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End-processing during non-homologous end-joining: a role for exonuclease 1

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ABSTRACT

Non-homologous end-joining (NHEJ) is a critical error-prone pathway of double strand break repair. We recently showed that tyrosyl DNA phosphodiesterase 1 (Tdp1) regulates the accuracy of NHEJ repair junction formation in yeast. We assessed the role of other enzymes in the accuracy of junction formation using a plasmid repair assay. We found that exonuclease 1 (Exo1) is important in assuring accurate junction formation during NHEJ. Like *tdp1Δ* mutants, *exo1Δ* yeast cells repairing plasmids with 5′-extensions can produce repair junctions with templated insertions. We also found that *exo1Δ* mutants have a reduced median size of deletions when joining DNA with blunt ends. Surprisingly, *exo1Δ pol4Δ* mutants repair blunt ends with a very low frequency of deletions. This result suggests that there are multiple pathways that process blunt ends prior to end-joining. We propose that Exo1 acts at a late stage in end-processing during NHEJ. Exo1 can reverse nucleotide additions occurring due to polymerization, and may also be important for processing ends to expose microhomologies needed for NHEJ. We propose that accurate joining is controlled at two steps, a first step that blocks modification of DNA ends, which requires Tdp1, and a second step that occurs after synapsis that requires Exo1.

INTRODUCTION

A critical type of DNA damage is DNA double strand break. Double strand breaks can be generated by environmental agents such as ionizing radiation, and also can occur when single strand lesions interfere with cellular processes such as replication. Two major pathways repair double strand breaks: homologous recombination and non-homologous end-joining. Homologous recombination pathways predominantly repair damage occurring in S and G2 when a sister chromatid is available to serve as a template for repair. In haploid yeast cells, non-homologous end-joining (NHEJ) is critical in G1 cells and stationary phase cells, when a homologous chromosome is unavailable to serve as a template for repair. Therefore, NHEJ has the advantage of being capable of repairing double strand breaks in many cellular contexts (1).

Most components of NHEJ are highly conserved throughout the eukaryotic kingdom. The key components of NHEJ include a DNA binding heterodimer composed of Ku70 and Ku80 (2–5), a specialized DNA ligase (DNA ligase IV) (6,7) and ligase accessory factors [XRCC4 in mammals, Lif1 in yeast (8,9)]. In yeast, the complex that includes Mre11, Rad50 and Xrs2 (the MRX complex, termed the MRN complex in mammalian cells) is also required, perhaps to bring broken ends in close proximity (10,11). The end-joining reaction includes recognition of broken ends by Ku70/Ku80, ultimately followed by ligation by ligase IV (12). A variety of proteins potentially participate in end-processing including a specialized nuclease Artemis (13,14) (apparently absent in yeast), DNA polymerases [polμ and polλ in mammals (15–17), in yeast *POL4* (18,19)], and additional factors that may regulate ligase (Xlf/Cernunnos) (9,20,21). Mammalian cells also express a PI-kinase family member termed DNA-dependent protein kinase (DNA-PK) that plays an important role in some NHEJ reactions (22).

A key requirement in NHEJ is to alter DNA ends so that both strands can be ligated. This can include removal of damaged nucleotides, as well as the addition or removal of a small number of undamaged nucleotides. In general, the junctions formed during NHEJ will contain alterations that will include nucleotide additions or deletions. Since NHEJ does not use a homologous template to carry out repair, inaccuracy in the repair reaction is unavoidable. Nonetheless, the repair reactions at DNA ends are likely regulated to minimize mutational alterations. We recently demonstrated that yeast tyrosyl DNA phosphodiesterase I (Tdp1) prevents additions at repair junctions arising from broken DNA with 5′-extensions. We hypothesized that the 3′-nucleosidase activity of Tdp1 temporarily blocks the

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action of polymerases and other enzymes by generating ends with a 3′-phosphate (23). In this article, we demonstrate that Exo1 also plays a role in processing ends during NHEJ, and independently of Tdp1, limits addition reactions during NHEJ. We also show that Exo1 plays a role in processing blunt DNA ends during NHEJ.

MATERIALS AND METHODS

Yeast strains

Saccharomyces cerevisiae strains used in this study were isogenic derivatives of the wild-type strain BY4741 (*MAT*a *hisΔ1 leu2Δ0 lys20 ura3Δ0*). Individual yeast KANMX4 open reading frame (ORF) deletion mutants in BY4741 (*exo1Δ*, *yku80Δ*, *tdp1Δ*, and *pol4*Δ) were purchased from Open Biosystems (Huntsville, AL, USA). For generating double-disruption strains, *URA3* marked deletions of *TDP1* (23) or EXO1 [plasmid pHT133 (24)] were introduced into appropriate BY4741 mutant derivatives. Some experiments were also repeated in appropriate mutant derivatives of JN362a (23). The plasmid pHT133 was used to generate *exo1Δ* derivatives of JN362a.

Plasmid purification

The YCplac111 plasmid (a centromeric plasmid that carries the *LEU2* gene) was digested with different restriction enzymes and used as an extrachromosomal NHEJ substrate (4). Products were separated on a 0.8% agarose gel and linearized DNA was extracted from gels (QIAquick Gel Extraction Kit, Qiagen, Valencia, CA, USA) and eluted in water in aliquots of 40 ng/μl and stored at 4°C.

Yeast transformation

For yeast transformation assays of NHEJ, yeast cells were grown overnight in yeast peptone dextrose adenine (YPDA) media. Cells were diluted the following morning to the OD of 0.4 and grown until the suspension reached an OD_{600} of ~1. Typically, 100 ng of linearized or supercoiled DNA was transformed into yeast by electroporation (25). All experiments were performed with this carrier-free procedure to eliminate plasmid reactions with carrier DNA (19,26–28). Electroporation was performed at 0.75 V, 25 μF and 200 Ω, using cuvettes with a 0.1-cm gap width (Bio-Rad, Hercules, CA, USA).

In all cases, transformation with an undigested plasmid was performed in parallel to determine repair efficiency. Repair efficiency was expressed as the number of transformed colonies obtained using linearized plasmids to the number of colonies obtained with circular plasmids ×100.

Breakpoint junction analysis

By design, 100 independent colonies were analyzed per experiment. Plates containing less than 100 colonies were chosen, and all colonies on several plates were used for DNA isolation to minimize bias in selecting colonies. Plasmid DNA was extracted from colonies after transformation, as described earlier (23). The region containing the restriction enzyme site originally used to linearize the plasmid was amplified. For YCplac111, the primers were 5′-TAGCCGTAGTTAGGCCAC CAC-3′ and 5′-ACCGCACAGATGCGTAAGGAG-3′. PCR products were then digested with the same restriction enzyme(s) initially used to linearize the plasmid. Digested products were scored as accurately repaired, and PCR products that were resistant to digestion were sequenced by using the primer 5′-CCAATACGCAAACCGCCT CTCC-3′.

For some substrates, experiments were repeated with independent DNA preparation. In those cases, results are shown for all colonies analyzed (a multiple of 100).

Statistical analyses

Significance of differences in the accuracy of repair (or the frequency of particular classes of events) was assessed using Fisher's exact test, and values were calculated using Instat version 3.0.

RESULTS

Efficiency of NHEJ in *exo1*-deleted strains is not impaired

We recently reported that yeast *tdp1Δ* mutants exhibited reduced accuracy of NHEJ with substrates that carried 5′-extensions (23). The reduced accuracy was not accompanied by a change in repair efficiency. We were interested in testing whether other end-processing activities impaired the accuracy of NHEJ. In particular, we were interested in assessing various nucleases that might process unpaired nucleotides and thereby minimize insertions at repair junctions. One obvious candidate is Exo1, a nuclease important for both homologous recombination and mismatch repair (29,30). Earlier work of Wu *et al.* (31) did not detect a role for Exo1 at repair junctions during NHEJ; however, they did not assess the accuracy of repair using DNA with 5′-extensions.

We tested potential roles for Exo1 in the accuracy of NHEJ. The plasmid YCplac111 was linearized with HindIII, PstI or SmaI to generate 5′-extensions, 3′-extensions or blunt ends. The digested plasmids were transformed into the haploid wild-type strain BY4741 or an isogenic *exo1Δ* strain. Uncut plasmid was transformed in parallel to normalize for transformation efficiency. The results, shown in Figure 1, express the repair efficiency as the ratio of transformants with linearized plasmid to those with uncut plasmid ×100. The efficiency of repair for HindIII, PstI or SmaI linearized plasmids was comparable between wild-type and *exo1Δ* strains, confirming that Exo1 does not play an essential role in end-joining with these types of DNA ends.

Exo1 plays a role in accurate joining of ends with 5′-extensions

To assess the accuracy of the repair reaction, plasmids were isolated from the transformed cells, and the repair junctions were amplified by PCR. Precise repair of the plasmid maintains the original restriction site; therefore,

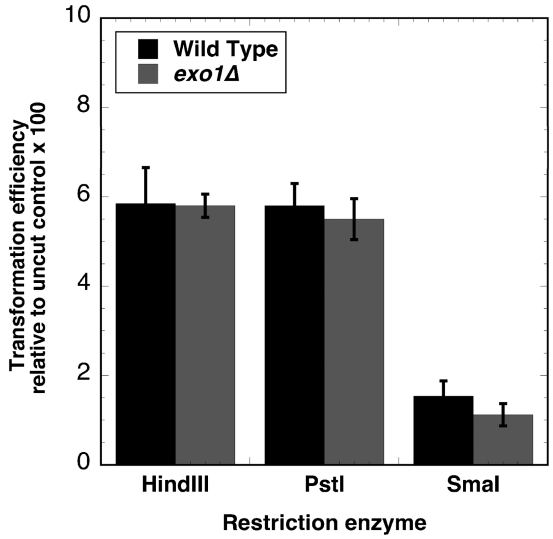


Figure 1. Plasmid repair efficiency in wild-type and *exo1Δ* strains. Plasmid YCplac111 was linearized with HindIII, PstI or SmaI, and transfected into BY4741 (WT) or in an *exo1Δ* derivative by electroporation. Repair frequencies for each genotype are expressed as the ratio of colonies obtained with linear DNA divided by colonies obtained with circular DNA ×100. The results shown are the mean of at least three independent transfections. The error bars indicate SEM.

A

HindIII digest (5′extension): 5′TACGCC**A** **AGCTT**GCATG 3′ 3′ATGCGG**TTCGA** **A**CGTAC 5′			**Overall frequency**
Wild-type	384★	TACGCC**A** **AGCTT**GCATG	**96%** accurate
	16	Deletions	**4%** deletions
exo1Δ	154★	TACGCC**A** **AGCTT**GCATG	**77%** accurate
	18	TACGCC**A**AGCT**AGCTT**GCATG	**19%** insertions
	12	TACGCC**A** GCT**AGCTT**GCATG	
	3	TACGCC**A** CT**AGCTT**GCATG	
	3	TACGCC**A** T**AGCTT**GCATG	
	2	TACGCC**A** C **AGCTT**GCATG	
	8	Deletions	**4%** deletions

B

EcoRI digest (5′extension): 5′GAGCTC**G** **AATTC**ACTGG 3′ 3′CTCGAG**CTTAA** **G**TGACC 5′			**Overall frequency**
Wild-type	196★	GAGCTC**G** **AATTC**ACTGG	**98%** accurate
	1	GAGCTC**G** TT**AATTC**ACTGG	**0.5%**insertions
	3	Deletions	**1.5%** deletions
exo1Δ	73★	GAGCTC**G** **AATTC**ACTGG	**73%** insertions
	14	GAGCTC**G**AATT**AATTC**ACTGG	**23%** insertions
	3	GAGCTC**G** ATT**AATTC**ACTGG	
	3	GAGCTC**G** T **AATTC**ACTGG	
	3	GAGCTC**GA** **AATTC**ACTGG	
	4	Deletions	**4%** deletions

C

PstI digest (3′extension): 5′TGC**CTGCA** **G**GTCGACTC 3′ 3′ACG**G** **ACGTC**CAGCTGAG 5'			**Overall frequency**
Wild-type	191★	TGC**CTGCA** **G**GTCGACTC	**95.5%** accurate
	9	Deletions	**4.5%** deletions
exo1Δ	186★	TGC**CTGCA** **G**GTCGACTC	**93%** accurate
	14	Deletions	**7%** deletions

Figure 2. Spectra of junctions recovered from the wild-type and *exo1Δ* strains. The accuracy of repair of plasmids of linearized DNA with 5′-extensions (HindIII, **A** and EcoRI, **B**) or 3′-extensions (PstI, **C**) was determined in WT and *exo1Δ* deleted strains. Plasmid DNA was isolated, and the repair junction was amplified by PCR. PCR products were digested using the same restriction enzyme used to linearize the DNA, and samples that could be digested were scored as accurately repaired. Samples that failed to digest were analyzed by DNA sequencing. The enzyme recognition sites are highlighted in bold. The junctions with insertions are underlined in gray. Stars denote accurately repaired junctions.

PCR products from accurate repair reactions can be digested with the same restriction enzyme used to linearize the plasmid. PCR products that could not be cut with the original restriction enzyme were sequenced to confirm that the repair was inaccurate, and to determine the nature of the misrepair events. In the wild-type strain, repair of the HindIII linearized plasmids is largely accurate (96% of colonies analyzed showed accurate repair in four independent experiments), and the remaining 4% that were misrepair events consisted of 1–2 nt deletions (Figure 2A). As we observed earlier with *tdp1Δ* strains, deletion of *exo1* also reduced the repair accuracy of linearized plasmids with 5′-extensions. In *exo1Δ* strains, 77% of colonies carried accurately repaired plasmids (Figure 2A). Of the 46 colonies carrying plasmids that were repaired inaccurately (23%), 38 isolates (19%) carried plasmids with insertions at the repair junction of 1–4 nt. In all cases, the insertions were templated and could be rationalized by partial or complete filling in of the 5′-extension. The eight other colonies carried small deletions, similar to what was observed with wild-type cells. The frequency of misrepair was significantly different between wild-type and *exo1Δ* strains ($P < 0.0001$ by Fisher's exact test). We also examined the frequency of misrepair of HindIII linearized plasmid in JN362a *exo1Δ*, an independently derived *exo1Δ* mutant. We observed 22/100 colonies carrying insertions, compared with 0/100 for JN362a EXO1^{+} (data not shown). This experiment demonstrates that the observed effect does not depend on the BY4741 strain background.

Similar results to those obtained with HindIII were obtained with plasmids linearized with *Eco*RI, which also generates 5′-extensions (Figure 2B). As observed in the HindIII-linearized plasmid, the misrepair events were largely templated insertions. These results demonstrate that *exo1Δ* deletion mutants carry out inaccurate NHEJ with substrates carrying 5′-extensions, a property also exhibited by *tdp1Δ* strains.

In *tdp1Δ* strains, misrepair during NHEJ is specific for substrates with 5′-extensions. To determine the accuracy of repair of DSBs with 3′-extensions, we examined transformation of wild-type and *exo1Δ* strains with *Pst*I linearized YCplac111. In the wild-type strain, repair of PstI linearized plasmids was accurate in 98% of colonies examined. In the first experiment in the *exo1Δ* strain, repair was accurate in 91/100 colonies, and the remaining 9/100 colonies showed inaccurate repair. One colony carried a deletion of 27 nt, and the other 8 inaccurate events were deletions of 1–3 nt. The difference between wild-type and *exo1Δ* strains is not quite statistically

significant ($P = 0.0582$). Since the deletion frequency was slightly elevated, we examined a second set of 100 colonies, and the pooled data from the two experiments are shown in Figure 2C. The pooled data from the two experiments showed 95.5% accurate repair events in wild-type cells, and 93% accurate repair events in *exo1Δ* strains. For the pooled data, the difference between wild-type and *exo1Δ* strains is not significant ($P = 0.1$). These results strongly suggest that *exo1Δ* strains do not show an appreciable increase in misrepair of substrates with 3′-extensions. Therefore, both *exo1Δ* and *tdp1Δ* strains show misrepair during NHEJ that is specific for 5′-extensions.

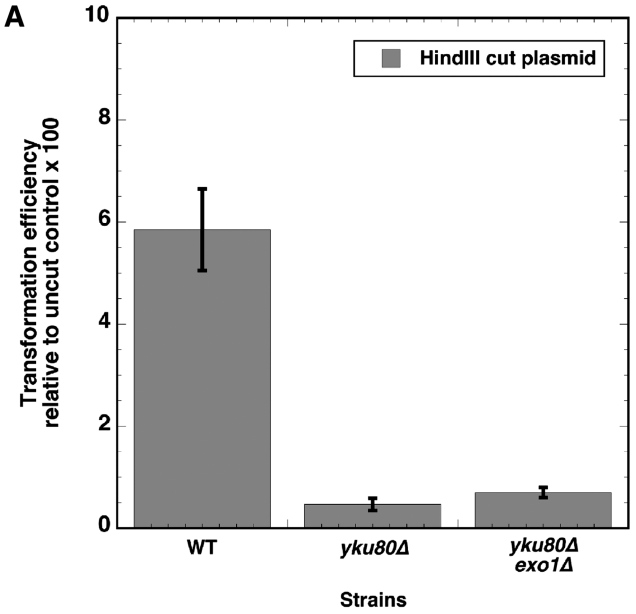
Additions in the absence of EXO1 require NHEJ factors and POL4

In our earlier work with misrepair that occurs in *tdp1Δ* strains, we found that the generation of templated additions requires NHEJ components including Ku70, Ku80 and DNA ligase IV. These results showed that the additions occurred through the NHEJ pathway. We carried out a similar analysis with *exo1Δ* strains. We generated *yku80Δ exo1Δ* strains, and examined efficiency of plasmid and accuracy of plasmid repair.

BY4741 derivatives lacking *YKU80* have a >10-fold decrease in repair of plasmids with HindIII ends compared with isogenic wild-type strains (Figure 3A). There was a similar decrease in the *yku80Δ exo1Δ* strain, with no significant difference in repair between the *yku80Δ* strain and the *yku80Δ exo1Δ* strain ($P = 0.24$).

We next examined the accuracy of repair of HindIII linearized plasmid in the NHEJ deficient strains. In *yku80Δ* single mutants, the HindIII linearized plasmid was accurately repaired in 199/200 colonies examined. As we and others noted earlier (23,32), the accuracy of repair of cohesive ends in NHEJ deficient mutants (such *YKU80*) has been controversial, with some reports indicating highly accurate repair, and other indicating substantial reductions in accuracy. In our experiments, we observed accurate repair in NHEJ deficient mutants. In the *yku80Δ exo1Δ* strain, 97/100 isolates showed accurate repair, and importantly, no insertions were recovered (Figure 3B). We conclude that the generation of insertions in *exo1Δ* strains occurs by the canonical NHEJ pathway.

In our earlier experiments, we showed that templated insertions occur in *tdp1Δ* strains, but not in *tdp1Δ pol4Δ* strains. To examine whether Pol4p was involved in the filling synthesis during NHEJ in *exo1Δ* strains, we analyzed the sequences of rejoined plasmids recovered from the *pol4Δ* strain and a *pol4Δ exo1Δ* double mutant. Daley *et al.* (19) reported that Pol4 is required for end-joining of 3′-extensions only if gaps need to be filled on both strands. For the junctions arising from the *Hin*dIII substrate with 5′-extensions, no significant difference was observed in the repair of *pol4* deleted strain (95.5% accurate, 3% carrying insertions) or *pol4 exo1* double deleted strain (95% accurate, 3% carrying insertion, Figure 3C). These findings indicate that Pol4 is



B

HindIII digest (5′extension): 5′TACGCCA AGCTTGCAT 3′ 3′ATGCGGTTCGA ACGTA 5′			Overall frequency
yku80Δ	199★	TACGCCA AGCTTGCATG	99.5% accurate
	1	Deletion	0.5% deletions
exo1Δ yku80Δ	97★	TACGCCA AGCTTGCATG	97% accurate
	3	Deletions	3% deletions

C

HindIII digest (5′extension): 5′TACGCCA AGCTTGCAT 3′ 3′ATGCGGTTCGA ACGTA 5′			Overall frequency
pol4Δ	191★	TACGCCA AGCTTGCATG	95.5% accurate
	1	TACGCCAAGCTAGCTTGCATG	3% insertions
	1	TACGCCAAGC AGCTTGCATG	
	3	TACGCCA GCTAGCTTGCATG	
	1	TACGCCA TAGCTTGCATG	
	3	Deletions	1.5% deletions
pol4Δ exo1Δ	95★	TACGCCA AGCTTGCATG	95% accurate
	1	TACGCCAAGCTAGCTTGCATG	3% insertions
	2	TACGCCAAGC AGCTTGCATG	
	2	Deletions	2% deletions

Figure 3. Insertions generated in *exo1Δ* strains occur through the NHEJ pathway and require *POL4*. (**A**) HindIII linearized YCplac111 was transfected into strains carrying a deletion of *yku80Δ or* of both *exo1Δ* and *yku80Δ*. As in Figure 1, transformation efficiency was normalized to undigested YCplac111. The efficiency of transformation was substantially reduced in the *yku80* strain compared with the wild-type strain and the *exo1Δ yku80Δ* strain showed a similar reduction compared with wild-type cells. (**B**) Accuracy of the repair of HindIII-linearized YCplac111 DNA was assessed in plasmids recovered from *yku80Δ* and *exo1Δ yku80Δ* strains. The data from the *yku80Δ* strain includes earlier published samples from our laboratory, as well as 100 additional isolates (23). The deletions in the *exo1Δ yku80Δ* strain ranged from 1 to 23 nt in length. (**C**) Accuracy of the repair of HindIII-linearized YCplac111 DNA was assessed in *pol4Δ* and *pol4Δ exo1Δ* strains. The data from the *pol4Δ* strain includes earlier published samples from our laboratory, as well as 100 additional isolates (23).

required for the inaccurate repair of 5′-cohesive extensions in *exo1Δ* strains.

Exo1 is involved in NHEJ repair of blunt ends

Consistent with earlier reports (33,34), we found that wild-type strains showed lower repair efficiency with blunt ends (SmaI-cut plasmid) than with 3′- or 5′-extensions (Figure 1). The repair efficiency of blunt ends in the wild-type was only ~10–20% of that seen with 5′- or 3′-extensions. The *exo1Δ* strain also showed inefficient repair with a slight decrease in efficiency compared with wild-type (Figure 1). This result is opposite of what we recently reported with *tdp1Δ* strains (23), where deletion of *TDP1* leads to an increase in the efficiency of repairing plasmid DNA carrying a blunt end (see also Figure 6A). Thus, *exo1Δ* and *tdp1Δ* strains have similar effects on the accuracy of repairing plasmid DNA with 5′-extensions; they have different effects on the efficiency of repairing plasmid DNA with blunt ends.

We next examined the accuracy of the repair junctions generated with SmaI digested plasmid DNA. Figure 4A shows the results obtained from the analysis of 100 colonies from wild-type and *exo1Δ* strains. The overall frequency of deletions was the same for both strains (61/100 repaired accurately). Interestingly, the size of the deletions differed between the two strains (Figure 4B). The median deletion size for wild-type cells was eight nucleotides, whereas the median deletion was 2 nt in the *exo1Δ* strain. Results of the spectrum of deletions seen in a *tdp1Δ* strain is also shown in Figure 4B (median deletion of 8 nt). It is clear that the *exo1Δ*strain has a greater frequency of deletions of 1–2 nucleotides, whereas both the wild-type and *tdp1Δ* strains have few small deletions and more isolates with deletions ≥10 nt.

Since we had observed that *POL4* was required for the stimulation of joining blunt ends in a *tdp1Δ* strain, we were interested in examining whether a deletion of *POL4* affected the size of deletions recovered with blunt ends. A *pol4Δ* strain had a minimal effect on the frequency of isolates with deletions (55/100 accurately repaired, $P = 0.47$ compared with wild-type), and the median deletion size was 8 nt (Figure 4B). Interestingly, the *pol4Δ exo1Δ* double mutant strain showed a large effect on the overall frequency of repair junctions with deletions. For the double mutant, 89/100 junctions exhibited accurate repair compared with 61/100 repaired accurately for the wild-type strain ($P < 0.0001$). Taken together these results show that the recovery of repaired plasmids with deletions can occur by two separable pathways, an *EXO1*-dependent pathway and a *POL4*-dependent pathway. Elimination of both pathways largely prevents the recovery of plasmids with deletions.

Genetic analysis of Tdp1 and Exo1 in end-joining pathways

As described earlier, we demonstrated that *tdp1Δ* strains carry out inaccurate end-joining with plasmids carrying 5′-extensions. Since *exo1Δ* strains exhibit a similar phenotype, we tested the genetic interaction between *tdp1Δ* and *exo1Δ*. There was no difference in efficiency of transformation of *exo1Δ* and *tdp1Δ exo1Δ* strains with HindIII linearized plasmid (data not shown). However, the two mutants showed an additive effect in the frequency of formation of junctions with templated insertions. The overall frequency of accurate repair of HindIII linearized DNA in an *exo1Δ* strain was 77 versus 67% in a *tdp1Δ exo1Δ* strain (Figure 5A, $P = 0.034$). A similar although greater effect was seen with EcoRI digested DNA (Figure 5B, $P = 0.008$). While there is a small increase in the number of deletions in *tdp1Δ exo1Δ* double mutants, the increase in deletion frequency is not significant ($P = 0.2$ for the comparison of HindIII digested DNA in *exo1Δ* versus *tdp1Δ exo1Δ* strains, $P = 0.4$ for the comparison of EcoRI digested DNA in *exo1Δ* vs. *tdp1Δ exo1Δ* strains). As was observed with both single mutants, there was no

A

SmaI digest blunt ends AGAGGATCCCC GGGTACCGAG TCTCCTAGGGG CCCATGGCTC				Overall frequency
Wild-type	61★	AGAGGATCCCC	GGGTACCGAG	61% accurate
	39	Deletions		39% deletions
exo1Δ	61★	AGAGGATCCCC	GGGTACCGAG	61% accurate
	39	Deletions		39% deletions
pol4Δ	55★	AGAGGATCCCC	GGGTACCGAG	55% accurate
	45	Deletions		45% deletions
pol4Δ exo1Δ	89★	AGAGGATCCCC	GGGTACCGAG	89% accurate
	11	Deletions		11% deletions

B

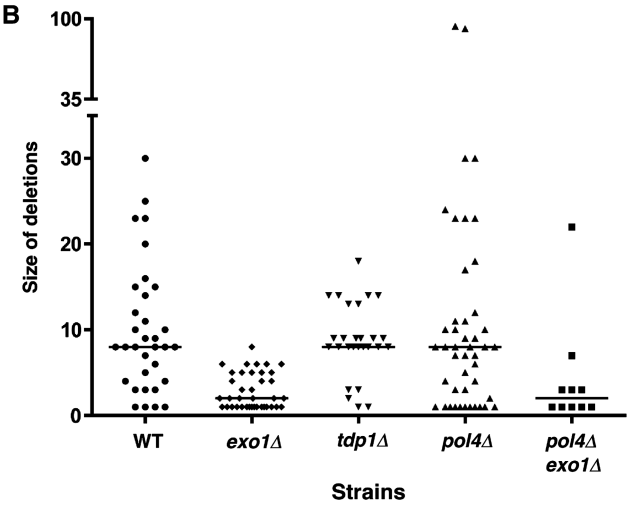


Figure 4. Repair of plasmid with blunt ends leads to smaller deletions in *exo1Δ* cells. (**A**) After transformation with YCplac111 DNA linearized with SmaI, plasmids were isolated from cells with the indicated genotypes. A total of 100 plasmids were analyzed from each genotype. The total numbers of accurately repaired and misrepaired plasmids are indicated. The number of misrepaired plasmids was not statistically different between any of the four genotypes examined. (**B**) The isolates carrying deletions of a given size is shown on the dot plot for wild-type, *exo1Δ*, *tdp1Δ*, *pol4Δ* and *exo1Δ pol4Δ* mutants. Each dot represents a single isolate with the indicated deletion size, and accurately repaired junctions are not presented. The median deletion size is shown as a solid gray line, and was calculated based on the colonies carrying deletions. Colonies with accurately repaired junctions are not included in the calculation of the median and are not shown on the dot plot.

A

HindIII digest (5′extension): 5′TACGCC**A** **AGCTT**GCATG 3′ 3′ATGCGG**TTCGA** **A**CGTAC 5′			**Overall frequency**
Wild-type	384★	TACGCCA **AGCTT**GCATG	**96% accurate**
	16	Deletions	**4% deletions**
exo1Δ	154★	TACGCC**A** **AGCTT**GCATG	**77% accurate**
	18	TACGCC**A**AGCT**AGCTT**GCATG	**19% insertions**
	12	TACGCC**A** GCT**AGCTT**GCATG	
	3	TACGCC**A** CT**AGCTT**GCATG	
	3	TACGCC**A** T**AGCTT**GCATG	
	2	TACGCC**A** C **AGCTT**GCATG	
	8	Deletions	**4% deletions**
tdp1Δ	77★	TACGCC**A** **AGCTT**GCATG	**77% accurate**
	9	TACGCC**A**AGCT**AGCTT**GCATG	**18% insertions**
	4	TACGCC**A** GCT**AGCTT**GCATG	
	1	TACGCC**A** CCT**AGCTT**GCATG	
	2	TACGCC**A** CT**AGCTT**GCATG	
	1	TACGCC**A** GC **AGCTT**GCATG	
	1	TACGCC**A**AG **AGCTT**GCATG	
	5	Deletions	**5% deletions**
exo1Δ tdp1Δ	134★	TACGCC**A** **AGCTT**GCATG	**67% accurate**
	38	TACGCC**A**AGCT**AGCTT**GCATG	**25.5% insertions**
	6	TACGCC**A**AGC **AGCTT**GCATG	
	5	TACGCC**A** GCT**AGCTT**GCATG	
	1	TACGCC**A** CT**AGCTT**GCATG	
	1	TACGCC**A** C **AGCTT**GCATG	
	15	Deletions	**7.5% deletions**

B

EcoRI digest (5′extension): 5′GAGCTC**G** **AATTC**ACTGG 3′ 3′CTCGAG**CTTAA** **G**TGACC 5′			**Overall frequency**
Wild-type	196★	GAGCTC**G** **AATTC**ACTGG	**98% accurate**
	1	GAGCTC**G** TT**AATTC**ACTGG	**0.5% insertions**
	3	Deletions	**1.5% deletions**
exo1Δ	73★	GAGCTC**G** **AATTC**ACTGG	**73% accurate**
	14	GAGCTC**G**AATT**AATTC**ACTGG	**23% insertions**
	3	GAGCTC**G** ATT**AATTC**ACTGG	
	3	GAGCTC**G** T **AATTC**ACTGG	
	3	GAGCTC**GA** **AATTC**ACTGG	
	4	deletions	**4% deletions**
tdp1Δ	74★	GAGCTC**G** **AATTC**ACTGG	**74% accurate**
	21	GAGCTC**G**AATT**AATTC**ACTGG	**22% insertions**
	1	GAGCTC**G** T**AATTC**ACTGG	
	4	Deletions	**4% deletions**
exo1Δ tdp1Δ	114★	GAGCTC**G** **AATTC**ACTGG	**57% accurate**
	52	GAGCTC**G**AATT**AATTC**ACTGG	**36% insertions**
	3	GAGCTC**G** ATT**AATTC**ACTGG	
	5	GAGCTC**G** TT**AATTC**ACTGG	
	12	GAGCTC**G** T**AATTC**ACTGG	
	14	Deletions	**7% deletions**

C

PstI digest (3′ extension): 5′TGC**CTGCA** **G**GTCGACTC 3′ 3′ACG**G** **ACGTC**CAGCTGAG 5′			**Overall frequency**
Wild-type	191★	TGC**CTGCA** **G**GTCGACTC	**95.5% accurate**
	9	Deletions	**4.5% deletions**
exo1Δ	186★	TGC**CTGCA** **G**GTCGACTC	**93% accurate**
	14	Deletions	**7% deletions**
exo1Δ tdp1Δ	96★	TGC**CTGCA** **G**GTCGACTC	**96% accurate**
	4	Deletions	**4% deletions**

Figure 5. Deletion of *EXO1* along with *TDP1* results in an additive increase in additions. The accuracy of repair of plasmids of linearized DNA with 5′-extensions (HindIII, **A** and EcoRI, **B**) or 3′-extensions (PstI, **C**) was determined in *exo1Δ tdp1Δ* deleted strains. Results with wild-type and *exo1Δ* are the data presented in figure 2, and the data from the *tdp1Δ* deleted strain was published earlier (23). The overall level of inaccurate repair for *exo1Δ tdp1Δ* was compared with *exo1Δ* using Fisher's exact test. For the HindIII digested DNA, $P = 0.034$; and for the EcoRI digested DNA, $P = 0.008$. The difference between any of the mutants with plasmid linearized with PstI was not significant.

significant effect with DNA carrying a 3′-extension (Pst1 digested, Figure 5C). We conclude that *tdp1Δ* and *exo1Δ* exert partly independent and additive effects on the accuracy of NHEJ.

Deletion of *TDP1* or *EXO1* have differing effects on joining blunt ends, with *tdp1Δ* strain exhibiting elevated repair while the *exo1Δ* strain changing the spectrum of repair junctions. Figure 6A shows the results obtained with repair efficiency for both single mutants and the *tdp1Δ exo1Δ* double mutant. Both *tdp1Δ* and *tdp1Δ exo1Δ* strains show elevated levels of transformation with SmaI digested DNA compared with wild-type (or the *exo1Δ* single mutant). However, the spectrum of deletions in the *tdp1Δ exo1Δ* double mutant is like the *exo1Δ* single mutant, with a median deletion size of 2 nt (Figure 6B). Deletion of *EXO1* is epistatic to deletion of *TDP1* for efficiency of repair, while the epistasis

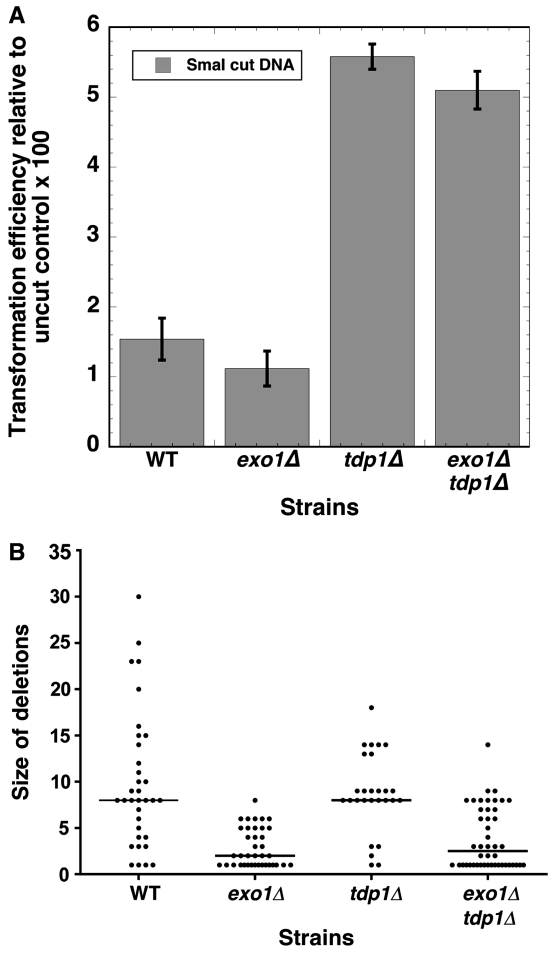


Figure 6. *EXO1* is not required for *tdp1Δ*mediated stimulation of joining blunt ends. (**A**) YCplac111 digested with SmaI was transfected into WT, *exo1Δ*, *tdp1Δ* or *exo1Δ tdp1Δ* strains. Repair frequencies were normalized with transfection of undigested YCplac111. The results shown are the mean of at least three independent transfections; error bars indicate SEM. (**B**) Plasmids were recovered from 100 independent colonies from wild-type, *exo1Δ*, *tdp1Δ* and *exo1Δ tdp1Δ* mutant strains. Data from the wild-type and single mutants were as presented in Figure 4B. As in Figure 4B, each dot represents a single isolate with the indicated deletion size, and accurately repaired junctions are not presented.

relationship is reversed for the median deletion size. As described in the discussion, we rationalize these results and propose that *TDP1* acts at an early step in the NHEJ pathway, and that *EXO1* has the potential to act at a subsequent step.

DISCUSSION

The major conclusion from this work is that yeast Exo1 plays a role in end-processing during non-homologous end-joining. The phenotypes of *exo1Δ* mutants are similar to what we observed for *tdp1Δ* mutants with substrates bearing cohesive ends, but differ for substrates with blunt ends. Like *tdp1Δ* mutants, *exo1Δ* mutants exhibit templated additions with substrates with 5′-overhangs, and neither mutant significantly affects the efficiency or accuracy of repair of plasmids terminating with 3′-overhangs. We found that *exo1Δ* mutants show smaller deletions with blunt ended substrates, and that deletion of both *EXO1* and *POL4* largely eliminates deletions that are recovered with blunt end substrates.

In this article we have used a plasmid repair assay, in which cells are transfected with linearized plasmid, and recovery of transformants depends on recircularization of the plasmid, predominantly by the NHEJ pathway. This assay has been used to demonstrate the role of yeast genes in NHEJ (5,6,35,36). While this assay does not completely reconstitute the conditions occurring when the substrates are broken chromosomes, it has the advantage of allowing the use of substrates with different types of DNA ends. The major alternate approach is to express homing endonucleases such as HO or Sce-I. Since all known homing endonucleases lead to ends with 3′-extensions, broken ends with 5′-extensions or blunt ends cannot be studied with this approach. The mutants studied here lead to phenotypes only with DNA with 5′-extensions or blunt ends; therefore, rare cutting nucleases have not been appropriate tools.

We recently proposed that Tdp1 controls the accuracy of NHEJ by its 3′-nucleosidase activity. We suggested that Tdp1 frequently removes a nucleoside leaving a 3′-phosphate. The 3′-phosphate blocks polymerases (and other DNA metabolic enzymes) until the phosphate is removed. Strong evidence for this model comes from the observation that over-expression of Tpp1, a phosphatase that is specific for 3′-PO_4 ends (37,38) leads to the same types of errors seen when Tdp1 activity is absent (23). Generation of additions in the absence of Tdp1 occurs through the canonical NHEJ pathway since additions are not seen when yKu80 or DNA ligase IV is absent. Furthermore, DNA polymerase IV is also required for the templated additions. The additions are specific for 5′-extensions, and no change in accuracy was noted for DNA ends carrying 3′-extensions.

We found that the same properties seen with *tdp1Δ* mutants are also seen with *exo1Δ* mutants. Transformation of DNA ends with 5′-extensions lead to templated insertions at the repair junctions in *exo1Δ* mutants. The insertions require yKu80 and DNA polymerase IV. While deletion of either *TDP1* or *EXO1* greatly increases the frequency of insertions at the repair junctions, the majority of junctions formed exhibit accurate repair. The insertions do not occur when the substrate DNA has a 3′-extension. Furthermore, the effects of deletion of Exo1 and Tdp1 are at least partly additive (Figure 5). These observations, summarized in the model shown in Figure 7, suggest that Tdp1 acts relatively early, and blocks the ability of polymerase IV to extend the end with a 5′-extension. If Tdp1 fails to prevent nucleotide insertions by polymerase, we suggest that Exo1 can remove the added nucleotides. This likely occurs after synapsis, and may arise form the generation of 5′-flaps during synapsis. This reaction of Exo1 is likely different from the resection of ends during homologous recombination because Ku has been shown to inhbiit Exo1-mediated synapsis (39). The added nucleotides would be removed by either direct 5′→3′-exonuclease activity or through 5′-flap endonuclease activity of Exo1 (40). Although we suggest that Exo1 acts after synapsis, we have no direct evidence that synapsis is required for Exo1 processing. This model rationalizes both the effects of *exo1Δ* single mutants, and the additive effect of *tdp1Δ exo1Δ* double mutants on accuracy.

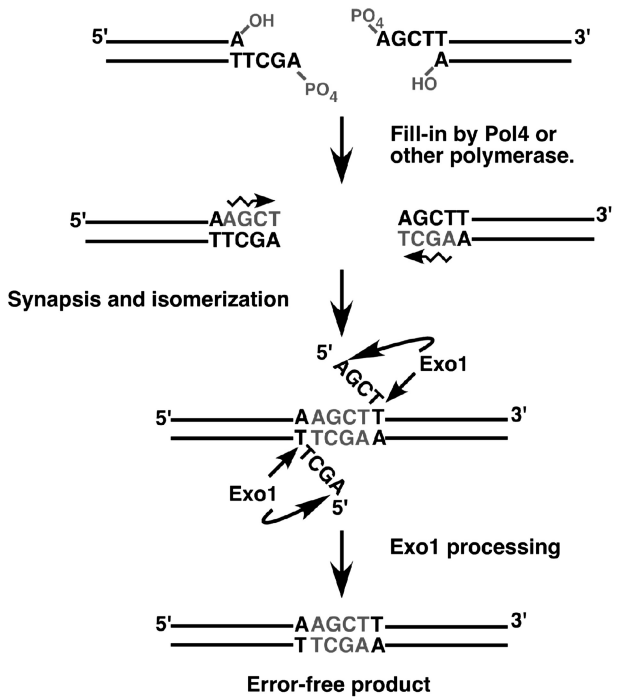


Figure 7. A model for the roles of Exo1 in accurate joining of cohesive ends. DNA ends with 5′-extensions are potentially substrates for filling in by Pol4 or another DNA polymerase. We previously suggested that the filling reaction might be largely prevented in the presence of active Tdp1, due to the removal of a nucleoside, and the generation of a 3′-phosphate (this reaction is not shown for simplicity). If Tdp1 does not act, and a DNA polymerase fills in the extension, synapsis and isomerization can lead to a structure with a 5′-flap. The flap can be removed by either the 5′→3′-exonuclease or the flap endonuclease activity of Exo1, leading to the recovery of an error-free product. The filling-in reaction cannot be directly reversed by Exo1, since it does not have 3′→5′-exonuclease activity.

While *tdp1Δ* and *exo1Δ* have similar effects on NHEJ with DNA substrates with cohesive ends, the mutants have distinct effects on substrates with blunt ends. While *tdp1Δ* mutants result in enhanced recovery of repaired plasmids that had been linearized with SmaI, there is no enhanced repair with this substrate seen in *exo1Δ* single mutants. For repair of blunt plasmid DNA, *exo1Δ* is epistatic to *tdp1Δ*. Therefore, the enhanced recovery of repaired plasmid in *tdp1Δ* strains does not require Exo1. However, for the size of deletions recovered in the mutants, the epistatic relationship is reversed, with *tdp1Δ exo1Δ* mutants showing the same reduced median deletion size as the *exo1Δ* single mutant. These observations suggest that the two genes perform distinct functions in processing blunt ends. We propose that Tdp1 inhibits processing of the 3′-end of blunt ends. This inhibition reduces the efficiency of the overall repair of blunt ends. The major determinant of the deletion size comes from the 5′→3′-resection by Exo1. Since *exo1Δ tdp1Δ* mutants show the same elevated efficiency of repair as *tdp1Δ* single mutants, we suggest that the processing of the 5′-ends by Exo1 is not critical for repair efficiency.

A striking finding from our work is that in the absence of both Exo1 and Pol4, almost all of the plasmids bearing blunt ends are repaired in a manner that does not lead to the deletion of any nucleotides. We would propose that these plasmids arise from a failure to process both the 5′- and 3′-ends. A plausible corollary is that efficient joining of blunt ends requires an obligatory intermediate with either a 3′- or 5′-tail. This hypothesis is consistent with the observation that joining of blunt ends is partially Ku independent and inefficient. However, joining of plasmids with either 3′- or 5′-extensions is more efficient in yeast than joining of blunt ends, even when the ends are not cohesive. The joining of mismatched ends requires canonical end-joining functions [see (31) for an example of the requirement of Ku70 for joining mismatched ends]. Therefore, end processing is likely to be the critical determinant in joining blunt ends. An interesting possibility is that end-processing occurs before recruitment of Ku to blunt DNA ends. Alternately, Ku binding inhibits the initial processing if the substrate DNA carries blunt ends.

An earlier examination of *exo1Δ* mutants did not observe a major effect on the efficiency of NHEJ (31). Since that study did not examine the effects of accuracy in detail, nor did it examine joining of DNA with blunt ends, it is not surprising that the effects we describe here were not detected.

The precise ordering of events that occur with different types of DNA ends will require the development of systems for introducing defined broken ends into cells. At present, rare cutting homing endonucleases such as Sce-1 or HO generate ends with 3′-extensions. Recent work by Lewis and colleagues have demonstrated the ability to use blunt cutting restriction enzymes such as PvuII in yeast (41), and other restriction enzymes generating 5′-extensions have been successfully applied to yeast for some time (42–44). It is interesting to note that expression of PvuII or EcoRV is much more poorly tolerated in yeast than expression of EcoRI (41). This suggests that efficient resection of blunt ends may be relatively deficient in yeast cells.

In conclusion, we have shown that Exo1 participates in some NHEJ reactions in yeast. Exo1 is required for accurate repair of 5′-extensions, and influences the nature of deletions in DNA with blunt ends. An important next step will be understanding how different end-processing factors are recruited to DNA ends, and how choices among different end-processing functions are regulated in physiological contexts.

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