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Mre11 and Ku Regulation of Double-Strand Break Repair by Gene Conversion and Break-Induced Replication

Sanchita Krishna[1], **Brant M. Wagener**[1], **Hui Ping Liu**[1], **Yi-Chen Lo**[1], **Rosa Sterk**[1], **John H.J. Petrini**[2], and **Jac A. Nickoloff**[1,*]

1 *Department of Molecular Genetics and Microbiology and Cancer Research and Treatment Center University of New Mexico School of Medicine Albuquerque, NM 87131*

2 *Molecular Biology Program Memorial Sloan-Kettering Cancer Center New York, NY 10021*

Abstract

The yeast Mre11-Rad50-Xrs2 (MRX) and Ku complexes regulate single-strand resection at DSB double-strand breaks (DSB), a key early step in homologous recombination (HR). A prior plasmid gap repair study showed that *mre11* mutations, which slow single-strand resection, reduce gene conversion tract lengths and the frequency of associated crossovers. Here we tested whether *mre11*Δ or nuclease-defective *mre11* mutations reduced gene conversion tract lengths during HR between homologous chromosomes in diploid yeast. We found that *mre11* mutations reduced the efficiency of HR but did not reduce tract lengths or crossovers, despite substantially reduced end-resection at the test (*ura3*) locus. End-resection is increased in *yku70*Δ, but this change also had no effect on tract lengths. Thus, heteroduplex formation and tract lengths are not regulated by the extent of end-resection during DSB repair in a chromosomal context. In a plasmid-chromosome DSB repair assay, tract lengths were again similar in wild-type and *mre11*Δ, but they were reduced in *mre11*Δ in a gap repair assay. These results indicate that tract lengths are not affected by the extent of end processing when broken ends can invade nearby sites, perhaps because MRX coordination of the two broken ends is dispensable when ends invade nearby sites. Although HR outcome was largely unaffected in *mre11* mutants, break-induced replication (BIR) and chromosome loss increased, suggesting that Mre11 function in mitotic HR is limited to early HR stages. Interestingly, *yku70*Δ suppressed BIR in *mre11* mutants. BIR is also elevated in *rad51* mutants, but *yku70*Δ did not suppress BIR in a *rad51* background. These results indicate that Mre11 functions in Rad51-independent BIR, and that Ku functions in Rad51-dependent BIR.

1. Introduction

Homologous recombination (HR) and non-homologous end joining (NHEJ) are the two major pathways by which DSB double-strand breaks (DSB) are repaired. DSB can be lethal if unrepaired, and misrepair can lead to genome rearrangements including deletions, inversions, amplifications, and translocations. Both DSB repair pathways operate in eukaryotic cells and play key roles in maintaining genome stability, although their relative efficiencies vary among organisms, by ploidy, and during the cell cycle [1,2]. In the yeast *Saccharomyces cerevisiae*, most DSB are repaired by HR, particularly in diploids and in S/G2-phase cells where homologous repair templates are readily available [3]. In yeast, HR is mediated by Rad51, the

*Corresponding author: Jac A. Nickoloff, Ph.D., Department of Molecular Genetics and Microbiology, University of New Mexico School of Medicine, Albuquerque, NM 87131. Tel. 505-272-6960, Fax. 505-272-6029, Email. JNickoloff@salud.unm.edu.

Rad51 paralogs Rad55 and Rad57, Rad54, and RPA. NHEJ involves yKu70, yKu80, Lig4, Lif1, and Lif2. Through Rad50 hook domains, the Mre11-Rad50-Xrs2 (MRX) complex promotes NHEJ by tethering DSB ends [4]. Mre11 has 3′-5′ exonuclease and endonuclease activities that modulate NHEJ outcome [5,6]. Mre11 also functions in HR, at least in part by modulating DSB end processing, as well as in meiotic DSB induction, DSB damage checkpoint signaling, and telomere stability [7,8]. All of these DSB repair proteins are highly conserved from yeast to man. HR is often accurate and conservative. NHEJ can be conservative when precise re-ligation is possible, i.e., when broken ends have complementary overhangs like those produced by nucleases. NHEJ can be mutagenic if imprecise and this outcome is rare with nuclease-induced DSBs in yeast. Imprecise NHEJ may be the only NHEJ outcome when ends are not complementary or require significant processing, such as those produced by ionizing radiation. In yeast, at least 25% of nuclease-induced DSB are repaired by precise NHEJ, but only ~0.1% are repaired by imprecise NHEJ [9,10].

In the early stages of HR, DSB are resected to long 3′ single-stranded tails (ssDNA) by a poorly understood process promoted by MRX and inhibited by the yKu70/yKu80 heterodimer. ssDNA is first bound by RPA, which is replaced by Rad51, producing a nucleoprotein filament that can search for and invade a homologous duplex DNA elsewhere in the genome. Strand invasion and branch migration produces heteroduplex DNA (hDNA), and mismatches in hDNA are repaired by the mismatch repair machinery. This transfers information from an unbroken donor locus to the broken, recipient locus, usually in continuous blocks termed gene conversion tracts that can extend from just a few bp to more than 12 kbp [11,12]. Thus, gene conversion tracts reflect both the extent of hDNA and mismatch repair activity, and represent localized regions of loss of heterozygosity.

Gene conversion is the most common DSB repair outcome in yeast. A fraction of conversions are associated with crossovers, and 50% of crossovers in G2 cells produce large-scale loss of heterozygosity, extending from the crossover point to the telomere [13]. Gene conversion is thought to occur by two related pathways. Synthesis-dependent strand annealing is a non-crossover pathway in which one end invades a donor, primes repair synthesis past the DSB site, then detaches and anneals to the (resected) non-invading strand. Crossovers may arise through two-ended invasions that produce double-Holliday junctions. In either case, failure to engage the second end can result in break induced replication (BIR) in which an invading strand is extended to the end of the chromosome >100 kbp distant. BIR was first identified in *rad51*Δ mutant cells and is rare or absent in wild-type cells [14–16]. Collapsed replication forks yield a single broken end, and BIR may be an important mechanism for restarting these forks [17]. However, BIR is not an optimum DSB repair pathway because it results in large-scale loss of heterozygosity or non-reciprocal translocations. BIR is thought to occur by an efficient Rad51-dependent strand invasion mechanism that also requires Rad52, Rad55, Rad57, and Rad54, and by an inefficient Rad51-independent mechanism that requires Rad59 (a Rad52 homolog), Tid1 (a Rad54 homolog), and Rad50; the requirement for Rad50 suggests requirements for the other MRX components Mre11 and Xrs2 [15,17,18]. On chromosome III, Rad51-independent BIR is also promoted by a 200 bp “FBI” sequence element located 34 kbp from *MAT* [19], but its mechanism of action is not clear, nor is it known whether FBI sequences exist on other chromosomes. Gene conversion can occur within 2 hours of DSB induction, but Rad51-dependent BIR requires 4–6 hours and is associated with G2 arrest [18].

Null mutations in any MRX subunit confers hypersensitivity to IR and methyl methanesulfonate (MMS) [20,21]. In mating-type switching, an intrachromosomal gene conversion event initiated by DSB induced in the *MAT* locus by HO nuclease, MRX defects reduce end-resection and delay, but do not eliminate DSB repair by HR [20,22]. Mre11 has 3′-5′ exonuclease activity on ssDNA and double-stranded DSB (dsDNA), and endonuclease activity on ssDNA with a preference for ssDNA/dsDNA junctions [20,23–27]. Mre11 has 4

conserved nuclease motifs and two classes of nuclease-defective mutations have been described that either prevent or allow MRX complex formation. Mre11 defects that prevent complex formation are similar or identical to *mre11*Δ, whereas those that form MRX complexes have a milder phenotype [26–28]. Together, the results suggest that Mre11 has nuclease-dependent roles, particularly in meiosis, whereas its ability to complex with Rad50 and Xrs2 is critical for several of its roles in mitotic DSB repair. In these latter roles MRX may be maintaining connections between broken ends ("end coordination") to facilitate NHEJ and HR [4,29,30].

Symington et al. [31] used a plasmid gap repair assay to show that gene conversion tracts are shorter in *mre11*Δ, consistent with the idea that reduced end-resection in *mre11*Δ limits hDNA formation. However, although end-resection is enhanced in *yku70*Δ mutants [32], conversion tract lengths were similar to wild-type during DSB-induced interchromosomal (allelic) HR [10]. These conflicting results could be explained by differences in ploidy, length of shared homology (i.e., ectopic vs. allelic HR), or interacting partners (plasmid-chromosome vs. interchromosomal). Here we show that end-processing is reduced in *mre11*Δ, and increased in *yku70*Δ, yet these changes do not alter allelic tract lengths, suggesting that end-processing does not regulate gene conversion tract lengths during interchromosomal events. We further show that *mre11* mutations do not reduce tract lengths when HR is stimulated by DSBs in chromosomal direct repeats, or during plasmid-chromosome HR. However, tract lengths were reduced in *mre11*Δ when HR was stimulated by a double-strand gap, suggesting that Mre11 regulation of tract lengths depends on whether broken ends invade sites located close or far from each other. DSB-induced allelic HR was reduced in *mre11* mutants, and both BIR and chromosome loss were increased, indicating a role for Mre11 in HR initiation. Interestingly, a *yku70*Δ mutation suppressed BIR, but not chromosome loss, in *mre11*Δ and nuclease-defective *mre11-H125N* mutants. Suppression of BIR by *yku70*Δ is specific to the *mre11* mutant background because *yku70*Δ did not suppress BIR in a *rad51* mutant. These results indicate that Mre11 functions in Rad51-dependent BIR, and that yKu70 functions in Rad51-independent BIR.

2. Materials and Methods

2.1. Plasmid DNA and yeast strains

Plasmids were manipulated and prepared as described [33,34]. Yeast culture and chromosome modification were described earlier [16,35,36]. Structures of all modified chromosomes were confirmed by Southern and PCR analyses. Plasmid pFH800 carries a *GAL1* promoter-driven HO nuclease gene (*GALHO*) on a *TRP1*/*ARS1*/*CEN4* (TAC) plasmid [37]. A pUC19 derivative carrying *ura3* inactivated by an *Eco*RI linker at *NcoI*, and *HIS3*/*ARS1*/*CEN4* (pUraRHAC-R), is identical to pUHAC described previously [38] except that *ura3* included 9 silent RFLP mutations. *MRE11* was deleted in appropriate strains by two-step transformation using pSK-*Scmre11*Δ [21]. The *mre11*Δ mutation was complemented with wild-type *MRE11* using pSK-ScMRE11, with derivatives carrying nuclease-defective alleles *mre11-2*, *mre11-3*, *mre11-4*, and *mre11-11* [21], and with a derivative of plasmid pScM11-314 [39] carrying an *mre11-H125N* mutation created with primer 5′-ATATCAGGTAATAATGATGATGCGTCGGGG [40]. Yeast strain genotypes are shown in Table 1. *yku70*Δ strains described previously [41] were mated with *mre11*Δ mutants, and diploids were sporulated to create *mre11*Δ *yku70*Δ double mutants. *RAD51* in pUC19 was mutated to *rad51-K191R* (hereafter *rad51KR*) using primer 5′-GAATTCAGGACCGGCCGTTCCCAGCTATGTC, *URA3* was inserted, and the plasmid was cleaved with *Bst*EII and transformed into appropriate strains. The resulting Ura^+ transformants were isolated, grown nonselectively, and Ura^- derivatives were selected on medium with 5-fluoro-orotic acid [42]. *rad51KR* was identified by mapping PCR products

with *Eag*I, and confirmed by DNA sequencing and by Southern hybridization analysis. *rad51KR yku70*Δ double mutants were created by mating/sporulation as above.

2.2. Chromosomal DSB repair assays

Allelic HR was analyzed as described [16] with the following modifications. Two-day old colonies from synthetic complete medium lacking tryptophan (-Trp plates) were transferred to 1.5 ml of YPGly (1.0% yeast extract, 2.0% peptone, 2.0% glycerol) and incubated for 24 h, split into two tubes per culture and the medium was replaced with 1.5 ml of YPD (2.0% dextrose; represses *GALHO*) or YPGal (2% galactose; *GALHO*-induced), incubated for 6 h before seeding to medium lacking tryptophan. The broken chromosome has a telomere-proximal *HIS3* (*HIS3*:telV) used to monitor crossovers, chromosome loss, and BIR. Ura^+ His^+, Ura^+ His^-, and Ura^- His^- products were identified with appropriate selective medium. Ura^- His^+ recombinants were distinguished from parental colonies by re-induction of *GALHO* on YPGal and transfer to uracil omission medium as Ura^+ papillae arise only in parental colonies [36,43]. A variety of sectored colonies arise (e.g., $Ura^{+/-}$ His^+ and Ura^+ $His^{+/-}$), most likely resulting from independent G2 events, and each sector was scored as a single colony. Some colonies have parental sectors and were scored as one parental and one recombinant colony. HR frequencies were calculated as the number of recombinants per YPD colony scored. For each determination, 3–4 independent populations were tested and 1000–1500 colonies were scored per population. The fraction of His^- products arising by G2 crossover is equivalent to the frequency of His^+ recombinants with two copies of *HIS3*:telV (termed His^{++}) identified by PCR among 60–100 His^+ products per strain. Total G2 crossover frequencies were calculated as double the fraction of His^- gene conversions among all HR products (doubling accounts for the fact that only half of crossovers result in *HIS3*:telV loss). Crossovers were also estimated from just Ura^+ products (i.e., the ratio of Ura^+ His^- : Ura^+ expressed as a percentage); this approach avoids uncertainties associated with loss of *HIS3*:telV by BIR or chromosome loss. Chromosome loss was determined directly as described [44], and BIR comprises non-loss, non-crossover His^- products [16]. Gene conversion tract lengths were estimated as the fraction of Ura^- recombinants among all (Ura^+ + Ura^-) recombinants (excluding BIR and chromosome loss products). In rare cases we identified Ura^- His^+ products that had lost the donor chromosome and retained a parental recipient chromosome, presumably as a result of spontaneous loss of the unbroken (donor) chromosome.

DSB-dependent cell killing was assayed with continuous *GALHO* induction by seeding equivalent numbers of cells to tryptophan omission medium with 2% glucose or 2% galactose and incubating for 3 days. DSB-dependent cell killing was calculated by dividing the number of galactose-grown colonies by the number of glucose-grown colonies and expressing the ratio as a percentage [41]. All statistical analyses were performed by using t tests unless noted otherwise.

2.3. Plasmid-chromosome HR assays

HR was analyzed between *Eco*RI digested pUraRHAC-R transformed by using lithium acetate [45] into wild-type and *mre11*Δ strains (DY3427 and BW3575, respectively), each with *ura3*-X764 on chromosome V. Pools of >100 His^+ transformants were collected and genomic DNA was isolated and cleaved with *Eco*RI prior to transformation into *E. coli* DH10B cells by electroporation [46]. Cleavage by *Eco*RI linearizes plasmids that circularized in yeast by precise NHEJ, and because linear DNA is ineffective at transforming *E. coli*, this eliminates NHEJ products from the analysis. For each transformation, 10% the *E. coli* transformants were seeded to LB-agar with ampicillin (to estimate the number of transformants), and the remainder were grown overnight as pools in 5 ml of liquid LB medium with ampicillin from which plasmid DNA was isolated and analyzed by agarose gel electrophoresis. Each pool comprised >100 transformants. The relative fractions of products with long and short gene conversion tracts

(co-conversion of X764 or not, respectively) were determined by densitometric analysis of specific gel bands using a NucleoTech gel analysis system. Gap repair was analyzed by cleaving pUHAC-R with *Ppu*MI and *Apa*I prior to selection of His^+ transformants as above. Tract lengths were estimated by determining fractions of Ura^+ and Ura^- colonies, and fractions of parental molecules were determined by *Eco*RI digestion of plasmid DNA pools from pools of His^+ transformants as above.

2.4. Analysis of single-strand DNA resection

Cells were cultured in YPGly as above, and harvested at various times after transfer to YPGal. Genomic DNA was prepared and DNA concentrations were measured by quantitative real-time PCR of *NDC1* as described [44]. Equal quantities of non-denatured DNA were spotted on a nylon membrane using a BioRad dot blot apparatus locus and hybridized with a denatured ^{32}P-labeled 660 bp *URA3* fragment amplified with primers 5′-CGCATATGTGGT GTTGAAGAA and 5′-TCTTTGTCGCTCTTCGCAAT.

3. Results

3.1. Mre11 defects reduce DSB-induced allelic HR efficiency, but do not alter HR outcome

Mating-type switching is delayed but not eliminated in *mre11*Δ cells [20]. Although mating-type switching is a well-studied form of DSB-induced HR, it measures only one outcome, namely gene conversion without an associated crossover. To determine whether Mre11 influences DSB-induced HR outcomes in a chromosomal context, we introduced *mre11*Δ mutations into diploid cells carrying one *ura3* allele inactivated by an HO site insertion at position 432, and a second allele inactivated by a +1 frameshift mutation 332 bp downstream (X764) (Fig. 1A). DSBs were created at the HO site following induction of *GALHO*, yielding Ura^+ products when gene conversion tracts do not reach X764, and Ura^- products when tracts extend beyond X764. Thus, changes in tract length alter the fractions of Ura^+ and Ura^- products. Additional phenotypically silent markers within and flanking *ura3* have been used to monitor tract lengths [16], but these are analyzed only if the Ura^+ : Ura^- ratio is altered. Crossovers, BIR, and chromosome loss are monitored with a telomere-proximal *HIS3* gene. This non-selective assay provides simultaneous measures of HR efficiency, and HR outcomes including gene conversion tract lengths and crossover frequencies, BIR, and chromosome loss. We also determined the effect of *mre11*Δ on chromosomal direct repeat HR using these same *ura3* alleles flanking *LEU2* (Fig. 1B), which provides measures of long and short-tract gene conversions, and deletions that result from crossovers or single-strand annealing.

We found that DSB-induced allelic HR product distributions were similar in wild-type, *mre11*Δ, and *mre11-H125N* (Fig. 2A), but total HR frequencies were reduced by ~1.5-fold in *mre11*Δ and *mre11-H125N* (Fig. 2B). These modest defects in DSB-induced allelic HR are consistent with the mild effects of MRX defects on mating-type switching [15,20,22], but contrast with the strong defects of *mre11*Δ, *mre11-2*, *mre11-4* mutants in the repair of IR-induced DSBs [21], and the strong defect of *mre11*Δ in IR-induced HR [39]. The modest reduction in allelic HR in *mre11*Δ contrasts with the nearly complete elimination of HR in *rad51* and *rad52* mutants [47] (unpublished results).

End resection is reduced in *mre11*Δ cells [32]. Symington et al. [31] examined HR between a transformed, gapped plasmid and a chromosomal donor locus and observed shorter gene conversion tracts in *mre11*Δ. These results led to the proposal that shorter ssDNA tails limit hDNA formation that is reflected in shorter tract lengths. In that study, *mre11*Δ also displayed reduced crossovers, consistent with the observation that crossovers are more frequent among products with long gene conversion tracts [48,49]. The allelic and direct repeat HR systems employed in this study gave a different result; allelic conversion tract lengths and crossovers

were not reduced in *mre11*Δ or *mre11-H125N* (Fig. 3A, B). In fact, total crossovers, and crossovers among Ura$^+$ products, increased in *mre11*Δ. In agreement with prior measurements of end-processing at *MAT*, ssDNA resection at *ura3* was significantly reduced in *mre11*Δ, and increased in *yku70*Δ (Fig. 3C), excluding the possibility of differential regulation of resection by Mre11 at *MAT* and *ura3*, or in the different genetic backgrounds. Allelic gene conversion tract lengths were also unaffected in *yku70*Δ and *mre11*Δ *yku70*Δ mutants (Fig. 3A) [10].

3.2. Mre11 defects do not alter DSB-induced direct repeat HR efficiency or outcome

IR-induced direct repeat HR is strongly reduced in *mre11*Δ [39]. In contrast, there was no difference in HO-induced direct repeat HR product spectra or total HR frequencies between wild-type and *mre11*Δ, or nuclease-defective *mre11-2*, *mre11-3*, *mre11-4*, and *mre11-11* mutants (Fig. 4A, B). Direct repeat conversion tract lengths were also unaffected in *mre11* mutants (Fig. 4C). These results indicate that gene conversion tract lengths (and by inference, hDNA formation) are not affected by the extent of single-strand resection in a chromosomal context.

3.3. mre11Δ reduces gene conversion tract lengths during double-strand gap repair but not DSB repair

To further investigate why Mre11 influences tract lengths in a transformed/gapped plasmid-chromosome assay [31] but not chromosomal HR, we tested whether tract lengths were reduced in *mre11*Δ in two plasmid-chromosome HR assays. In the first assay, HR was initiated by a DSB in the plasmid-borne copy of *ura3* prior to transformation into haploid wild-type or *mre11*Δ cells. As in the chromosomal assays above, the plasmid-chromosome assay was non-selective for long or short tract gene conversions; selection was for plasmid transformation using a *HIS3* gene, and against NHEJ products (see Fig. 5A and Materials and Methods). As shown in Fig. 5B and C, *mre11*Δ did not reduce the fraction of long tract products, but instead there was a slight increase. Thus, reduced end-processing in *mre11*Δ does not decrease tract lengths during chromosomal or plasmid-chromosome gene conversion induced by a DSB. Because the plasmid included a *CEN* element, crossovers produce lethal dicentric chromosomes and are not detected. However, this limitation does not account for the lack of an effect of *mre11*Δ on tract lengths (see Discussion). In the second assay, we introduced a 222 bp double-strand gap into the plasmid-borne *ura3* prior to transformation (Fig. 5D), similar to the 238 bp gap tested by Symington et al. [31]. As above, selection was for His$^+$ transformants; we did not select against NHEJ because the two NHEJ events required to recreate parental molecules were expected to be rare. Parental molecules were recovered and may have arisen from incompletely digested plasmid DNA prior to transformation. However this did not confound the interpretation because the fractions of parental molecules were similar in wild-type and *mre11*Δ (~10%; data not shown). The remaining 90% were recombinants distributed among short- and long-tract gene conversions. We scored 199 and 202 colonies from wild-type and *mre11*Δ, respectively, and consistent with Symington et al. [31], long-tracts were significantly reduced in *mre11*Δ (Fig. 5E; $P = 0.005$, Fisher exact test). Together, the results indicate that Mre11 influences tract lengths during gap repair, but not DSB repair.

3.4. Mre11 defects increase BIR, chromosome loss, and DSB-dependent cell killing

HR is the major DSB repair pathway in yeast, particularly in diploid cells, and is critical for cell survival of DSB damage [3]. In *mre11*Δ, allelic HR is reduced (Fig. 2B) and mating-type switching is delayed [20,50], suggesting that the limited end-processing in *mre11*Δ inhibits an initial stage of HR. In agreement with this view, BIR and chromosome loss were increased in diploid *mre11*Δ and *mre11-H125N* cells (Fig. 6A, B). BIR and chromosome loss were higher in *mre11*Δ than *mre11-H125N*, consistent with prior studies showing that this nuclease mutant, which is capable of forming of an MRX complex, has a weaker phenotype than *mre11*Δ [26–

28]. It is likely that any mutation that prevents MRX complex formation, such as *rad50*Δ, would also show high levels of BIR and chromosome loss. We observed about 25% more DSB-dependent cell killing in *mre11*Δ than wild-type, but this was fully suppressed by the nuclease-defective *mre11-H125N* allele, indicating that this function requires the MRX complex but not the Mre11 nuclease (Fig. 6C). The enhanced BIR, chromosome loss, and cell killing in *mre11* mutants, together with the lack of change in HR outcome, indicates that Mre11 promotes DSB-induced HR at an early stage (see Discussion).

3.5. yku70Δ suppresses BIR, but not chromosome loss in mre11 mutants

Ku and Mre11 have opposing roles in end-processing, and *yku70*Δ partially suppresses the radiosensitivity of *mre11*Δ cells [39]. The latter result suggests that Ku may interfere with the repair of IR-induced DSBs when Mre11 is absent. In addition, *yku70*Δ mutation in otherwise wild-type cells increases the frequency of DSB-induced HR, most likely because those DSBs normally repaired by precise NHEJ are shunted to HR [10]. We therefore tested whether Ku interferes with DSB repair by HR in *mre11* mutant backgrounds by assaying HR in diploid *mre11*Δ *yku70*Δ and *mre11-H125N yku70*Δ cells. Surprisingly, DSB-induced HR was reduced to a small, but significant degree by *yku70*Δ mutation in both *mre11* backgrounds (Fig. 2B), and *yku70*Δ enhanced DSB-dependent cell killing in *mre11*Δ, but not in *mre11-H125N* (Fig. 6C). Thus, in *mre11* mutants, *yku70*Δ enhances cell survival after IR exposure, but reduces HR and cell survival after nuclease-induced DSBs. The reduced repair/survival in *yku70*Δ depends on the formation of MRX but not Mre11 nuclease activity. Gene conversion tract lengths and crossover frequencies were unaffected by *yku70*Δ alone, or in combination with *mre11*Δ or *mre11-H125N* [10] (Fig. 3A and data not shown).

Interestingly, BIR in the *mre11*Δ background was strongly suppressed by *yku70*Δ, and a similar suppression was observed in *mre11-H125N* (Fig. 6A), but *yku70*Δ had little or no effect on chromosome loss in either *mre11* mutant (Fig. 6B). To determine whether *yku70*Δ is a general BIR suppressor, we examined the effect of *yku70*Δ in a *rad51KR* (ATPase-defective) background (Fig. 7). *rad51KR* is similar to *mre11*Δ in showing a modest reduction in DSB-induced HR, and increased levels of BIR and chromosome loss. However, unlike *mre11* mutants, *yku70*Δ did not suppress BIR in *rad51KR*. Thus, *yku70*Δ suppression of BIR is specific to the *mre11* mutant background. Prior studies suggest that BIR in *mre11* mutants is Rad51-dependent [15], and our results indicate that yKu70 also functions in the Rad51-dependent BIR pathway. Note that in wild-type cells, Ku suppresses HR, presumably by shunting DSBs to NHEJ [10]. However, in *rad51KR* mutant cells, *yku70*Δ reduces HR (Fig. 7), similar to its effect in *mre11*Δ (Fig. 2B). Thus, Ku appears to promote HR in cells with modest HR defects.

4. Discussion

4.1. End-processing regulates gene conversion tract lengths during double-strand gap repair but not DSB repair

The MRX complex is involved in several aspects of the cellular DNA damage response including DSB repair by NHEJ and HR, meiotic DSB formation, telomere maintenance, checkpoint activation, and checkpoint adaptation [8,32]. In this study we focused on Mre11 regulation of HR efficiency and outcome. *mre11* mutations include those that eliminate MRX complex formation, like *mre11*Δ and *mre11-D16A*, and less severe mutations like *mre11-H125N* that permit complex formation but lack nuclease activity [27]. Although *mre11* mutants show profound sensitivity to IR and severe reductions in IR-induced HR [21,39], HR-mediated *MAT* switching is delayed but not eliminated [20], and allelic and direct repeat HR stimulated by HO nuclease is mildly reduced or unaffected (Figs. 2 and 4). These results indicate that the strong defect in IR-induced HR [39] reflects a requirement for Mre11 to process “ragged” ends at DSBs produced by IR, rather than an HR defect *per se*. We found that both *mre11*Δ and

mre11-H125N reduced HO-induced allelic HR, and increased BIR and chromosome loss, but the phenotype was stronger in *mre11*Δ. In addition, *mre11* mutations did not affect HR outcome. These results indicate that MRX function in DSB repair by allelic HR is limited to early HR stages, and that this depends on MRX complex formation, and to a lesser extent, on Mre11 nuclease activity. The Mre11 nuclease is critical for meiotic HR, presumably reflecting its role in removing covalently bound Spo11 from broken ends [20,51].

Although 5′-3′ end-processing is reduced in *mre11*Δ [20,32,52] (Fig. 3C), the Mre11 exonuclease is unlikely to be involved in end-processing because it has the opposite (3′-5′) polarity. Instead, the Mre11 ssDNA endonuclease might act on DNA unwound by MRX or a helicase, or MRX might promote other exonuclease activities, such as Exo1 [50]. Such regulatory roles for MRX are consistent with Mre11 being among the first proteins recruited to DSBs [53–55]. We recently showed that Rad51 recruitment to DSBs is delayed for at least two hours after DSB induction in *mre11*Δ [56]. Here we shown that this results in only a modest (1.5-fold) reduction in HR when assayed over a period of days (Fig. 2), suggesting that in the absence of MRX, sufficient Rad51 is eventually recruited to broken ends to catalyze HR. The modest reduction in HR and increases in BIR and chromosome loss may reflect HR failure at early times after DSB induction. Defects in other proteins that act early in HR, such as Rad51, also reduce HR and increase BIR and chromosome loss [14,15] (see below).

Crossovers are more frequently associated with long gene conversion tracts [31,48,49]. Thus, tract length regulation is important because it affects both the extent of localized loss of heterozygosity, and the frequency of large-scale loss of heterozygosity associated with crossovers. Yeast proteins known to regulate tract lengths and/or crossovers during DSB repair include Sgs1, Top3, Srs2, and Rad9 [49,57] (unpublished results). Gene conversion tract lengths reflect both the extent of hDNA formation, and mismatch repair within hDNA. hDNA can form directly during strand invasion and by branch migration of joint molecules. *mre11*Δ displayed shorter gene conversion tracts and reduced crossovers in a plasmid-chromosome gap repair assay, suggesting that hDNA formation is proportional to the extent of end resection [31]. However, tracts were not shorter in *mre11*Δ when gene conversion was stimulated by DSBs in allelic, direct repeat, or plasmid-chromosome systems, nor did *mre11*Δ decrease allelic crossovers (Figs. 3 and 5). Our plasmid-chromosome HR assay does not detect crossovers, but this limitation cannot explain why tract lengths were not reduced in *mre11*Δ because crossovers occur less often among products with short tracts [31,48,49]. DSB-induced gene conversion tract lengths are increased in *sgs1*Δ [49] and it was recently shown that Sgs1 and Mre11 are in a large complex in unstressed cells, and in a smaller complex when the DNA damage checkpoint is activated [58]. Thus, a tract length reduction due to decreased end processing in *mre11* might be balanced by an increase if Sgs1 function is compromised in *mre11*. However, this model does not explain why *mre11* reduces tract lengths during gap repair but not DSB repair [31] (Fig. 5).

Our data indicate that Mre11 does not regulate tract lengths when ends can invade nearby sequences in a donor duplex. In addition to regulating end-processing, MRX (through its Rad50 subunit) is thought to play a role in end-coordination during DSB repair. MRX may have a lesser role in coordinating ends when ends invade nearby sites. When ends are forced to invade distant sites (during gap repair), hDNA formation and tract length may be controlled to a greater degree by the extent of end-processing. The lack of correlation between tract lengths and end-processing for DSB-initiated events suggests that in this situation, hDNA forms primarily by branch migration after strand invasion occurs in a limited region, rather than by strand invasion over the full length of a single-stranded tail. In this view, hDNA formation by branch migration will be similar whether both a donor duplex and an invading duplex must be unwound, or if only the donor duplex must be unwound (because the invading strand has been resected to ssDNA), which is consistent with branch migration between two duplexes being an energy-

neutral process that proceeds by random walk [59,60]. Although branch migration might be expected to be impeded in a chromatin environment, nucleosome eviction by the INO80 chromatin remodeling complex is sharply reduced in *mre11*Δ [56], yet hDNA formation in *mre11*Δ (reflected in tract lengths) appears unaffected. This suggests that nucleosomes do not inhibit branch migration.

Symington et al. [31] suggested that shorter gene conversion tracts in *mre11*Δ could explain its spontaneous hyperrecombination phenotype [61] as co-conversion of heteroallelic markers is less likely when tracts are short. There are three other ways to explain spontaneous hyperrecombination in *mre11*Δ that are independent of tract length changes. First, spontaneous DSBs may be shunted from NHEJ to HR, although this is inconsistent with the fact that Ku defects do not increase spontaneous HR [62], and the HR deficiency of *mre11*Δ (Fig. 2B). Second, the Rad50 subunit of MRX is a member of the SMC protein family that functions in a manner analogous to cohesin to promote repair between sister chromatids, rather than between homologs. Thus, spontaneous hyperrecombination in *mre11*Δ could reflect a shift in repair from sister chromatid to homolog interactions [8]. Finally, MRX is important for S-phase checkpoint arrest [63,64], and hyperrecombination is a common phenotype of checkpoint defective cells [65].

4.2. Mre11 and Ku function in distinct BIR pathways

Gene conversion requires strand invasion and coordination of the two broken ends. If strand invasion is inefficient, or if the two ends are not coordinated, BIR or chromosome loss can result. Through its Rad50 subunit, MRX might play an important role in coordinating ends both for NHEJ and gene conversion. However, this cannot explain the fact that both BIR and chromosome loss are elevated in *mre11-H125N* (Fig. 6A, B), yet the MRX complex forms normally in this mutant [27]. It is more likely that the reduced recruitment of Rad51 to DSBs in *mre11*Δ [56] reduces the efficiency of strand invasion, with the failure of one or both ends to invade the donor resulting in BIR or chromosome loss.

Two BIR pathways have been defined, including an efficient Rad51-dependent pathway that also requires Rad52, Rad55, Rad57, and Rad54, and an inefficient Rad51-independent pathway that requires Rad50, Rad59, and Tid1 [15,17]. [Note that gene conversion is much more efficient than either BIR pathway [18]]. The requirement for Rad50 in Rad51-independent BIR implicates Mre11 and Xrs2 in this pathway. Thus, the BIR observed in *mre11* mutants is probably Rad51-dependent, and the suppression of BIR in *mre11*Δ by *yku70*Δ suggests that Ku has an important role in Rad51-dependent BIR. This conclusion gains support from the observation that *yku70*Δ does not affect BIR in *rad51KR*. Thus, Ku and Mre11 function in distinct BIR pathways; these proteins also have opposing roles in checkpoint adaptation to a persistent DSB [32].

In wild-type cells, DSB repair by HR is increased in *yku70*Δ because Ku promotes DSB repair by NHEJ [10]. However, NHEJ is defective in *mre11*Δ [8] and in *mre11*Δ and *mre11-H125N*, Ku promotes both HR and BIR (Fig. 2B and 6A). How might Ku promote these strand invasion-dependent processes? At *MAT*, BIR in *rad51* mutants preferentially initiates in or near a “facilitator of BIR” (FBI) site and Ku regulation of end-processing might promote BIR in *mre11* mutants by controlling access to a similar site near *ura3*. However, this is unlikely because BIR in *mre11* mutants is Rad51-dependent which does not require the FBI site [18], and because *mre11*Δ and *mre11*Δ *yku70*Δ show similar rates of end-processing [32] but different levels of BIR. Ku can tether DNA ends [66] and potentially promote HR in *mre11*Δ by coordinating ends, but this does not explain its positive role in BIR because BIR does not require end-coordination, and because Ku lacks affinity for ssDNA and intact duplex DNA [67]. Although Ku does not bind ssDNA, it somehow regulates end-processing even after long ssDNA tails have been produced [32], perhaps because Ku migrates inward after initial

binding to dsDNA ends [68]. Ku may therefore remain associated with ssDNA-dsDNA junctions, and this could affect Rad51 loading because the *Escherichia coli* Rad51 homolog, RecA, is known to load at ssDNA-dsDNA junctions [69]. Finally, the end-binding/protection properties of Rad52 are reminiscent of Ku [67,70], end-resection is slowed by yeast Rad52 [71], and human Rad52 binds to 3′ termini of resected DNA [70]. Ku might promote BIR and HR in *mre11*Δ by protecting a fraction of ends from degradation and "handing off" these ends to Rad52, thereby promoting BIR and HR. Such a role might be evident only when Ku is unable to fulfill its normal role in NHEJ, as in NHEJ-defective *mre11* mutants. *yku70*Δ suppresses BIR but not chromosome loss in *mre11*Δ and in *mre11-H125N*, perhaps because both strand invasion (HR/BIR) and NHEJ are compromised in *yku70*Δ.

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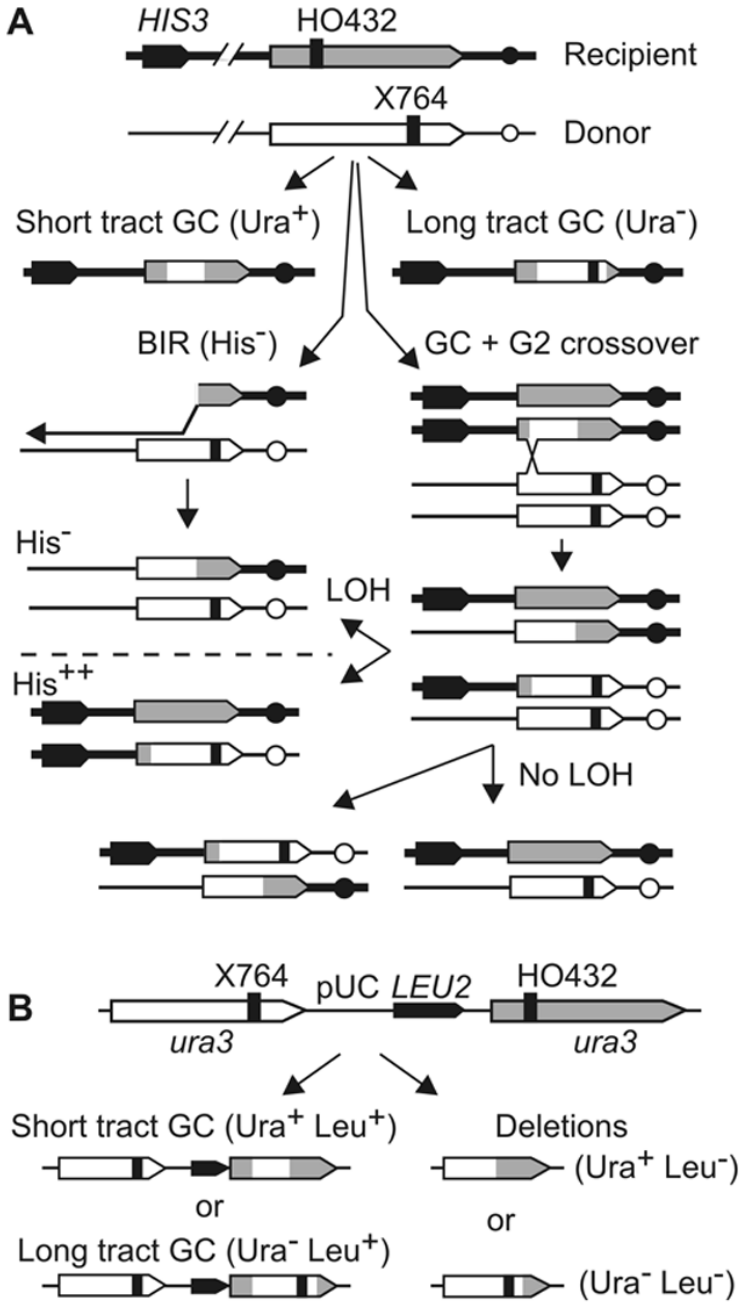

Fig 1.

Allelic and direct repeat HR substrates and outcomes. (A) Allelic HR substrate has *ura3* alleles inactivated by frameshift mutations comprising an HO site (HO432) and X764. The chromosome carrying the HO site is marked with *HIS3*:telV. HR produces short- and long-tract gene conversions (GC); only the converted (recipient) chromosome is shown. Some conversions have associated crossovers and in G2 cells, half of crossovers result in LOH at *HIS3*:telV (His^{-} or His^{++} products) and half remain heterozygous. Some G2 cells experience only a single DSB (as shown) due to limited access of HO to its site in *ura3*. BIR produces only His^{-} products (shown above the dotted line). His^{-} products also arise by chromosome loss (not shown) that are also Ura^{-}. (B) Direct repeat substrate has the same *ura3* alleles

flanking pUC19 and *LEU2*. The four principal HR product types (with associated phenotype) are shown below.

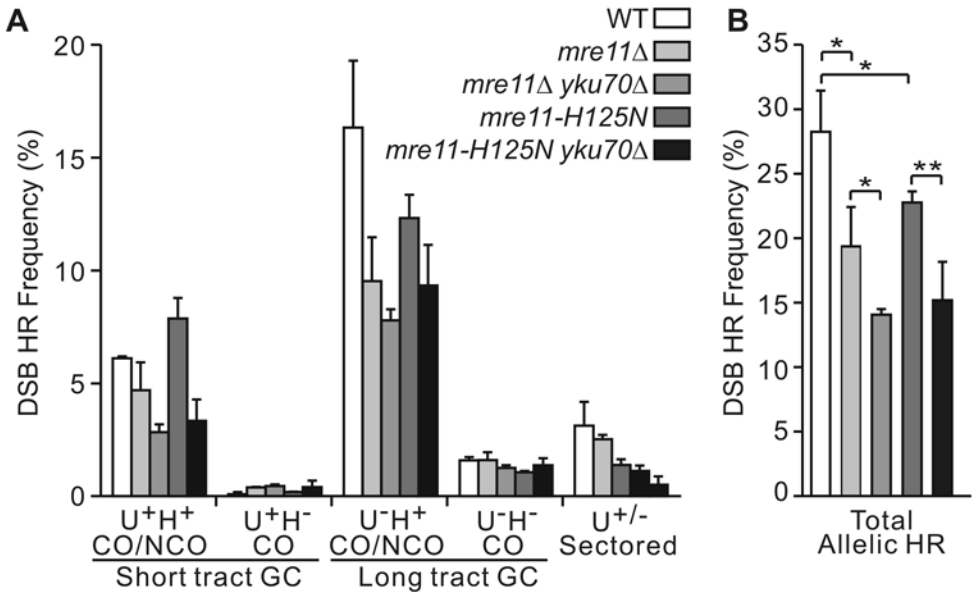

Fig 2.

DSB-induced allelic HR product spectra. (A) Gene conversion (GC) frequencies are shown for wild-type (WT), *mre11*Δ, and *mre11-H125N* with or without *yku70*Δ. Values for each product class are averages ±SD for 4 determinations. His^- products are crossovers (CO) but His^+ may be crossover or non-crossover (NCO) products. These values do not include BIR or chromosome loss events. (B) Total allelic HR frequencies from panel A. * indicates $P < 0.05$; ** indicates $P < 0.01$.

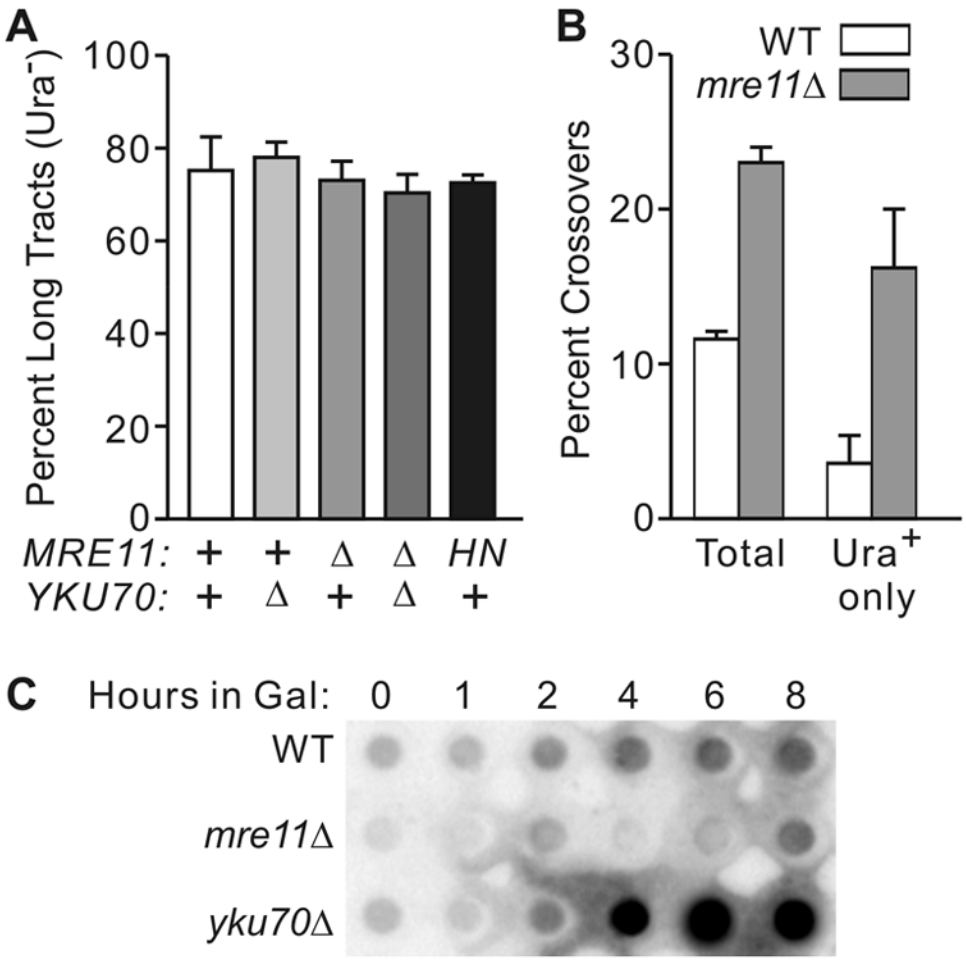

Fig 3.

Allelic gene conversion tract lengths do not correlate with extent of end-processing. (A) Tract lengths were estimated as the percentage of long-tract products (Ura^-) in wild-type (+), deletion mutants of *mre11* or *yku70* (Δ), or *mre11-H125N* (*HN*). Values are averages ±SD for 3–8 determinations. (B) Percent crossovers estimated as His^- fractions among all products, or just Ura^+ classes. (C) End-processing measured by dot-blot of native genomic DNA from wild-type, *mre11*Δ, and *yku70*Δ diploid cells isolated before and at indicated times after *GALHO* induction.

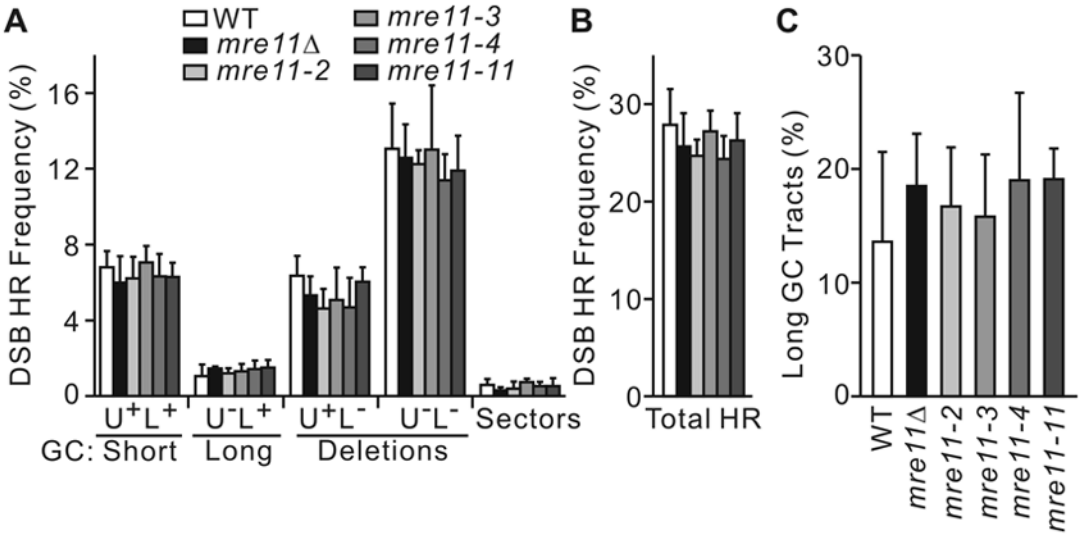

Fig 4.

Mre11 has little or no role in DSB-induced direct repeat HR. (A) Direct repeat HR product spectra in *mre11*Δ cells with wild-type complementing vector (*mre11*Δ/*MRE11*), empty vector control (*mre11*Δ), or vectors expressing nuclease-defective *mre11* alleles. Values are averages ±SD for 4 determinations. There were no significant differences in frequencies of individual HR product classes, or total HR.

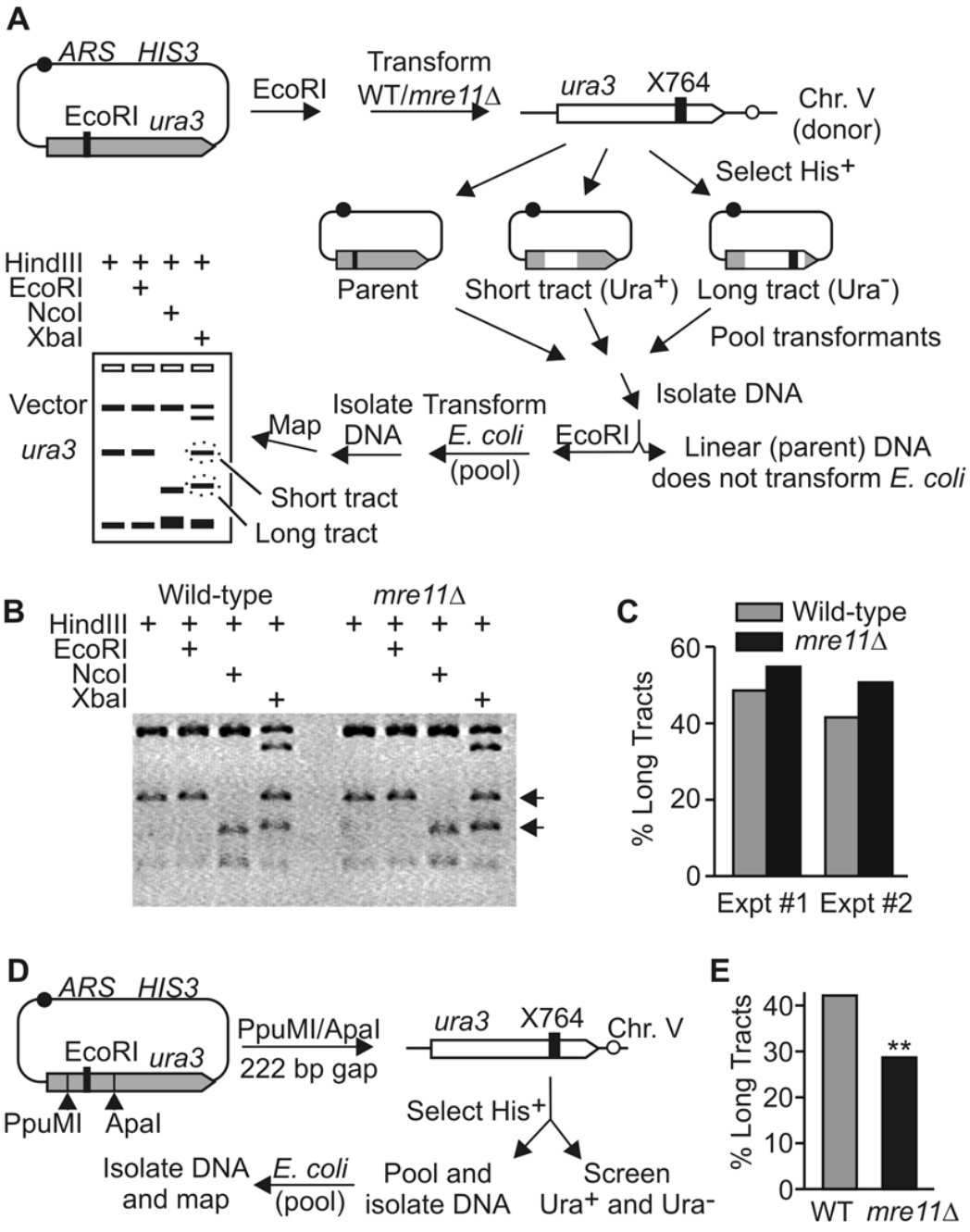

Fig 5.

Plasmid-chromosome HR. (A) HR between chromosome V and a linear, transformed plasmid carrying *ura3* inactivated by an *Eco*RI linker in *Nco*I (position 432). Selected His^+ transformants were pooled, and isolated genomic DNA was digested with *Eco*RI. This linearizes parental molecules, and only the circular gene conversion products efficiently transform *E. coli*. Ampicillin-resistant *E. coli* transformants were pooled and mapped to determine the ratio of long and short tract HR products. The first three lanes are controls: *Hin*dIII excises a 1.2 kbp *ura3* fragment from the vector; smaller fragments result from *Hin*dIII sites in *HIS3*. The *Hin*dIII/*Eco*RI digest confirms that no parental molecules are present. The *Hin*dIII/*Nco*I digest confirms that all molecules arose by gene conversion (*ura3*

cleaved by *Nco*I). The *Hin*dIII/*Xba*I digest reveals the relative fractions of molecules with long or short tracts (*ura3* cleaved by *Xba*I or not cleaved, respectively). (B) *mre11*Δ does not reduce plasmid-chromosome tract lengths. The fourth lane of each set (*Hin*dIII/*Xba*I) reveals similar fractions of long and short tract HR products. (C) Scanning densitometric analysis of agarose gels from two independent experiments (Expt #1 data are from gel shown in panel B). (D) Gap repair assay; symbols are described in panel A. (E) Percentage of long tracts among gap repair products, calculated as the ratio of Ura^- recombinants per total recombinants (Ura^+ + Ura^-).

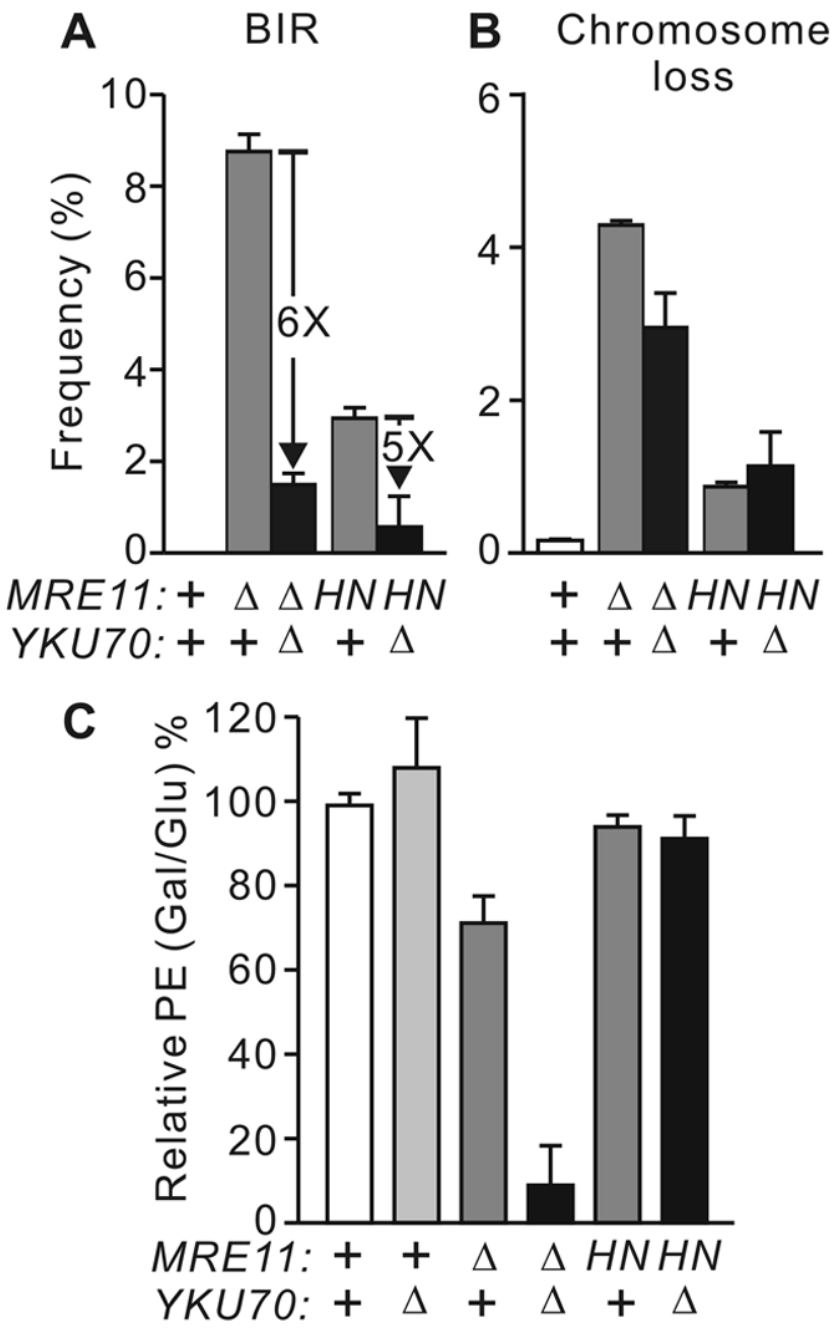

Fig 6.

mre11 mutations increase BIR, chromosome loss, and DSB-induced cell killing. All values are averages ±SD for 3–4 determinations. (A) BIR in *mre11* mutants is suppressed 5- to 6-fold by *yku70*Δ. (B) *yku70*Δ does not suppress chromosome loss in *mre11* mutants. (C) DSB-dependent cell killing is enhanced in *mre11*Δ and further enhanced in this background by *yku70*Δ.

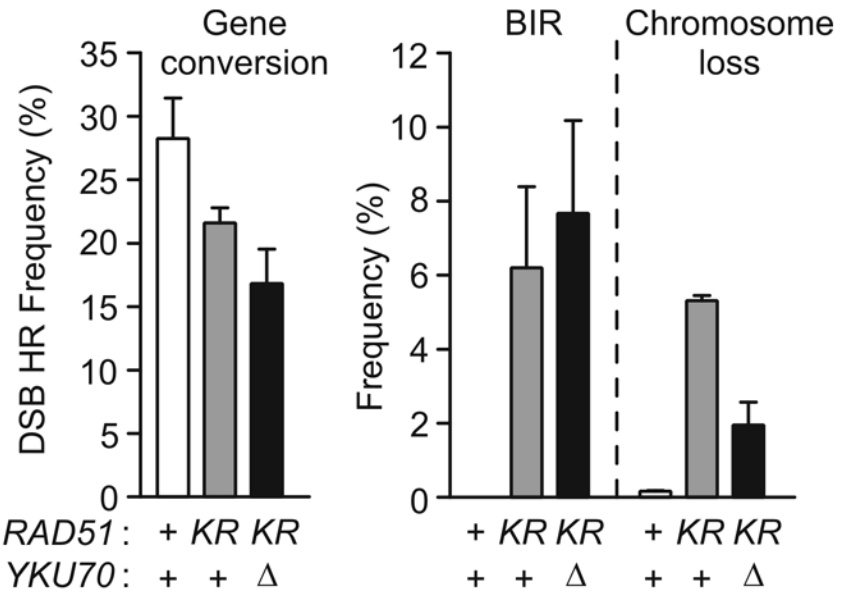

Fig 7.

*yku70*Δ does not suppress BIR in *rad51KR*. Data are presented as shown in Figs. 2B, 6A, and 6B.

Table 1

Yeast strains.

Name	Genotype	Source
JW3082	*MAT***a**-*inc ade2-101 his3-200 lys2-801*::pHSS*GALHO::LYS2 trp1-Δ1 leu2-Δ1 ura3*-X764- *LEU2-ura3*R-HO432	[36]
BW3566	JW3082 *mre11*Δ	This study
JC3441	*MAT***a**-*inc ade2-101 his3-200:HIS3*:telV *lys2-801*::pHSS*GALHO::LYS2 trp1-Δ1 leu2-Δ1* RscRI-*ura3*R-HO432-*LEU2*	[16]
DY3427	*MATα ade2-101 his3-200 lys2-801 trp1-Δ1 leu2-Δ1* RscBam-*ura3*-X764-*LEU2*	[16]
JC3517-13	Diploid product of DY3427 × JC3441	[16]
BW3575	JC3441 *mre11*Δ	This study
BW3575	DY3427 *mre11*Δ	This study
BW3592	JC3517-13 *mre11*Δ	This study
JC3548	JC3441 *yku70*Δ	[41]
JC3549	DY3427 *yku70*Δ	[41]
JC3550	JC3517-13 *yku70*Δ	[41]
BW3591	JC3441 *mre11*Δ *yku70*Δ	This study
SK3799	DY3427 *mre11*Δ *yku70*Δ	This study
SK3800	JC3517-13 *mre11*Δ *yku70*Δ	This study
JC3654	JC3441 *rad51KR*	This study
JC3655	DY3427 *rad51KR*	This study
JC3656	JC3517-13 *rad51KR*	This study
SK3801	JC3441 *rad51KR yku70*Δ	This study
SK3802	DY3427 *rad51KR yku70*Δ	This study
SK3803	JC3517-13 *rad51KR yku70*Δ	This study