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Requirement of *POL3* and *POL4* on non-homologous and microhomology-mediated end joining in *rad50/xrs2* mutants of *Saccharomyces cerevisiae*

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Abstract

Non-homologous end joining (NHEJ) directly joins two broken DNA ends without sequence homology. A distinct pathway called microhomology-mediated end joining (MMEJ) relies on a few base pairs of homology between the recombined DNA. The majority of DNA double-strand breaks caused by endogenous oxygen species or ionizing radiation contain damaged bases that hinder direct religation. End processing is required to remove mismatched nucleotides and fill in gaps during end joining of incompatible ends. *POL3* in *Saccharomyces cerevisiae* encodes polymerase δ that is required for DNA replication and other DNA repair processes. Our previous results have shown that *POL3* is involved in gap filling at 3′ overhangs in *POL4*-independent NHEJ. Here, we studied the epistatic interaction between *POL3*, *RAD50*, *XRS2* and *POL4* in NHEJ using a plasmid-based endjoining assay in yeast. We demonstrated that either *rad50* or *xrs2* mutation is epistatic for end joining of compatible ends in the *rad50 pol3-t* or *xrs2 pol3-t* double mutants. However, the *pol3-t* and *rad50* or *pol3-t* and *xrs2* mutants caused an additive decrease in the end-joining efficiency of incompatible ends, suggesting that *POL3* and *RAD50* or *POL3* and *XRS2* exhibit independent functions in NHEJ. In the *rad50 pol4* mutant, end joining of incompatible ends was not detected. In the *rad50* or *xrs2* mutants, NHEJ events did not contain any microhomology at the rejoined junctions. The *pol3-t* mutation restored MMEJ in the *rad50* or *xrs2* mutant backgrounds. Moreover, we demonstrated that NHEJ of incompatible ends required *RAD50* and *POL4* more than *POL3*. In conclusion, *POL3* and *POL4* have differential functions in NHEJ, independent of the *RAD50*-mediated repair pathway.

Introduction

Eukaryotic cells have evolved two main mechanisms to repair DNA double-strand breaks (DSBs): homologous recombination and non-homologous end joining (NHEJ). Homologous recombination requires extensive sequence homology on a homologous template. NHEJ rejoins two broken ends without extensive homology. NHEJ is a minor DSB repair pathway in yeast and is evident only in the absence of the genes involved in homologous recombination (1), or in the absence of a homologous template (2). The main genetic factors of NHEJ in yeast include the end-binding Hdf1/Hdf2 (yKu70/yKu80) heterodimer, the *MRE11/RAD50/XRS2(MRX)* complex and the *Dnl4/Lif1* ligase complex (3). A majority of DSBs caused by endogenous oxygen species or ionizing radiation contain damaged bases that cannot be ligated directly, as end processing of DSBs is required before religation (3,4). Some end-joining events, which show a few base pairs of homology at end-joining junctions, are the

result of a repair process termed microhomology-mediated end joining (MMEJ). MMEJ is distinct from the typical NHEJ pathway, as it is independent of the end-binding yku70/yku80 heterodimer and is usually associated with deletion of the sequences spanning one of the microhomology regions and the intervening sequence (5–7). In the absence of classical NHEJ repair functions such as DNA ligase 4 (*Lig4*), Ku70-Ku80, DSB repair occurs MMEJ that requires base pairing of microhomologous sequences (8,9). In *Saccharomyces cerevisiae* lacking the NHEJ protein Lig4 or any member of the MRX complex is able to join linearised plasmids using MMEJ (10–12). The *MRX* complex is required for both homologous recombination and NHEJ (13). It is also involved in formation and end resection of DSBs in meiotic recombination (14,15). The MRX complex exhibits end-bridging activity and possesses exonuclease and endonuclease activities that can process hairpin structures (16,17). Rad50 binds to DNA and unwinds DNA in an ATP-dependent manner (18), and Xrs2 targets Rad50 and Mre11 onto DNA ends (19). It has been shown that the nuclease activity of Mre11 is not required for NHEJ (20,21). On the other hand, Mre11 exhibits 3′ to 5′ exonuclease activity that stimulates degradation of DNA ends until microhomologies are revealed between two ends of a DSB and exposes microhomologies for end alignment (22). The MRX complex stimulates intermolecular ligation by Dnl4/Lif1 ligase in yeast (23) and is required for NHEJ of incompatible ends (10,24). The *rad50* mutation caused a 100-fold decrease in the frequency of nonhomologous integration of transforming DNA (25). Mutation of one gene of the complex causes a 10–100-fold decrease in the efficiency of end joining of compatible DSBs in yeast and the rejoined plasmids derived from these mutants did not contain any microhomology at the junctions (10,26). In addition, the *MRX* complex is involved in checkpoint activation in response to DSBs and suppression of gross chromosomal rearrangements (27–29).

The *S.cerevisiae POL3/CDC2* gene, encodes the large catalytic subunit of polymerase δ which is required for DNA replication (30,31) and other DNA repair processes (32–35). The *pol3-t* allele is a temperature-sensitive mutant with an amino-acid substitution D641N in the vicinity of the polymerase motif VI (36). The *pol3-t* allele exhibits a slow rate of lagging strand synthesis that leads to accumulation of single-stranded DNA on the template and is associated with S phase arrest (37,38). The *pol3-t* allele was initially isolated as *tex1* mutant that exhibited a 20–100-fold increase in Tn5 excision of a bacterial transposon inserted within the yeast *LYS2* gene (37). In addition, the *pol3-t* mutation causes a 100-fold increase in the frequency of deletions between short direct repeats (39) and destabilises microsatellite and minisatellite DNA sequences (40). Other *pol3* alleles exhibited an elevated frequency of spontaneous intrachromosomal recombination (41). Previous results have shown that the *pol3-t* mutation is epistatic to the *rad50* mutant for methyl methanesulfonate (MMS) sensitivity, suggesting that *POL3* may work together with *RAD50* in DSB repair (42).

The *S.cerevisiae POL4* gene encodes for the DNA polymerase IV a member of the Pol X family of DNA polymerases sharing homology with DNA polymerases λ and β (43). Deletion of *POL4* is not lethal to the cells, but confers higher sensitivity to DNA damaging agent such as MMS (44,45). These results suggest that Pol4 may have a role in the maintenance of genome stability during repair of MMS-induced alkylation damage (45). Pol4 has been found to interact with *S.cerevisiae* Rad27 and Dnl1/Lif1 and to play a role in repair of DSBs by NHEJ (46,47). In addition, *POL4* has been shown to be required for MMEJ of linear plasmids and in NHEJ-mediated repair of chromosomal DSBs promoting chromosomal translocations (48–51).

We have examined the effect of the *pol3-t* mutation in NHEJ and found that *POL3* is required for gap filling at 3′ overhangs in *POL4*-independent NHEJ (52). In addition, the *pol3-t* mutation exhibited a 2–3-fold decrease on the overall efficiency of end joining that did not require gap filling at 3′ overhangs, suggesting that Pol3 has a generalised function in NHEJ. However, whether *POL3* belongs to the same NHEJ pathway as the *MRX* complex has not been investigated. Moreover, the role and the genetic requirements of *POL3*- and *POL4*- MMEJ and NHEJ is not completely clear. In this study, we examined the epistatic interaction between *POL3* and *RAD50/XRS2*, and between *POL4* and *RAD50* in NHEJ and MMEJ using a plasmid-based end-joining assay in yeast. We also examined the effect of *rad50 pol3-t, xrs2 pol3-t* and *pol4 rad50* double mutations on direct end joining and MMEJ by sequence analysis of the rejoined junctions.

Materials and methods

Yeast strains

Experiments were performed in the haploid *S.cerevisiae* wild-type RSY12 strain (*MAT***a** *leu2-3,112 his3-11, 15 ura3Δ::HIS3*), in which the entire *URA3* open reading frame and promoter sequence was replaced by the *HIS3* gene (53). The construction of the isogenic *rad50* mutant was described previously (25). The *xrs2* disruption cassette (*xrs2::LEU2*) was released from plasmid pEI40 by digestion with BamHI and HindIII (kindly provided by Ivanov and Haber) (54), and used to transform into the RSY12 strain to generate the isogenic *xrs2* derivative. The *pol3-t* isogenic mutant was constructed by two-step gene replacement, in which the HpaI-linearised plasmid p171 was transformed into the RSY12 strain and the *pol3-t* allele was isolated after FOA selection (40). The same plasmid was transformed into the *rad50* and *xrs2* mutants to construct the isogenic *rad50 pol3-t* and *xrs2 pol3-t* double mutant strains. *Escherichia coli* strain DH5α was used for maintenance and amplification of plasmid DNA. To construct the isogenic *rad50 pol4* double mutant, the *pol4* strain was used and the *rad50* mutation was generated as described previously (25).

Plasmids

YEp*lac*195 contains the *URA3* marker, Amp^R gene for selection and the 2-micron origin of replication (55).

Plasmid end-joining assay

Plasmid YEp*lac*195 was digested with restriction enzymes HindIII and/or KpnI and purified by phenol:chloroform extraction. Yeast strains were grown at 30°C overnight (25°C for temperature-sensitive strains), diluted to 1×10^{-7} cells/ml into 50 ml Yeast extract, bacto Peptone. plus Adenine, Dextrose (YPAD) and incubated for 4 h at 30°C under constant shaking. About 100–200 ng of the linearised plasmid YEp*lac*195 was transformed into yeast strains using the transformation protocol as described previously (56). Circular plasmid YEp*lac*195 was transformed into the yeast strains to measure the transformation efficiency. *URA3*$^+$ transformants were selected after 4 days of growth. The efficiency of NHEJ was calculated as dividing the number of *URA3*$^+$ colonies arisen after transformation with linearised plasmid in comparison to that of the circular plasmid. Yeast plasmid DNA was purified and transformed into *E.coli* DH5α (52). Plasmid DNA was isolated from the *E.coli* strain using the QIAGEN miniprep kit (Qiagen, Valencia, CA, USA). The isolated DNA was digested with BamHI to exclude any uncut plasmids and with HindIII to estimate the size of the rejoined plasmid. The junction sequences of the rejoined plasmids were obtained by

sequencing using primers upstream and downstream of the multi-cloning sites, 195-8-FW 5′-ATACGCAAACCGCCTCTCC-3′, 195-690-RC 5′-ATGCGACGTGCAAGA TTACC-3′ and 195-4583-FW 5′ACTGGCTTCAGCAGAGCGCAGATACC-3′.

Statistical analysis

As previously reported, NHEJ efficiencies were statistically analyzed using Student's *t*-test. Comparison of microhomology usage between different mutants was made using Chi-Square test or Fisher's exact test (52). The normalised frequency of end-joining events was calculated by multiplying the overall efficiency of end joining presenting in Table 2 with the respective fraction of direct end joining or MMEJ events obtained from sequencing.

Results

The *pol3-t* and *pol4* mutation did not affect NHEJ of compatible ends in *rad50* or *xrs2* mutant background

We examined the interaction between *pol3-t* and *rad50* or *xrs2*, and *pol4* and *rad50* on NHEJ of compatible ends. Both *rad50 pol3-t* and *xrs2 pol3-t* double mutant strains were generated in the isogenic RSY12 background (described in methods). A plasmid-based end-joining assay in yeast was carried out as described previously (52). YEp*lac*195 was linearised with HindIII or KpnI to generate 5′ or 3′ overhangs and transformed into the haploid wild-type, *pol3-t, rad50, xrs2, pol4, rad50 pol3-t*, *xrs2 pol3*-t and *rad50 pol4* isogenic mutant strains. In parallel, circular YEp*lac*195 plasmid was transformed into each strain to measure transformation efficiency. After 4 days of growth, *URA3*$^+$ transformants were counted. NHEJ efficiency was measured by dividing the number of transformants counted after transformation with the linearised plasmid by the number of that transformed with circular plasmid. NHEJ efficiency in the *pol3-t* mutant was 81% for rejoining of the HindIII-linearised plasmid and 76% for rejoining of KpnI-linearised plasmid, similar to that of the wild-type cells (Table 1, data on wild-type and *pol3-t* strain were taken from (52) for comparison). By comparison, NHEJ efficiency in the *pol4* mutant was 76% for rejoining KpnI-linearised plasmid and 77% for rejoining HindIII-linearised plasmid. In *rad50* or *xrs2* mutant backgrounds, NHEJ efficiency decreased by 1012-fold, similar to previous results (26,57). Similarly, the *rad50 pol3-t*, or *xrs2 pol3-t* double mutation caused a 10–14-fold decrease in the NHEJ efficiencies of compatible ends (*P* values shown in Table 1). Again, the *rad50 pol4* caused a 9- and 17-fold decrease in the NHEJ efficiencies of compatible ends (Table 1). Therefore, in the *rad50* or *xrs2* mutant, the overall NHEJ efficiency of compatible ends is about the same as in the double mutant *rad50 pol3-t*, *xrs2 pol3-t* or *rad50 pol4* indicating that the either *rad50* or *xrs2* mutation is epistatic in the double mutant background.

The *pol3-t* and *pol4* caused additive effect in *rad50* or *xrs2* mutations on NHEJ of incompatible ends

We then examined the epistatic interaction between *pol3-t* and *rad50 pol3-t* and *xrs2*, and *pol4* and *rad50* on NHEJ of incompatible ends. Plasmid YEp*lac*195 was linearised with HindIII and KpnI to generate 5′ and 3′ incompatible ends and transformed into the wild-type RSY12, *pol3-t, rad50, xrs2, rad50 pol3-t* and *xrs2 pol3-t* isogenic mutant strains. The NHEJ efficiency of wild-type cells was about 31%. In the *pol3-t* mutant, the NHEJ efficiency was 11.7%, a 2.7-fold decrease ($P < 0.0005$); in the *pol4* mutant the NHEJ efficiency was 8.7%, a 3.6-fold decrease ($P < 0.0001$) (Table 2, data was taken from (52)). In the *rad50* and *xrs2* mutants, the efficiencies of NHEJ were 9.8 and 9.0%, respectively, significantly different from that of wild-type cells ($P < 0.0001$ for both) (Table 1), which complements data reported previously (10). NHEJ efficiencies in the *rad50 pol3-t* and *xrs2 pol3-t* double mutants were markedly reduced to 4.9 and 5.8%, respectively (*P* values shown in Table 2). In the *rad50 pol4* double mutant, no *URA3*$^+$ colonies were recovered indicating that NHEJ of incompatible end is abolished in this mutant. These results indicate that the *pol3-t* and *rad50* or *pol3-t* and *xrs2* mutants decreased the end-joining efficiency of incompatible ends additively, suggesting that *POL3* and *RAD50* or *XRS2* exhibit independent functions in NHEJ. Moreover, *pol4* is required for NHEJ of incompatible ends in the *RAD50* mutant background.

POL3 and *POL4* are not required for MMEJ

Previously, we have shown by sequence analysis that *POL3* is actually involved in NHEJ by facilitating gap filling at 3′ overhang but not 5′ overhang during *POL4*-independent end joining (52). To further analyze the role of *POL3* and *POL4* in end joining, we investigated the MMEJ events in *pol3-t* and *pol4* mutant. The sequence analysis of the junctions showed that 11 out of 37 (30%) junctions in the wild-type strain involved 2–5 bp microhomology, whereas 10 out of 28 (36%) junctions in the *pol3-t* mutant contained 2–4 bp of microhomologies, this difference between mutant and wild type

Table 1. NHEJ of compatible ends

Yeast strain	Total *URA3* colonies			Total *URA3* colonies	
	Circular	KpnI linearised	% Joined	HindIII linearised	% Joined
Wild type	8576	7632	88.9 ± 5.4	7334	85.8 ± 4.9
pol3-t	9572	7787	81.2 ± 9.2	7244	75.7 ± 10.6
pol4	12 048	6811	55.5 ± 5.9	8529	69.9 ± 4.7
rad50	7132	630	8.8 ± 0.7***	593	8.3 ± 2.3***
xrs2	5556	448	8.0 ± 1.7***	418	7.4 ± 1.5***
rad50 pol3-t	4956	386	7.7 ± 1.8***	401	8.1 ± 2.5***
rad50 pol4	11 061	576	5.2 ± 0.7***	2160	9.4 ± 1.4***
xrs2 pol3-t	6141	385	6.3 ± 1.8***	417	6.7 ± 1.2***

Results are the mean of four or five independent experiments ± standard deviation. Data were statistically analysed using the Student's '*t*' test. Data referring to *rad50*, *xrs2* and *pol3-t* mutants were compared to wild-type strain. Data referring to *rad50 pol3-t* and *xrs2 pol3-t* were compared to both wild-type and *pol3-t* strain. Data referring to *rad50 pol4* were compared to wild-type and *pol4* strain.

***$P < 0.001$.

Table 2. NHEJ of incompatible ends

Yeast strain	Total *URA3* colonies			Statistics					
	Circular	HindIII, KpnI linearised	% Joined						
					Wild type	*pol3-t*	*rad50*	*xrs2*	*pol4*
Wild type	10 572	3327	31.4 ± 7.5						
pol3-t	9880	1160	11.7 ± 4.8	*pol3-t*	0.0003				
pol4	17 850	1560	8.7 ± 1.1	*pol4*	<0.0001				
rad50	9863	972	9.8 ± 1.5	*rad50*	<0.0001				
xrs2	10 058	906	9.0 ± 2.9	*xrs2*	<0.0001				
rad50 pol3-t	16 078	794	4.9 ± 1.8	*rad50 pol3-t*	<0.0001	0.0087	0.0004		
xrs2 pol3-t	17 730	607	5.8 ± 2.3	*xrs2 pol3-t*	<0.0001	0.0019		0.0011	
rad50 pol4	14 223	0	0	*rad50 pol4*	ND		ND		ND

Results are the mean of six independent experiments ± standard deviation. Data were statistically analyzed using Student's *t*-test. The NHEJ efficiency of each mutant was compared to that of wild-type strain and the efficiencies of double mutant were compared to that of wild-type and each of single mutant (*P* values are shown on the right columns).

is not significantly different (Figures 1 and 2A). Interestingly, in the *pol4* mutant, 13 out of 18 junctions (77%) contained 2–5 bp microhomology (52). Finally, 5 out of 37 (14%) junctions in wild-type cells contained 4 or more bp compared to 8 out of 28 (29%) junctions in the *po3-t* mutant that is also not significantly different. These results indicate that *POL3* and *POL4* are not required for MMEJ.

RAD50 and *XRS2* are required for MMEJ

None of the rejoined plasmids recovered from *rad50* or *xrs2* mutant clones contained microhomologies at the junctions (Figure 1), similar to previous results (10,26). Since *RAD50*, *MRE11* and *XRS2* work as a protein complex (58) and the end-joining efficiencies observed in the *rad50* and *xrs2* mutants were very low, we combined the rejoined plasmids isolated from the *rad50* and *xrs2* mutants and compared the junctions to those from the wild-type cells. None of the 13 junctions from the *rad50* and *xrs2* mutants contained microhomologies, whereas 11 out of 37 (30%) clones in the wild-type cells utilised microhomologies of 2–5 bp, showing a significant difference in the fraction of MMEJ events ($P < 0.05$, Fisher's exact test). These results are consistent with previous studies, reporting that *RAD50* and *XRS2* are required for MMEJ (10,26).

pol3-t mutation partially restores MMEJ in *rad50* and *xrs2* mutants

One out of two events in the *rad50 pol3-t* double mutant used 2 bp of microhomology, and four out of nine events in the *xrs2 pol3-t* double mutant contained microhomology (Figure 1). The rejoined plasmids isolated from the *rad50* and *xrs2* single mutants were combined and compared to that from the *rad50 pol3-t* and *xrs2 pol3-t* double mutants. In the *rad50 pol3-t* and *xrs2 pol3-t* double mutants, 5 out of 11 (46%) junctions contained 2–4 bp of microhomology, whereas none of 13 junctions from the *rad50* and *xrs2* single mutants involved microhomology ($P < 0.05$, by Fisher's exact test, Figure 2B). The fraction of MMEJ events in the *rad50 pol3-t* and *xrs2 pol3-t* double mutants is not significantly different from that of the *pol3-t* mutant (Figure 2C) or the wild-type strain. These results indicate that the *pol3-t* mutation partially restores MMEJ events in the *rad50* and *xrs2* mutants.

The actual effect of *POL3* and *POL4* on direct end joining and MMEJ

To assess the actual effect of each mutation on direct end joining (non-MMEJ) and MMEJ of incompatible ends, we determined the normalised frequency of direct end joining or MMEJ (Figure 3). This was calculated by multiplying the overall efficiency of end joining (Table 2) with the respective fraction of direct end joining or MMEJ events obtained from sequencing results. In the *pol3-t* and *pol4* mutants, there were, respectively, a 2.9-fold and a 3.3-fold decreases in direct end joining and a 2.2-fold and a 4.4-fold decreases in MMEJ relative to the wild-type strain. In the *rad50* and *xrs2* mutants, the normalised frequency of direct end joining decreased by 2.3- and 2.5-fold, respectively, and there was no MMEJ event. In the *rad50 pol3-t* and *xrs2 pol3-t* double mutants, the normalised frequency of direct end joining decreased additively by 8.2 and 6.9-fold, respectively, and the normalised frequency of MMEJ decreased by 4.2- and 3.6-fold, respectively, relative to the wild-type strain. These observations indicate that *POL3* and *RAD50* or *POL3* and *XRS2* exhibit independent functions on direct end joining, and MMEJ events were restored in the *rad50 pol3-t* and *xrs2 pol3-t* double mutants. Finally, in the case of *rad50 pol4* mutant, we observed neither MMEJ nor NHEJ events. The result of this experiment demonstrates that MMEJ and NHEJ require both *POL4* and *RAD50*.

Discussion

The MRX complex plays important functions in NHEJ being involved in DSBs processing and resection (59,60). In addition, MRX complex has been previously shown to be essential for NHEJ of incompatible ends using a plasmid transformation assay (10). Here, we examined the effect of *POL3* and *POL4* in the *rad50* and *xrs2* mutant background to assess their role in NHEJ and MMEJ of incompatible ends.

Our results showed in the *pol3-t rad50* and *pol3-t xrs2* mutants NHEJ efficiency of incompatible ends decreased additively indicating that *rad50* or *xrs2* mutation is not epistatic to *pol3-t* for NHEJ of DSBs containing incompatible ends. When the DNA ends of the DSB possess compatible end structures, *rad50* and *xrs2* mutation lead to a significant reduction of NHEJ that is not affected by the presence of *pol3-t* or *pol4* mutation suggesting that *pol3-t* and *rad50*/*xrs2* are epistatic. In the absence of *RAD50* or *XRS2*, the NHEJ efficiencies of compatible ends decreased by 10-fold, similar to previous results (26,57). It has been shown that the majority of products in the *rad50* or *xrs2* mutant were merely religations of compatible ends that did not require end processing (26,57). Our results showed that *rad50* or *xrs2* mutation is epistatic for NHEJ of compatible ends in the *rad50 pol3-t* or *xrs2 pol3-t* double mutant background, whereas the *pol3-t*

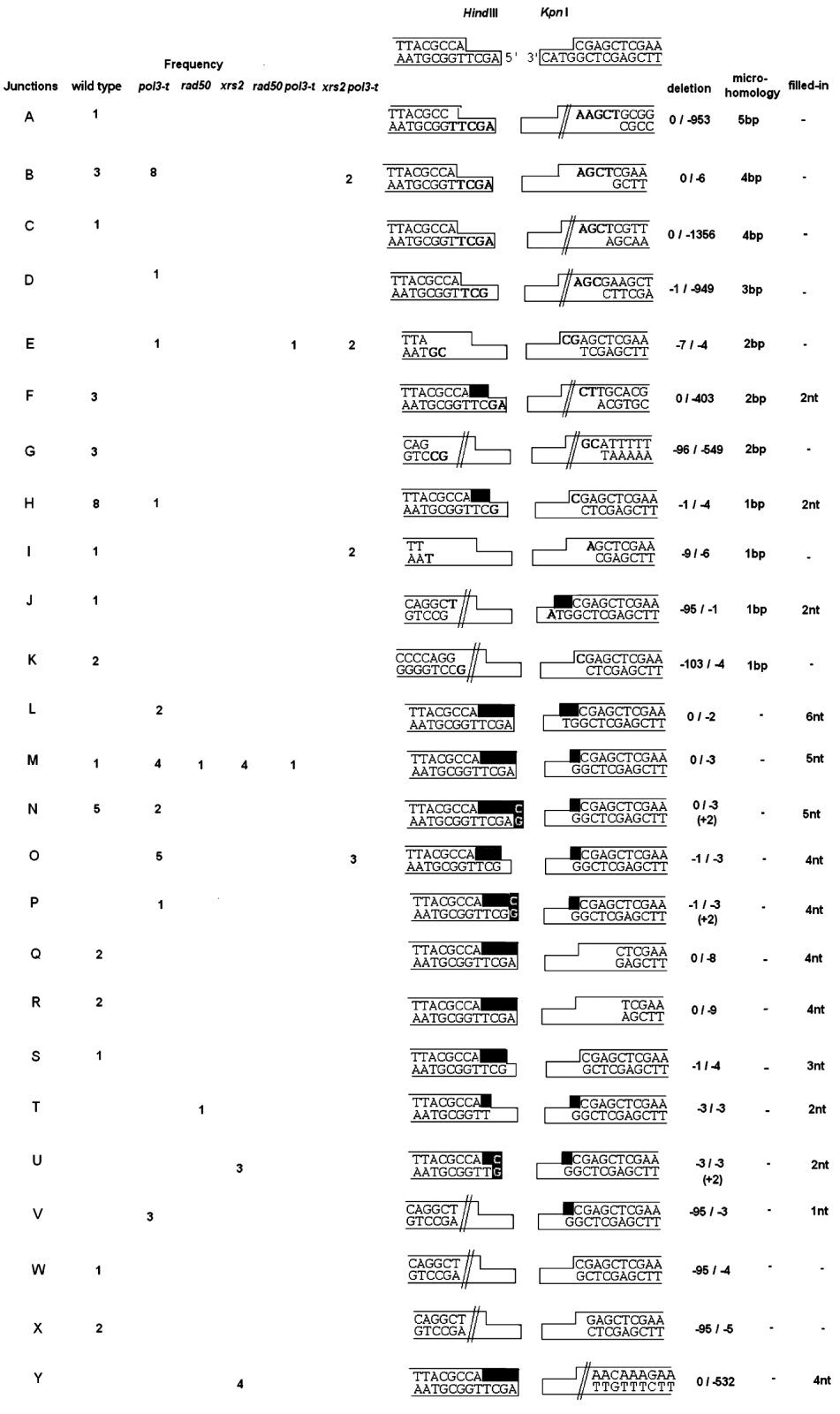


Figure 1. HindIII–KpnI junction sequences recovered from the wild-type RSY12 strain, *pol3-t, rad50, xrs2, rad50 pol3-t* and *xrs2 pol3-t* mutants. Junctions from wild-type strain and the *pol3-t* mutant were taken from previous publication and included for comparison (52). The plasmid YEp*lac*195 linearised with HindIII and KpnI is shown. The number of clones recovered from each strain for each junction is shown on the left. The nucleotides required to be filled-in are highlighted in grey. Microhomology utilised for end joining is shown in bold letters. Deleted nucleotides are presented as blank at the ends of the break and the number of deleted nucleotides is shown as '-'. '//' represents the end has large deletion. Inserted nucleotides are shown in white letters and highlighted in grey, the number of inserted nucleotides is shown as '+' in parenthesis. Overall, data reported in the table refer to a total of 37 and 28 clones from wild-type and *pol3-t* strain, respectively (52); a total of 13 clones from *rad50*/*xrs2* strain and 11 clones from *rad50 pol3-t*/*xrs2 pol3-t* were analysed.

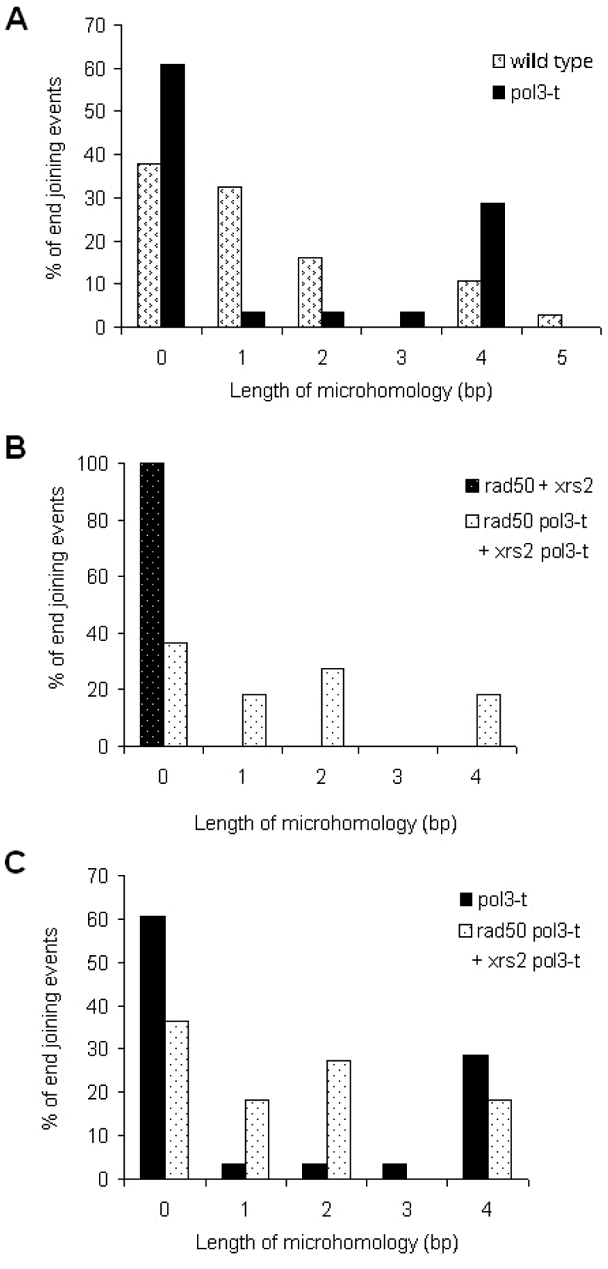


Figure 2. The distribution of microhomology usage in the end-joining events. (**A**) Wild-type strain compared to *pol3-t,* (**B**) *rad50* + *xrs2* compared to *rad50 pol3-t* + *xrs2 pol3-t* and (**C**) *pol3-t* compared to *rad50 pol3-t* + *xrs2 pol3-t* mutant strains.

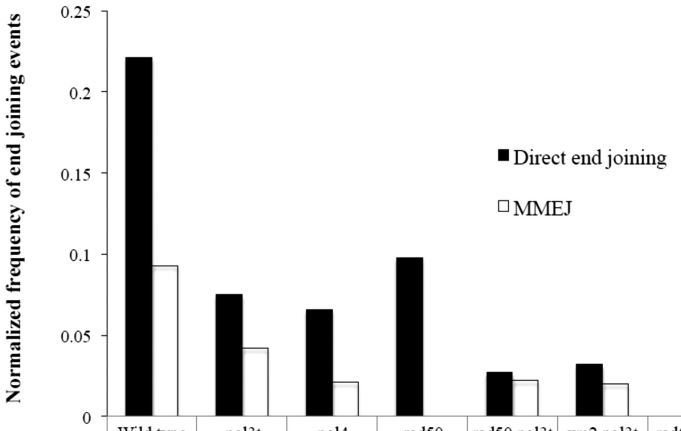
mutation by itself had no effect. It may be that *POL3* does not play any role in NHEJ of compatible ends. However, we have previously shown that the *pol3-t pol4* double mutant significantly decreased the NHEJ efficiency of compatible ends (52), suggesting that either one of the polymerases is required for end joining of compatible ends.

Sequence analysis of junctions indicated a lack of MMEJ events in the *rad50* or *xrs2* mutants. The *pol3-t* mutation restores MMEJ events in the *rad50* and *xrs2* mutants. Previously, we reported that in the absence of *POL4* when NHEJ is seriously compromised repair of DSBs is channelled into *POL3*-mediated NHEJ (52); similarly, in the MRX mutants where the DSBs processing is not occurring, most DSBs could be repaired by MMEJ. Our results also show that NHEJ of incompatible ends required both *RAD50* and *POL4*. Recently, *pol4* deletion mutant has been demonstrated to be completely defective in gap filling-mediated repair events (51). Importantly, overexpression of *POL4* gene restored the gap filling activity and DSB repair (51). Therefore, our results confirm that *POL4* is required for gap filling-mediated DSB repair events.

After normalisation with the overall NHEJ efficiency (Figure 3), the normalised frequencies of direct end joining in the double mutants also decreased additively, suggesting that *POL3* and *RAD50* or *XRS2* exhibit independent functions on NHEJ (direct end joining). Previous results have implicated that the *pol3-t* mutation has a generalised effect on NHEJ, in which the efficiencies of NHEJ that did not require gap filling at 3′ overhangs were significantly decreased by 2–3-fold in the *pol3-t* mutant (52). Since *RAD50* and *XRS2* belong to the same NHEJ pathway as *yKu70/yKu80* and *Dnl4/Lif1*, it appears that such putative function of *POL3* in NHEJ is independent of Ku and *RAD50*. Previous studies on *in vitro* end-joining reactions by the Klenow Fragment of DNA Polymerase I in *E.coli* (61) have implicated a bridge pathway of DNA polymerase-mediated end joining, in which a polymerase by itself can mediate NHEJ. The bridging activity mediated by a polymerase was further demonstrated in a crystal structure of *Mycobacterium tuberculosis* polymerase domain of *LigD* mediating synapsis of two noncomplementary DNA ends (62). Similarly to other polymerases such as human Pol β, it is possible that in NHEJ yeast mutants, *POL3* may help *POL4* in NHEJ (63); for instance, *POL3* may be capable of bringing two DNA ends into close proximity, thus enhancing the efficiency of NHEJ. Further work is required to examine the biochemical function of *POL3* in NHEJ.

Our results showed that the rejoined plasmids (HindIII–KpnI) recovered from the *rad50* or *xrs2* mutant did not contain any microhomology at the junctions, consistent with previous results (26,64). The role of *MRE11* in MMEJ has been shown in *in vitro* studies indicating that the nuclease activity of *MRE11* degrades DNA ends until microhomologies are revealed between the two ends of a DSB which exposes microhomologies for end alignment to facilitate MMEJ (22). Our results indicate that the *pol3-t* mutation partially restores MMEJ events in the *rad50* or *xrs2* background. This may be due to the replication defects associated with the *pol3-t* mutant. It has been shown that the *pol3-t* mutant exhibits a slow rate of lagging-strand synthesis and leads to generation of long stretches of single-stranded DNA (ssDNA) on the lagging–strand template (37). Similar to chromosomal replication, replication of a 2-micron plasmid is initiated at the ARS sequence and proceeds bidirectionally (65). In the *pol3-t* mutant, a reduced rate of replication of the transformed 2-micron plasmid may result in formation of ssDNA on the replicating plasmid and exposes microhomology sequences for end alignment that facilitates MMEJ. Thus, the replication defects associated with the *pol3-t* mutant may compensate for loss of nuclease activity and partially restores MMEJ in the *rad50* and *xrs2* mutants. In fact, there were more MMEJ events containing four or more bp in the *pol3-t* mutant compared to wild-type cells (29% in *pol3-t* vs. 14% in wild type), although the difference was not significant.

Alternatively, it has been shown that microhomology-mediated illegitimate recombination is induced by DSBs caused by ionizing radiation and restriction enzymes (66). It is possible that the MMEJ events seen in the *rad50 pol3-t* or *xrs2 pol3-t* mutant could be induced by DSBs. Previous results have shown that the hyperrecombinational phenotype of the *pol3-t* mutant are abolished in the absence of *RAD50* and the DNA-damage induced intrachromosomal recombination in the *pol3-t* mutant is reduced in the *rad50* mutant, suggesting that *RAD50* may be involved in resolution of stalled



	Wild type	pol3t	pol4	rad50	rad50 pol3t	xrs2 pol3t	rad50 pol4
Direct end joining	0.221	0.075	0.066	0.098	0.027	0.032	0
MMEJ	0.093	0.042	0.021	0	0.022	0.02	0

Figure 3. Direct end joining and MMEJ in wild-type and mutant strains. The frequency of direct end-joining and MMEJ events of incompatible ends was normalised in wild-type and mutant strains for comparison. The overall NHEJ efficiency is multiplied by the fraction of direct end joining or MMEJ events.

replication forks in the *pol3-t* mutant and induction of homologous recombination (42). Consistent with these results, *MRE11* has been shown to prevent formation of DSBs during DNA replication by processing of secondary structures that block progression of replication forks (67,68), and is involved in resolution of stalled replication forks in mammalian cells (69). In the *pol3-t* mutant, slow rate of lagging strand synthesis accumulates single-strand gaps on the template can lead to stalling of replication forks, which by itself can be converted to DSBs (70). It is likely that in the absence of *RAD50*, stalling of replication forks is resolved by illegitimate recombination, in which formation of DSBs resulted from stalled replication forks could induce the MMEJ pathway in the *rad50 pol3-t* or *xrs2 pol3-t* double mutant.

Taken together, *POL3* does not function in the classical *RAD50*-mediated NHEJ pathway and has a minor role as compared to that of *POL4*. In addition, we can also conclude that *POL3* acts independently in the NHEJ.

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