Trp+ colonies from the blunt end or 12-bp MH substrates and then sequenced.

Mating-type switching and end-resection assays. The mating type–switching assay was performed as previously described21. Briefly, HO endonuclease expression was induced by addition of 2% galactose to cells grown in YPL. After 1 h, cells were collected and resuspended in YPL and 2% glucose to prevent HO expression and allow repair by gene conversion. Samples were collected at the indicated time points, and genomic DNA was extracted and digested by Styl for Southern blot analysis. To detect the HO-cut fragment and repaired products, a probe was generated by PCR amplification of MAT sequences distal to the HO cut site (coordinates 201176–201580 on the chromosome 3 sequence). The assays were performed twice, and a representative gel is shown in Supplementary Figure 4. End resection was measured by a similar protocol except that the strains used lack the HML and HMR loci, thus preventing gene conversion repair, and HO was expressed continuously through the time course. The MAT 2.6-kb probe was used (coordinates 201184–204089 on chromosome 3) to detect the fragment 2.6 kb distal to the HO cut site. A POX1 probe (coordinates 108631–109001 on chromosome 12) was used for normalization of band intensities using ImageJ (NIH). DSBR end resection for each time point was estimated as a ratio of the signal intensity corresponding to that before induction and represents the mean of three independent experiments.

Plasmid end-joining assay. The linear end-joining substrate was amplified from the pL111 plasmid using primers that anneal to sequences 320 bp upstream and 340 bp downstream of the TRPI ORF and the associated ARS416 (ref. 61). The primers were designed with the desired length of microhomology at the ends of the PCR fragment or with no homology at the ends62. Phusion polymerase (New England BioLabs) was used to avoid 3′ overextensions in the PCR fragment or with no homology at the ends62. Plasmid end-joining assay. The linear end-joining substrate was amplified from the pL111 plasmid using primers that anneal to sequences 320 bp upstream and 340 bp downstream of the TRPI ORF and the associated ARS416 (ref. 61). The primers were designed with the desired length of microhomology at the ends of the PCR fragment or with no homology at the ends62. Plasmid end-joining assay. The linear end-joining substrate was amplified from the pL111 plasmid using primers that anneal to sequences 320 bp upstream and 340 bp downstream of the TRPI ORF and the associated ARS416 (ref. 61). The primers were designed with the desired length of microhomology at the ends of the PCR fragment or with no homology at the ends62. Plasmid end-joining assay. The linear end-joining substrate was amplified from the pL111 plasmid using primers that anneal to sequences 320 bp upstream and 340 bp downstream of the TRPI ORF and the associated ARS416 (ref. 61). The primers were designed with the desired length of microhomology at the ends of the PCR fragment or with no homology at the ends62.
RPA-eGFP variants were determined using the extinction coefficients $8.8 \times 10^4$ and $1.16 \times 10^6$, respectively, at 280 nM.

**Strand annealing assays.** Annealing of the $^{32}$P-labeled 48-mer oligonucleotide (oligo-25) with the complementary unlabeled oligonucleotide (oligo-26) was performed as previously described. Briefly, the reaction buffer contained 30 mM Tris-Cl (pH 7.5), 5 mM MgCl₂ and 1 mM DTT, DNA concentrations were 200 nM, and the RPA concentration was 30 nM in all reactions. Reactions were initiated by the addition of unlabeled oligo-26 and were quenched by the addition of excess unlabeled oligo-25. Annealing was monitored by separation through 12% polyacrylamide in 1× Tris-borate-EDTA buffer. Results were visualized by phosphoimager and quantified with ImageJ software.

**Flow cells and DNA curtains.** Chromium barriers were fabricated on fused silica microscope slides using electron-beam lithography, as described previously. Flow cells and lipid bilayers were prepared as described. Single-stranded DNA substrates were generated by rolling-circle replication, as described. The ssDNA was coupled to the bilayer through a biotin-streptavidin linkage and aligned at the barriers by application of buffer flow.

DNA curtain experiments were performed using a prism-type total internal reflection fluorescence microscope (Nikon) with two back-illuminated iXon electron multiplying charge-coupled devices (Andor Technology). Illumination was provided by a 200-mW, 488-nm laser and a 200-mW, 561-nm laser (Coherent, Inc.). The intensities at the prism faces were ~14 mW and ~25 mW for the 488-nm and 561-nm lasers, respectively. Fluorescence signals were separated by a filter cube equipped with a dichroic mirror (ZT561rdc), band-pass filter (ET525/50m) and long-pass filter (ET575lp) (Chroma Technology Corp.).

For visualizing the RPA-ssDNA complexes, RPA-eGFP or mutant RPs (100 pM) were injected at 30 °C in HR buffer (30 mM Tris-acetate (pH 7.5), 5 mM Mg-acetate, 50 mM KCl, 1 mM DTT, 2.5 mM ATP and 200 µg ml⁻¹ BSA). Reactions were allowed to continue for 20 min and were then chased with RPA-mCherry (100 pM). Throughout the experiments, 100-ms images were captured at 2-s intervals, and data collection continued for a period of 40 min. The resulting videos were used to generate kymographs, and integrated signal intensities were measured over the entire length of the ssDNA molecules. For quantification, all data were normalized and corrected for background using a region of the slide surface without any ssDNA.

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