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Pol3 is involved in nonhomologous end-joining in *Saccharomyces cerevisiae*

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ABSTRACT

Nonhomologous end joining connects DNA ends in the absence of extended sequence homology and requires removal of mismatched DNA ends and gap-filling synthesis prior to a religation step. Pol4 within the Pol X family is the only polymerase known to be involved in end processing during nonhomologous end joining in yeast. The *Saccharomyces cerevisiae POL3/CDC2* gene encodes polymerase δ that is involved in DNA replication and other DNA repair processes. Here, we show that *POL3* is involved in nonhomologous end joining using a plasmid-based end-joining assay in yeast, in which the *pol3-t* mutation caused a 1.9- to 3.2-fold decrease in the end-joining efficiency of partially compatible 5′ or 3′ ends, or incompatible ends, similar to the *pol4* mutant. The *pol3-t pol4* double mutation showed a synergistic decrease in the efficiency of NHEJ with partially compatible 5′ ends or incompatible ends. Sequence analysis of the rejoined junctions recovered from the wild-type cells and mutants indicated that *POL3* is required for gap filling at 3′ overhangs, but not 5′ overhangs during *POL4*-independent nonhomologous end joining. We also show that either Pol3 or Pol4 is required for simple religation of compatible or blunt ends. These results suggest that Pol3 has a generalized function in end joining in addition to its role in gap filling at 3′ overhangs to enhance the overall efficiency of nonhomologous end joining. Moreover, the decreased end-joining efficiency seen in the *pol3-t* mutant was not due to S-phase arrest associated with the mutant. Taken together, our genetic evidence supports a novel role of Pol3 in nonhomologous end joining that facilitates gap filling at 3′ overhangs in the absence of Pol4 to maintain genomic integrity.

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1. Introduction

DNA double-strand breaks (DSBs) are produced exogenously by ionizing radiation and chemical DNA damaging agents or by endogenous free radicals generated during cellular metabolism. DSBs are mainly repaired by homologous recombination in yeast that requires extensive sequence homology between the damaged DNA and the donor template. Alternatively, nonhomologous end joining (NHEJ), the main mechanism of DSB repair in mammalian cells, directly

Abbreviations: DSB, double-strand break(s); NHEJ, nonhomologous end-joining; MMEJ, microhomology-mediated end-joining; bp, basepair; nt, nucleotide.

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joins two broken DNA ends with no or little homology [1]. A subset of NHEJ events called microhomology-mediated end-joining (MMEJ) relies on a few base pairs of sequence homology that facilitates alignment of DNA ends [2–4]. NHEJ is usually associated with gain or loss of genetic material, nonetheless accurate end joining of broken ends is important in maintaining continuity of chromosomal DNA and cell survival. Defects in NHEJ are implicated in aberrant chromosomal rearrangements, cell transformation, and immunodeficiency [5,6].

The main genetic factors of the NHEJ pathway in yeast include the end-binding Hdf1/Hdf2 (yKu70/yKu80) heterodimer, the Mre11/Rad50/Xrs2 complex and Dnl4/Lif1 ligase complex [7]. A reconstituted NHEJ system *in vitro* has shown that the Hdf1/Hdf2 heterodimer and Dnl4/Lif1 ligase complex can promote rejoining of complementary ends [8]. However, the majority of DSBs generated by endogenous oxygen species or ionizing radiation contain incompatible ends that render DNA ends unusable for direct ligation. End joining of such events requires nucleolytic processing and possibly polymerization prior to the religation step [9].

The *S. cerevisiae POL3*/*CDC2* gene, encodes the large catalytic subunit of polymerase δ, which possesses the polymerase and proofreading 3′–5′ exonuclease activities that is required for leading strand and lagging strand synthesis in DNA replication [10,11] and other DNA repair processes [12–15]. The *pol3-t* allele is a temperature-sensitive mutant with an amino acid substitution D643N in the vicinity of the polymerase motif VI [16]. At the restrictive temperature, the *pol3-t* mutant exhibited replication defects and cell cycle arrest in the S phase [17]. 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 [18]. A replication slippage mechanism was most likely responsible for the enhanced excision events. Furthermore, some *pol3* alleles exhibited an elevated frequency of spontaneous intrachromosomal recombination [19]. The *pol3-t* mutation causes a 100-fold and a 40-fold increase in the frequency of deletions between short direct repeats [20] and longer repeats [21], respectively, as well as destabilizes microsatellite and minisatellite DNA sequences [22].

In the current literature, only the polymerases within the Pol X family are known to be involved in NHEJ. In yeast, Pol β encoded by *POL4* is required for NHEJ of incompatible ends, in which efficient gap-filling synthesis and removal of mismatches are required [23,24]. Further studies showed that physical and functional interactions among Pol4, Dnl4/Lif1 and Fen-1 (a 5′ flap endonuclease) coordinate between DNA ends processing and NHEJ enzymes [25,26]. The mammalian homologues Pol μ and Pol λ have similar functions in gap filling during NHEJ. *In vitro* data demonstrate the association between Pol μ, Ku and the Ligase IV/XRCC4 [27]. Similarly, interaction between Pol λ and Ligase IV/XRCC4 facilitates the gap-filling activity of Pol λ [28].

In this paper, we examined the effect of the *pol3-t* mutation on NHEJ using a plasmid-based end-joining assay in yeast. We also examined the epistatic interaction between *POL3* and *POL4* in NHEJ. We report here that Pol3 is involved in NHEJ and is required for gap-filling synthesis at 3′ overhangs during Pol4-independent NHEJ.

2. Materials and methods

2.1. Yeast strains

Experiments were performed in the haploid *Saccharomyces cerevisiae* strain RSY12 (*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 [29]. The *pol3-t* isogenic mutants were constructed by two-step gene replacement using the plasmid p171 cut with HpaI and FOA selection [21]. The *pol4* isogenic mutant was constructed by PCR-mediated disruption, in which the disruption cassette was generated by PCR using primers POL4-LEU2-FW 5′AAGGATAAACATGCGACCTGTTAGACAAATCGCACAT GTCTCTAAAGGGTACCGTCATCACCGAAACG-3′, POL4-LEU2-RC 5′-CCCATTCG ATATTTCTATGTTCGGGTTCAGCGTATTTTAAGTTTAATAACATAGGCGTATCA CGAGGC-3′ and plasmid YEp*lac*128 as a template, the cassette was then transformed into the RSY12 strain to generate the *pol4* mutant. The same disruption cassette was transformed into the *pol3-t* mutant to generate the isogenic *pol3-t pol4* double mutant. *E. coli* strain DH5α was used for maintenance and amplification of plasmid DNA.

2.2. Plasmids

YEp*lac*195 contains the *URA3* marker, *Amp*^{r} gene for selection and the 2-μm origin of replication [30].

2.3. Plasmid-based end-joining assay

Plasmid YEp*lac*195 was digested with restriction enzymes and purified by phenol:chloroform extraction. Yeast strains were grown at 30 °C overnight (25 °C for temperature-sensitive strains) and diluted to 1×10^{-7} cells/ml into 50 ml YPAD and incubated for 4 h at 30 °C under constant shaking. 100–200 ng of the linearized plasmid YEp*lac*195 was transformed into yeast strains using the transformation protocol previously described [31]. Circularized plasmid YEp*lac*195 was transformed into the yeast strains to measure for the transformation efficiency. Ura$^+$ transformants were selected after 4 days of growth. The efficiency of NHEJ was calculated as dividing the number of Ura$^+$ colonies arisen after transformation with linearized plasmid in comparison with that to the uncut plasmid. Yeast plasmid DNA was purified by glass beads [32], transformed into *E. coli* DH5α and selected on Ampicillin plates. Plasmid DNA was isolated from the *E. coli* strain using the QIAGEN miniprep kit (Qiagen, Valencia, CA). The isolated DNA was first digested with BamHI to exclude any uncut plasmid or single cut plasmid that was rejoined by simple religation, in which the BamHI site is located between PstI and SacI sites or between HindIII and KpnI sites. About 5–10% of rejoined plasmids recovered from the wild-type and mutant strains could be digested by BamHI and were excluded from the calculation of transformation efficiency and sequencing analysis. The isolated DNA was further digested with HindIII to estimate the size of the rejoined plasmid. The junction sequences of the rejoined plasmids were determined by sequencing using primers upstream and downstream of the multi-cloning sites, 195-8-FW 5-ATACGCAAACCGCCTCTCC-3′,

195-690-RC 5′-ATGCGACGTGCAA GATTACC-3′ and 195-4583-FW 5′ACTGGCTTCAGCAGAGCGCAGATACC-3′.

2.4. *Treatment with hydroxyurea*

Cells were diluted 1:50 from an overnight culture (RSY12 was pre-grown at 30 °C and *pol3-t* at 25 °C) in warm (30 °C) YPAD and treated with 75 mM hydroxyurea. After 4 h at 30 °C, cells were counted and small budded cells were considered to be in S-phase. The percent of S-phase cells was 89.4 ± 3.6 for RSY12 and 83.2 ± 10.3 for the *pol3-t* mutant.

2.5. *Statistical analysis*

Data on the efficiencies of NHEJ were statistically analyzed using Student's *t*-test. The percentages of end-joining events that involved gap filling in different background were compared using Chi-square test or Fisher's exact test.

3. Results

3.1. *Either Pol3 or Pol4 is required for simple religation of compatible or blunt ends during NHEJ*

To examine any effect of the *pol3-t* mutation on NHEJ, we utilized a plasmid-based end-joining assay in yeast. The YEp*lac*195 plasmid contains the *URA3* gene and a 2-μm region which allows the plasmid to replicate episomally [30]. First, we tested whether Pol3 was required for simple religation of compatible ends during NHEJ. Since Pol4 is currently the only polymerase known to be involved in NHEJ in yeast [23], we also examined the epistatic interaction between Pol3 and Pol4 on rejoining of compatible ends. Plasmid YEp*lac*195 was first linearized with either HindIII or KpnI to generate 5′ or 3′ overhangs, and transformed into the haploid wild-type RSY12 strain and the isogenic *pol3-t*, *pol4* and *pol3-t pol4* mutants lacking the *URA3* gene, in the absence of any homology between the transformed plasmid and the yeast genome. In parallel, the uncut YEp*lac*195 plasmid was transformed into each strain to control for the transformation efficiency. After 4 days of growth, Ura^+ transformants were selected. The NHEJ efficiency was measured as normalizing the number of transformants arisen after transformation with the linearized plasmid in comparison to that with the uncut plasmid.

The efficiencies of end joining with HindIII or KpnI-linearized ends in the *pol3-t* and *pol4* mutant strains were similar to that of the wild-type cells (Table 1), this lack of effect was also found previously for the *pol4* mutant [23]. However, the *pol3-t pol4* double mutation caused a significant 3.0-fold decrease in the NHEJ efficiency of 3′ overhangs (KpnI-linearized) and a significant 1.8-fold decrease in end joining of 5′ overhangs (HindIII-linearized) (Table 1). These results suggest that either one of the polymerases is required for simple religation of compatible ends during NHEJ.

We also tested the effect of *pol3-t*, *pol4* and *pol3-t pol4* mutations on end joining with blunt ends. SmaI-linearized plasmid was transformed into each strain and the NHEJ efficiencies were measured. Both *pol3-t* and *pol4* mutants exhibited a small decrease in the NHEJ efficiencies that are not significantly dif-

Table 1 – NHEJ of compatible or blunt ends

Yeast strain		Ura^+ colonies		Ura^+ colonies		Ura^+ colonies		
	Circular	KpnI-restricted	% Joined	HindIII-restricted	% Joined	Circular	SmaI-restricted	% Joined
RSY12	8576	7632	88.9 ± 5.4	7334	85.8 ± 4.9	18,904	12,553	65.8 ± 12.4
pol3-t	9572	7787	81.2 ± 9.2	7244	75.7 ± 10.6	21,271	9,784	46.0 ± 11.5
pol4	9293	7069	76.1 ± 9.3	7178	77.5 ± 16.6	19,448	11,283	58.0 ± 4.3
pol3-tpol4	7054	2061	29.3 ± 9.6^{***a,b,c}	3308	46.9 ± 9.8^{***a,*b,c}	10,496	2,674	25.5 ± 9.4^{**a,*b,***c}

Results are the mean of 4–5 independent experiments ± standard deviation. Data were statistically analyzed using the Student's *t*-test. Data referring to *pol3-t pol4* were compared with RSY12[a], *pol3-t*[b] and *pol4*[c]. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

ferent from the wild-type cells (Table 1). However, the *pol3-t pol4* double mutation caused a 2.6-fold decrease in the NHEJ efficiency that was significantly different from the wild-type cells or either of the single mutants (Table 1). These results suggest that either one of the polymerases is also required for NHEJ with blunt ends.

To further examine whether end joining of the HindIII, KpnI or SmaI-linearized substrates involved end processing, we amplified the rejoined junctions from the wild-type cells and all of the mutant strains by PCR and tested whether the rejoined plasmids retained the restriction sites after end joining. We amplified five rejoined junctions from each strain and digested the PCR products with the respective restriction enzymes (HindIII, KpnI or SmaI). All of the junctions recovered from the wild-type cells and all mutant strains restored the restriction sites for the rejoining of the HindIII, KpnI and SmaI-linearized substrates (data not shown), indicating that the compatible ends or blunt ends were religated directly without any end processing. These results suggest that either Pol3 or Pol4 is required for simple religation of compatible ends or blunt ends during NHEJ without addition or deletion of nucleotides.

3.2. Pol3 is involved in NHEJ of partially compatible 5′ or 3′ ends or incompatible ends

We then tested the effect of the *pol3-t* mutation on the repair of NHEJ substrates with partially compatible 5′ and 3′ ends, and incompatible ends. The PstI–SacI substrate presented in Fig. 1 juxtaposes 3′ overhangs with a compatible "GC" within the 4-bp overhang and the BamHI–EcoRI substrate presented in Fig. 2 contains 5′ overhangs with a compatible "AT" within the 4-bp overhang, in which these substrates are useful in exploring gap filling after end alignment of the compatible 2 bp. The HindIII–KpnI substrate shown in Fig. 3 juxtaposes 5′ and 3′ incompatible ends that are useful in mixed joining in the absence of any compatible bps. The linearized and uncut YEp*lac*195 plasmids were transformed into the wild-type RSY12 strain and the *pol3-t* mutant independently and the NHEJ efficiencies were determined.

The *pol3-t* mutation caused a significant 1.9- or 3.2-fold decrease, respectively in the NHEJ efficiency with the BamHI–EcoRI or PstI–SacI substrates that contain partially compatible 5′ or 3′ ends, and a 2.7-fold decrease in the efficiency of mixed joining with the HindIII–KpnI substrate (Table 2). These results suggest that Pol3 is involved in NHEJ with partially compatible 5′ or 3′ ends or incompatible ends.

3.3. *pol3-t* is synergistic with *pol4* in NHEJ of partially compatible 5′ ends or incompatible ends

To examine the epistatic interaction between the *pol3-t* and *pol4* mutants on NHEJ with partially compatible ends or incompatible ends, the same linearized substrates and the uncut plasmid were transformed into the isogenic *pol4*

Junctions	RSY12	*pol3-t*	*pol4*	*pol3-tpol4*	PstI	SacI	Deletions	Filled-in	Microhomology
	Frequency				TTGCATGCCTGCA 3' AACGTACGG	CGAATTCACT 3' TCGAGCTTAAGTGA			
A	3	10			TTGCATGCCTGC AACGTACGGA	TCGAATTCACT CGAGCTTAAGTGA	-1 / -1	2nt	2bp
B		3	1		TTGCATGC AACGTA	TCGAATTCACT CGAGCTTAAGTGA	-5 / -1	1nt	2bp
C			3		TTGCAT AACGTA	AGCTCGAATTCACT TCGAGCTTAAGTGA	-7 / 0	4nt	-
D		1			TTGGG AACCC	GAATTCACT CTTAAGTGA	-10 / -5 (+4)	-	-
E	4		1		AGCT	CGAATTCACT TCGAGCTTAAGTGA	-12 / 0	-	4bp
F	1	3	6	18	TTGCATGCCT AACGTACG	CGAATTCACT GAGCTTAAGTGA	-3 / -2	-	2bp
G			1	2	TTGCA AAC	CT GTGA	-8 / -10	-	2bp
H		1			TTGC AACGT	AATTCACT TAAGTGA	-8 / -6	-	1bp
I			2		TTGCATGCC AACGTACG	GAATTCACT GCTTAAGTGA	-4 / -4	-	1bp
J			1		TTGCATGC AACGTAC	GAATTCACT GCTTAAGTGA	-5 / -4	-	1bp
K			1		CTCGTATGT // GAGCATAC //	CGAATTCACT AGCTTAAGTGA	-79 / -3	-	1bp
L	2				TCTGACTT // AGACTGA //	CGAATTCACT AGCTTAAGTGA	-479 / -3	-	1bp

Fig. 1 – PstI–SacI junction sequences recovered from the wild-type RSY12 strain, *pol3-t*, *pol4* and *pol3-tpol4* mutants. The plasmid YEp*lac*195 linearized with PstI and SacI was 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. Microhomologies utilized for end joining are 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 DNA 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.

BamHI: ACTCTAGAG / TGAGATCTCCTAG 5' — EcoRI: 5' AATTCACTGGCCG / GTGACCGGC

Junctions	RSY12	pol3-t	pol4	pol3-tpol4	BamHI end	EcoRI end	Deletion	Filled-in	Microhomology
A		1			ACTCTAGAGGATC / TGAGATCTCCTAG	AATTCACTGGCCG / TTAAGTGACCGGC	0 / 0	8nt	-
B			1	1	ACTCTAGAGGATC / TGAGATCTCCTAG	CACTGGCCG / GTGACCGGC	0 / -4	4nt	-
C	1				ACTCTA / TGAGAT	AATTCACTGGCCG / TTAAGTGACCGGC	-7 / 0	4nt	-
D	1	1		1	ACTCTAGAGG / TGAGATCTCCT	AATTCACTGGCCG / TAAGTGACCGGC	-2 / 0	4nt	1bp
E	2		1		ACTCTAG / TGAGATCT	AATTCACTGGCCG / TAAGTGACCGGC	-5 / -0	3nt	1bp
F			1		ACTCTAGAGGAT / TGAGATCTCCTA	GCCG / CGGC	-1 / -9	3nt	-
G	2	6	9	5	ACTCTAGAGG / TGAGATCTCCTA	ATTCACTGGCCG / AGTGACCGGC	-1 / -1	2nt	2bp
H	2	4	8	10	ACTCTAGAGGA / TGAGATCTCCTAG	TCACTGGCCG / TGACCGGC	0 / -3	2nt	2bp
I			1		ACTCTAG / TGAGATCT	ATTCACTGGCCG / AAGTGACCGGC	-5 / -1	2nt	1bp
J	1	1			ACTCTAGA / TGAGATCTCC	GGCCG / GGC	-3 / -8	-	2bp
K		1		1	ACTCTAG / TGAGATCT	ACTGGCCG / GACCGGC	-5 / -5	-	1bp
L			1		ACTCTAGAG / TGAGATCTCC	GT / A	-3 / -12	-	1bp
M	1				ACTCTAGA / TGAGATCTC	GCCG / GGC	-4 / -9	-	1bp
N	1				ACTCTA / TGAGATC	GCCG / GGC	-6 / -9	-	1bp
O		1			ACT / TGAG	CTGGCCG / ACCGGC	-9 / -6	-	1bp
P				2	ACT / TGAG	CGTCGTTT / CAGCAAA	-9 / -11	-	1bp
Q			1		ACTC / TGAG	GTCGTTTT / CAGCAAAA	-9 / -12	-	-

Fig. 2 – BamHI–EcoRI junction sequences recovered from the wild-type RSY12 strain, *pol3-t*, *pol4* and *pol3-tpol4* mutants. Description of junctions is the same as Fig. 1.

mutant and the *pol3-t pol4* double mutant independently and the NHEJ efficiencies were measured. Similar to the *pol3-t* mutant, the *pol4* mutation caused a significant 1.9- or 1.8-fold decrease, respectively in the efficiencies of NHEJ with the BamHI–EcoRI or PstI–SacI substrates, and a significant 3.6-fold decrease in the efficiency of mixed joining with the HindIII–KpnI substrate (Table 2). Intriguingly, the *pol3-t pol4* double mutation caused a synergistic decrease in the NHEJ efficiency of the BamHI–EcoRI or HindIII–KpnI substrate, a 7.3-fold and 7.2-fold decrease respectively, which are significantly different from either one of the single mutant and the wild-type cells (Table 2). For the end joining with the PstI–SacI substrate, the *pol3-t pol4* double mutant caused a 2.9-fold decrease in the NHEJ efficiency, similar to that of *pol3-t*, and was significantly different from that of *pol4* or wild-type cells.

3.4. *Pol3 is involved in Pol4-independent NHEJ that requires gap-filling synthesis on 3′ ends, but not 5′ ends*

To further examine if Pol3 was involved in gap-filling synthesis during NHEJ in the presence and absence of Pol4, we sequenced the rejoined plasmids recovered from the wild-type strain, *pol3-t*, *pol4* and *pol3-t pol4* double mutants and inferred the number of nucleotides needed to be filled-in in each clone from the sequence of the rejoined junctions. We first analyzed the junctions arisen from rejoining of the PstI–SacI substrate that contains partially compatible 3′ ends. It has been shown that Pol4 is only required for end joining of 3′ overhangs that necessitates gap filling on both strands, but is dispensable for joints with a gap on one strand [24]. Thus, we compared the frequency of end-joining event that requires gap filling on both strands (junction A in Fig. 1) among

the wild-type cells and all of the mutants. 30% (3 out of 10) junctions in the wild-type compared to 56% (10 out of 18) junctions in the *pol3-t* mutant that is not significantly different (Fig. 1). However, in the *pol4* mutant none of 16 junctions had gap filling on both strands, that is significantly different from the wild-type ($p < 0.05$) as well as the *pol3-t* mutant ($p < 0.0005$). Similarly, none of 20 junctions in the *pol3-t pol4* double mutant had gap filling on both strands, that is significantly different from the wild-type cells ($p < 0.05$) and the *pol3-t* mutant ($p < 0.0001$). These results showed that Pol4 is required for end joining of 3′ overhangs that requires gap filling on both strands, which is consistent with previous results [24]. Other end-joining events that require gap filling on one strand are junctions B and C that were observed in the *pol3-t* and *pol4* mutants (Fig. 1). When comparing the frequency of events that require gap filling on either one or both strands, 30% (3 out of 10) rejoined plasmids in the wild-type required gap-filling synthesis compared to 72% (13 out of 18) rejoined plasmids arisen from the *pol3-t* mutant ($p < 0.05$) (Figs. 1 and 4). In the *pol4* mutant, 25% (4 out of 16) rejoined plasmids required gap filling, that is not significantly different from the wild-type. Intriguingly, none of the 20 rejoined plasmids from the *pol3-t pol4* double mutant involved gap filling that is significantly different from the wild-type cells ($p < 0.05$), *pol3-t* ($p < 0.000005$) or *pol4* mutant ($p < 0.05$, by Fisher's exact test) (Fig. 4). These results showed that Pol3 is required

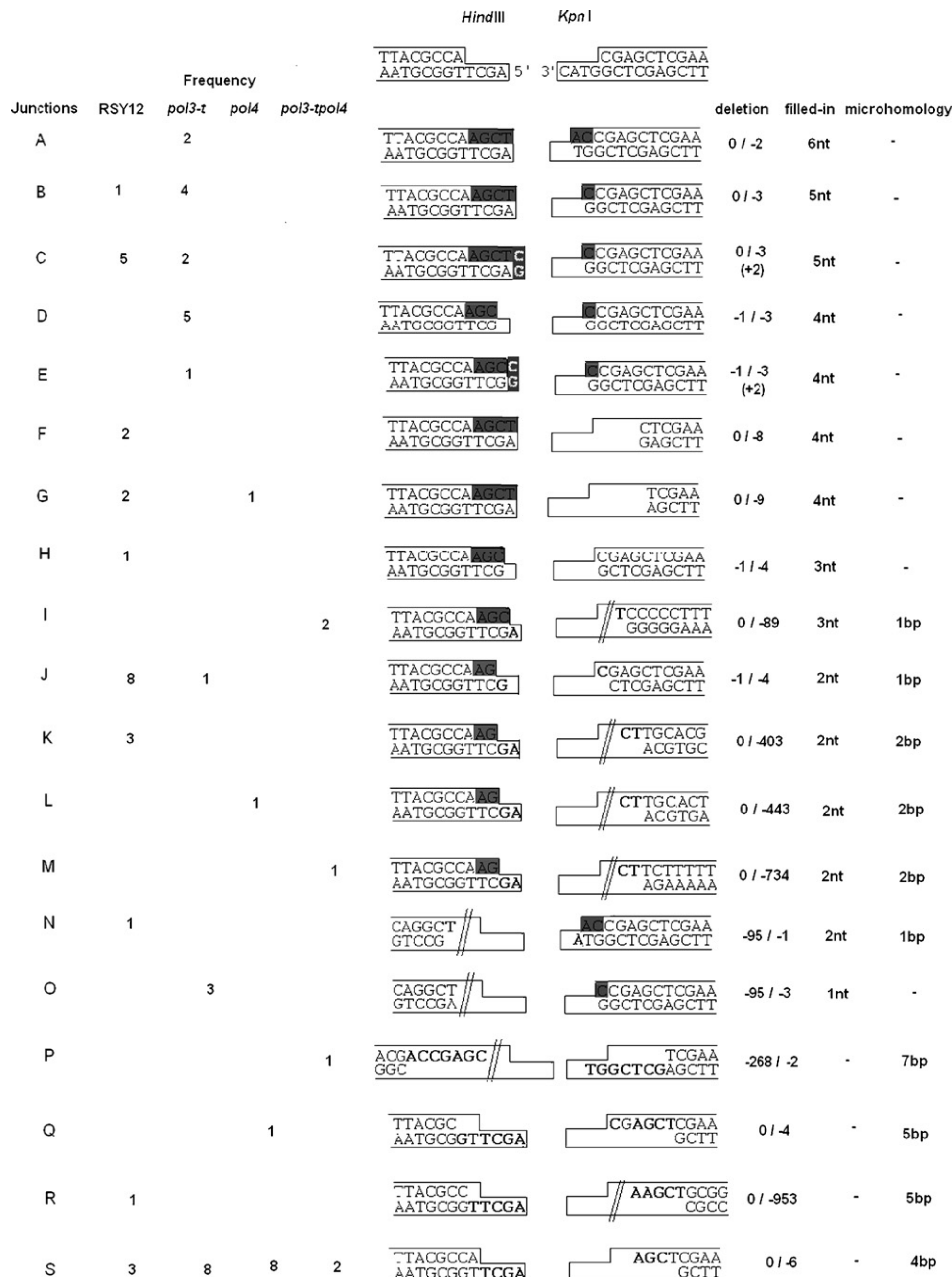

Fig. 3 – HindIII–KpnI junction sequences recovered from the wild-type RSY12 strain, *pol3-t*, *pol4* and *pol3-tpol4* mutants. Description of junctions is the same as Fig. 1. Junction O has one mismatch within the microhomology region.

					Left end	Right end			
T				1	CACATGTTC / GTGTA	ATTTT / CAAGTAAAA	-356 / -419	-	4bp
U	1				TTACGCCA / AATGCGGTTCGA	AGCTCGTT / AGCAA	0 / -1356	-	4bp
V			1		CATGATTAC / GTACTA	CGAGCTCGAA / ATGGCTCGAGCTT	-8 / -1	-	3bp
W				1	TTAGGCACC / AATCCG	GAGCTCGAA / TGGCTCGAGCTT	-102 / -2	-	3bp
X		1			TTACGCCA / AATGCGGTTCG	AGCGAAGCT / CTTCGA	-1 / -949	-	3bp
Y		1			TTA / AATGC	CGAGCTCGAA / TCGAGCTT	-7 / -4	-	2bp
Z			1		TTAC / AATGCG	GCTCGAA / AGCTT	-6 / -7	-	2bp
A1			2		TGATTAC / ACTAA	CGATCGC / TGGCTAGCG	-8 / -129	-	2bp
A2	3				TTACGCC / AATGCGGTT	AATCTTGC / AGAACG	-3 / -400	-	2bp
A3	1				CAG / GTCCG	GCATTTTT / TAAAAA	-96 / -549	-	2bp
A4				2	GCTATGACC / CGATACTG	TCGAA / GAGCTT	-16 / -8	-	1bp
A5	2				CCCCAGG / GGGGTCCG	CGAGCTCGAA / CTCGAGCTT	-103 / -4	-	1bp
A6				1	AACGCAAT / TTGCGTTAA	TTCACTGGCC / AGTGACCGG	-131 / -14	-	1bp
A7			1		GCTTCCAGG / CGAAGGTCCC	GAGCTCGAA / TCGAGCTT	-518 / -5	-	1bp
A8			2		GCTTCCAG / CGAAGGTCC	GCTCGAA / GAGCTT	-519 / -7	-	1bp
A9	1				CAGGCT / GTCCGA	CGAGCTCGAA / GCTCGAGCTT	-95 / -4	-	-
A10	2				CAGGCT / GTCCGA	GAGCTCGAA / CTCGAGCTT	-95 / -5	-	-

Fig. 3 – (*Continued*).

for gap filling at 3′ overhangs during Pol4-independent NHEJ.

Comparison of junctions recovered from the wild-type cells and the *pol3-t* mutant indicated that there is a higher frequency of junction A that requires gap filling on both strands in the *pol3-t* mutant relative to the wild-type cells, although it is not significant (56% in *pol3-t* vs. 30 wt.%). These results suggest that in the absence of Pol3 DSB repair is channeled into the Pol4-mediated end joining that mediates gap filling on both strands.

Comparison of junctions from the *pol3-t* and *pol4* mutants demonstrated the absence of junction A in the *pol4* mutant, indicating that Pol4 is required for end joining of 3′ overhangs that requires gap filling on both strands. However, both mutants can mediate gap filling on one strand (junctions B and C), suggesting that either Pol3 or Pol4 is capable of gap filling on one strand at 3′ overhangs. Comparison of junctions recovered from the *pol4* and *pol3-t pol4* mutants indicated the absence of joints that involved any gap filling in the double mutant. These results suggest that junctions B and C recovered from the *pol4* mutant were mediated by Pol3, and repair of DSBs is channeled into Pol3-mediated NHEJ in the absence of Pol4.

For the BamHI–EcoRI substrate that contains partially compatible 5′ ends, 73% (8 out of 11) rejoined plasmids recovered from the wild-type strain required gap-filling synthesis at 5′ overhangs, compared to 80% (12 out of 15) from the *pol3-t* mutant and 91% (21 out of 23) from the *pol4* mutant, both are not significantly different from the wild-type cells (Figs. 2 and 4). In the *pol3-t pol4* double mutant, 85% (17 out of 20) rejoined plasmids involved gap filling at 5′ overhangs that is not significantly different from the wild-type cells, *pol3-t* or *pol4* mutant. These results indicate that neither Pol3 nor Pol4 are required for gap filling at 5′ overhangs during NHEJ.

Table 2 – NHEJ of partially compatible 5′ or 3′ ends or incompatible ends

Yeast strain	Ura⁺ colonies			Ura⁺ colonies			Ura⁺ colonies		
	Circular	BamHI–EcoRI	% Joined	Circular	PstI–SacI	% Joined	Circular	HindIII–KpnI	% Joined
RSY12	18,349	7008	38.2 ± 12.5	12,020	4454	37.1 ± 5.7	10,572	3327	31.5 ± 9.4
pol3-t	15,888	3144	$19.8 \pm 4.2^{*}$	12,117	1396	$11.5 \pm 3.0^{*}$	9,880	1160	$11.7 \pm 4.4^{**a}$
pol4	25,622	5268	$20.6 \pm 7.2^{*}$	17,850	3751	$21.0 \pm 1.8^{***}$	17,850	1560	$8.7 \pm 1.1^{***a}$
pol3-tpol4	15,404	808	$5.2 \pm 1.3^{**a,b,c,}$	14,068	1797	$12.8 \pm 0.46^{***a,NS,b,*c,}$	14,068	616	$4.4 \pm 1.0^{**a,*b,*c}$

Results are the mean of 4–6 independent experiments ± standard deviation. Data were statistically analyzed using the Student's t-test. Data referring to the *pol3-t* and *pol4* mutants were compared to RSY12. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ^{NS}not significant; ^{a}compared with the wild-type RSY12, ^{b}with *pol3-t* strain and ^{c}with *pol4*.

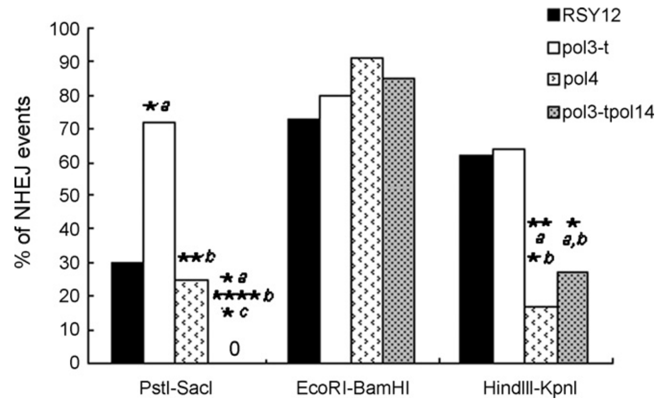

Fig. 4 – Percentage of NHEJ events that require gap filling synthesis. Data were statistically analyzed using the Chi-square test. Data referring to *pol3t*, *pol4* and *pol3-tpol4* mutants were compared with [a]RSY12, *pol3-t*[b] and *pol4*[c]. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{****}p < 0.00005$.

For the HindIII–KpnI substrate that contains 5′ and 3′ incompatible ends, 62% (23 out of 37) rejoined plasmids recovered from the wild-type strain required gap-filling synthesis, compared to 64% (18 out of 28) from the *pol3-t* mutant that is not significantly different and 17% (3 out of 18) from the *pol4* mutant which is significantly different from the wild-type strain ($p < 0.005$) or the *pol3-t* mutant ($p < 0.05$) (Figs. 3 and 4). These results showed that Pol4 is required for gap filling on mixed joints containing incompatible ends, consistent with previous results that Pol4 facilitates gap filling at mixed joins in conjugation with removal of mismatched nucleotides [23]. In the *pol3-t pol4* double mutant, 27% (3 out of 11) rejoined plasmids involved gap filling that is significantly different from wild-type cells or *pol3-t* mutant ($p < 0.05$ for both), but not significantly different to the *pol4* mutant, indicating that the *pol4* mutation is dominant in the *pol3-t pol4* background for mixed joining. In addition, the junctions that involved gap filling in the *pol3-t pol4* mutant (junctions I and M in Fig. 3) were comparable to that in the *pol4* mutant (junctions G and L in Fig. 3). These results suggest that Pol3 is not required for gap filling on mixed joints with incompatible ends.

Taken together, these results demonstrate that Pol3 is a minor polymerase that is required for gap-filling synthesis at 3′ overhangs during Pol4-independent NHEJ. Our results also indicate that neither Pol3 nor Pol4 is required for gap filling at 5′ overhangs during NHEJ and suggest that another polymerase or enzyme is essential for 5′ overhang processing.

3.5. *Decreased NHEJ efficiency in the pol3-t mutant was not due to S phase arrest*

Since the *pol3-t* mutant exhibits replication defects and cell cycle arrest at S phase [17], the decreased NHEJ efficiency seen in the *pol3-t* mutant could be due to S phase arrest associated with the mutant. To test this possibility, the wild-type strain and isogenic *pol3-t* mutant were first treated with hydroxyurea for 4 h to inhibit DNA replication and then transformed with the HindIII, KpnI-linearized plasmid. The NHEJ efficiencies were determined. In the wild-type strain, the NHEJ efficien-

Table 3 – Effect of S-phase arrest on NHEJ in RSY12 wild-type and *pol3-t* mutant

Yeast strain	Ura$^+$ colonies		
	Circular	HindIII–KpnI linearized	% Joined
RSY12 growing	15,062	4559	30.3 ± 6.0
RSY12 S-phase[a]	14,719	5401	36.7 ± 2.8
pol3-t growing	14,305	1449	10.1 ± 3.2
pol3-t S-phase[a]	11,180	2931	26.2 ± 6.6*

Results are the mean of five independent experiments ± standard deviation. Data were statistically analyzed using the Student's *t*-test. $^*p < 0.05$.

cies were 30.3% in the growing cells and 36.7% in the S-phase arrested cells (Table 3), indicating that S-phase arrest slightly increased the NHEJ efficiency. In the *pol3-t* mutant, the NHEJ efficiencies were 10.1% in the growing cells and increased to 26.2% in the S-phase arrested cells ($p < 0.05$). These results exclude the possibility that the decreased NHEJ efficiency observed in the *pol3-t* mutant was due to S-phase arrest associated with the mutant.

4. Discussion

In this paper, we demonstrate that Pol3 is involved in NHEJ using a plasmid-based end-joining assay in yeast. The *pol3-t* mutation displayed a 1.9- to 3.2-fold decrease in the NHEJ efficiency with partially compatible 5′ or 3′ ends, or incompatible ends. The *pol4* mutation also caused a 1.9- to 3.6-fold decrease in the NHEJ efficiency, similar to previous results. The *pol3-t pol4* double mutation showed a synergistic decrease in the efficiency of NHEJ with partially compatible 5′ ends or incompatible ends. Sequence analysis of the junctions recovered from the wild-type cells and all of the mutants indicate that Pol3 is required for gap filling at 3′ overhangs during Pol4-independent NHEJ. However, both Pol3 and Pol4 are dispensable for gap filling at 5′ overhangs during NHEJ. We also demonstrate that either one of the polymerases is required for simple religation of compatible or blunt ends.

Pol3 has polymerization activity and low processivity in DNA replication [10,11] and is involved in other DNA repair processes [12–15]. As the *pol3-t* allele exhibited a diminished rate of lagging-strand synthesis in DNA replication [18], it is possible that the *pol3-t* mutant exhibits a decreased activity in gap filling synthesis that may account for the decreased end-joining efficiency in the *pol3-t* mutant. *In vitro* experiments examining the biochemical activities of Pol δ purified from eggs of the teleost fish *Misgurnus fossilis* have shown that Pol δ can fill in small gaps in a processive manner [33]. In addition, the observations that Pol δ binds efficiently to gapped DNA and DNA duplex containing 3′ single-stranded tails [33] further supports that these DNA intermediates present in NHEJ can be recognized by Pol δ.

Pol4 is responsible for a substantial amount of end-joining events that require efficient gap filling and removal of mismatches in yeast [23,24], thus we further examined the epistatic interaction between Pol3 and Pol4 in NHEJ. Based on sequence analysis of the rejoined plasmids recovered from the wild-type cells and all of the mutant strains, our results showed that Pol3 is required for gap filling at 3′ overhangs during Pol4-independent NHEJ since none of the PstI–SacI junctions recovered from the *pol3-t pol4* double mutant involved gap filling at 3′ overhangs compared to 72% of junctions from the *pol3-t* mutant and 27% of junctions from the *pol4* mutant. These results suggest that Pol4 is the major polymerase promoting gap-filling synthesis at 3′ overhangs while Pol3 is a minor polymerase that facilitates similar activities. Our results also showed that Pol4 is strongly required for joining of DSBs when the 3′ overhangs contain gaps on both strands, but is dispensable for joining with a gap on only one strand, which is consistent with previous results shown by Daley et al. [24]. According to our results, in the absence of Pol4, repair of DSBs is channeled into Pol3-mediated NHEJ that can fill-in gaps at 3′ overhangs. In contrast, our results showed that both Pol3 and Pol4 are dispensable for gap filling at 5′ overhangs, suggesting that other polymerases or enzymes are essential for 5′ end processing. Previous studies have shown that Pol4 is exclusively required for gap filling at 3′ overhangs during NHEJ, in which Pol4 is a specialized polymerase that has a decreased dependence on a stable template-primer pairing for DNA synthesis [24]. Our data indicated that Pol3 does not compensate for the loss of Pol4 in processing 5′ overhangs during NHEJ. Moreover, our results show that Pol3 is not required for gap filling at mixed joints containing fully incompatible ends in the absence of Pol4, in which the HindIII–KpnI junctions that involved gap filling recovered from the *pol3-t pol4* double mutant were comparable to that from the *pol4* mutant.

The *pol3-t* mutation caused a 1.9- to 3.2-fold decrease in the NHEJ efficiencies of partially compatible 5′ or 3′ ends, or fully incompatible ends, in which the spectrum of products recovered from the *pol3-t* mutant was comparable to the wild-type cells. These results suggest that the *pol3-t* mutation has a generalized effect on NHEJ, regardless of the necessity of gap filling at 3′ overhangs. This is also supported by the fact that the *pol3-t* mutant in the *pol4* mutant background has a significantly lower efficiency of end joining of compatible ends and of blunt ends (Table 1).

One possibility is that Pol3 might bridge the two ends in close proximity or stimulate the activity of the ligase complex for DNA religation. A bridge pathway of DNA polymerase-mediated end-joining has been previously proposed by King et al. based on *in vitro* end-joining reactions by the Klenow Fragment of DNA Polymerase I in *E. coli* [34], in which a DNA polymerase binds to a 3′ recessed or blunt end, joins to the other 3′ end and utilizes it as a template for DNA synthesis. Further evidence on a polymerase-mediated NHEJ is based on the crystal structure of *Mycobacterium tuberculosis* polymerase domain of LigD-mediating synapsis of two noncomplementary DNA ends [35], supporting that a polymerase is capable of bridging two DNA ends together. It is possible that Pol3 in yeast may contain bridging activity to juxtapose two ends together in addition to its role in gap filling at 3′ overhangs, thus increasing the overall efficiency of NHEJ. Alternatively, it was proposed that a polymerase can act as an alignment protein to stabilize base-pairing of short homologous sequences between two ends of a DSB and facilitates microhomology-mediated end joining [34]. However, it is unlikely that Pol3 acts as an alignment protein as our data showed that the *pol3-*

t mutant has no defect in utilizing microhomologies during NHEJ. Further work is required to examine the biochemical functions of Pol3 in NHEJ.

Our data also showed that either Pol3 or Pol4 is required for simple religation of compatible ends or blunt ends, in which the *pol3-t pol4* double mutation caused a 3.0- or 1.8-fold decrease in the end-joining efficiency of KpnI or HindIII-linearized ends and a 2.6-fold decrease in the efficiency of SmaI-linearized ends, whereas neither of the *pol3-t* nor *pol4* mutations by themselves has any effect on rejoining of compatible or blunt ends. However, it has been shown that the polymerization activity is not required for rejoining of compatible ends in mammalian cells as there was no effect on *in vitro* end-joining when dNTP was omitted in the nuclear cellular extract [36]. This is also supported by the fact that all our religation products of compatible or blunt ends restored the restriction sites and did not involve any gap filling. It is known that Ku binds to DNA ends and bridges two broken ends together [37,38]. Ku further recruits the Dnl4/Lif1 ligase complex to the DSB ends and activates the Dnl4/Lif1 complex for DNA religation [8,39]. It is possible that either one of the polymerases may interact with Ku and enhance its bridging activity to juxtapose two broken ends, thus increasing the overall efficiency of NHEJ with compatible ends or blunt ends. In addition, our data showed that neither Pol3 nor Pol4 is required for microhomology-mediated end joining in which repair of DSBs were shifted to the microhomology-mediated end-joining pathway in the *pol3-t pol4* double mutant, suggesting that the putative bridging activity of Pol3 or Pol4 is exclusively required for mediating direct end-joining events without any microhomology.

Since the *pol3-t* mutation exhibited replication defects that arrested cells at S phase [17], S-phase arrest associated with the mutant might possibly have led to decreased NHEJ efficiency seen in the *pol3-t* mutant. To test this possibility, we pretreated cells with hydroxyurea before transformation of the linearized plasmid and found that S-phase arrest slightly increased the NHEJ efficiency in the wild-type cells and caused a 2-fold increase in the *pol3-t* mutant, indicating that decreased NHEJ efficiency observed in the *pol3-t* mutant was not due to S-phase arrest associated with the mutant. The 2.0-fold increase in the NHEJ efficiency in the arrested *pol3-t* mutant could possibly be due to higher amounts of Pol3 protein available for NHEJ during replication arrest.

In higher eukaryotes, Pol δ activity increased significantly after induction of preB cell differentiation while other polymerase activities remained at low level or decreased, suggesting that Pol δ may be involved in DNA synthesis to fill-in gaps created by opening of hairpin intermediates during V(D)J recombination [40]. As V(D)J recombination require all NHEJ factors [41], our data that Pol3 is involved in NHEJ is in agreement with a potential role of Pol δ in gap filling during V(D)J recombination.

In yeast, Pol3 is known to be involved in gap filling or repair synthesis in a variety of DNA repair processes such as DSB-induced gene conversion, nucleotide excision repair, base excision repair, mismatch repair and translesion DNA synthesis [12–15]. Our genetic evidence shows a novel role of Pol3 in NHEJ that mediates gap-filling synthesis at 3′ overhangs in the absence of Pol4. It is plausible that eukaryotic cells contain redundant polymerases to enhance NHEJ capacity for the maintenance of genomic integrity.

Conflict of interest statement

There is no conflict of interest.

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REFERENCES

[1] M.K. Derbyshire, L.H. Epstein, C.S. Young, P.L. Munz, R. Fishel, Nonhomologous recombination in human cells, Mol. Cell. Biol. 14 (1994) 156–169.

[2] S.J. Boulton, S.P. Jackson, *Saccharomyces cerevisiae* Ku70 potentiates illegitimate DNA double-strand break repair and serves as a barrier to error-prone DNA repair pathways, EMBO J. 15 (1996) 5093–5103.

[3] R.H. Schiestl, M. Dominska, T.D. Petes, Transformation of *Saccharomyces cerevisiae* with nonhomologous DNA: illegitimate integration of transforming DNA into yeast chromosomes and in vivo ligation of transforming DNA to mitochondrial DNA sequences, Mol. Cell. Biol. 13 (1993) 2697–2705.

[4] K.M. Kramer, J.A. Brock, K. Bloom, J.K. Moore, J.E. Haber, Two different types of double-strand breaks in *Saccharomyces cerevisiae* are repaired by similar RAD52-independent, nonhomologous recombination events, Mol. Cell. Biol. 14 (1994) 1293–1301.

[5] K.K. Khanna, S.P. Jackson, DNA double-strand breaks: signaling, repair and the cancer connection, Nat. Genet. 27 (2001) 247–254.

[6] A.J. Pierce, M. Jasin, NHEJ deficiency and disease, Mol. Cell 8 (2001) 1160–1161.

[7] Z. Dudasova, A. Dudas, M. Chovanec, Non-homologous end-joining factors of *Saccharomyces cerevisiae*, FEMS Microbiol. Rev. 28 (2004) 581–601.

[8] L. Chen, K. Trujillo, W. Ramos, P. Sung, A.E. Tomkinson, Promotion of Dnl4-catalyzed DNA end-joining by the Rad50/Mre11/Xrs2 and Hdf1/Hdf2 complexes, Mol. Cell 8 (2001) 1105–1115.

[9] J.S. Critchlow, SE DNA end-joining: from yeast to man, Trends Biochem. Sci. Rev. 10 (1998) 394–398.

[10] A. Boulet, M. Simon, G. Faye, G.A. Bauer, P.M. Burgers, Structure and function of the *Saccharomyces cerevisiae* CDC2 gene encoding the large subunit of DNA polymerase III, EMBO J. 8 (1989) 1849–1854.

[11] R. Hindges, U. Hubscher, DNA polymerase delta, an essential enzyme for DNA transactions, Biol. Chem. 378 (1997) 345–362.

[12] A.M. Holmes, J.E. Haber, Double-strand break repair in yeast requires both leading and lagging strand DNA polymerases, Cell 96 (1999) 415–424.

[13] M.E. Budd, J.L. Campbell, DNA polymerases required for repair of UV-induced damage in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 15 (1995) 2173–2179.

[14] Z. Wang, X. Wu, E.C. Friedberg, DNA repair synthesis during base excision repair in vitro is catalyzed by DNA polymerase epsilon and is influenced by DNA polymerases alpha and delta in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 13 (1993) 1051–1058.
[15] L. Giot, R. Chanet, M. Simon, C. Facca, G. Faye, Involvement of the yeast DNA polymerase delta in DNA repair in vivo, Genetics 146 (1997) 1239–1251.
[16] H.T. Tran, N.P. Degtyareva, D.A. Gordenin, M.A. Resnick, Genetic factors affecting the impact of DNA polymerase delta proofreading activity on mutation avoidance in yeast, Genetics 152 (1999) 47–59.
[17] J. Culotti, L.H. Hartwell, Genetic control of the cell division cycle in yeast. 3. Seven genes controlling nuclear division, Exp. Cell Res. 67 (1971) 389–401.
[18] D.A. Gordenin, A.L. Malkova, A. Peterzen, V.N. Kulikov, Y.I. Pavlov, E. Perkins, M.A. Resnick, Transposon Tn5 excision in yeast: influence of DNA polymerases alpha, delta, and epsilon and repair genes, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 3785–3789.
[19] A. Aguilera, H.L. Klein, Genetic control of intrachromosomal recombination in *Saccharomyces cerevisiae*. I. Isolation and genetic characterization of hyper-recombination mutations, Genetics 119 (1988) 779–790.
[20] H.T. Tran, N.P. Degtyareva, N.N. Koloteva, A. Sugino, H. Masumoto, D.A. Gordenin, M.A. Resnick, Replication slippage between distant short repeats in *Saccharomyces cerevisiae* depends on the direction of replication and the RAD50 and RAD52 genes, Mol. Cell. Biol. 15 (1995) 5607–5617.
[21] A. Galli, T. Cervelli, R.H. Schiestl, Characterization of the hyperrecombination phenotype of the *pol3-t* mutation of *Saccharomyces cerevisiae*, Genetics 164 (2003) 65–79.
[22] R.J. Kokoska, L. Stefanovic, H.T. Tran, M.A. Resnick, D.A. Gordenin, T.D. Petes, Destabilization of yeast micro- and minisatellite DNA sequences by mutations affecting a nuclease involved in Okazaki fragment processing (rad27) and DNA polymerase delta (*pol3-t*), Mol. Cell. Biol. 18 (1998) 2779–2788.
[23] T.E. Wilson, M.R. Lieber, Efficient processing of DNA ends during yeast nonhomologous end joining. Evidence for a DNA polymerase beta (Pol4)-dependent pathway, J. Biol. Chem. 274 (1999) 23599–23609.
[24] J.M. Daley, R.L. Laan, A. Suresh, T.E. Wilson, DNA joint dependence of pol X family polymerase action in nonhomologous end joining, J. Biol. Chem. 280 (2005) 29030–29037.
[25] H.M. Tseng, A.E. Tomkinson, A physical and functional interaction between yeast Pol4 and Dnl4-Lif1 links DNA synthesis and ligation in nonhomologous end joining, J. Biol. Chem. 277 (2002) 45630–45637.
[26] H.M. Tseng, A.E. Tomkinson, Processing and joining of DNA ends coordinated by interactions among Dnl4/Lif1, Pol4, and FEN-1, J. Biol. Chem. 279 (2004) 47580–47588.
[27] K.N. Mahajan, S.A. Nick McElhinny, B.S. Mitchell, D.A. Ramsden, Association of DNA polymerase mu (pol mu) with Ku and ligase IV: role for pol mu in end-joining double-strand break repair, Mol. Cell. Biol. 22 (2002) 5194–5202.
[28] W. Fan, X. Wu, DNA polymerase lambda can elongate on DNA substrates mimicking non-homologous end joining and interact with XRCC4-ligase IV complex, Biochem. Biophys. Res. Commun. 323 (2004) 1328–1333.
[29] P. Manivasakam, R.H. Schiestl, Nonhomologous end joining during restriction enzyme-mediated DNA integration in *Saccharomyces cerevisiae*, Mol. Cell. Biol. 18 (1998) 1736–1745.
[30] R.D. Gietz, A. Sugino, New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites, Gene 74 (1988) 527–534.
[31] D. Gietz, A.St. Jean, R.A. Woods, R.H. Schiestl, Improved method for high efficiency transformation of intact yeast cells, Nucleic Acids Res. 20 (1992) 1425.
[32] G.D.E. Adams, A. Kaiser, C.A.T. Stearns, Methods in Yeast Genetics, 1997 Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1997.
[33] N.P. Sharova, E.B. Abramova, S.B. Dmitrieva, D.D. Dimitrova, V.S. Mikhailov, Preferential interaction of loach DNA polymerase delta with DNA duplexes containing single-stranded gaps, FEBS Lett. 486 (2000) 14–18.
[34] J.S. King, C.F. Fairley, W.F. Morgan, DNA end joining by the Klenow fragment of DNA polymerase I, J. Biol. Chem. 271 (1996) 20450–20457.
[35] N.C. Brissett, R.S. Pitcher, R. Juarez, A.J. Picher, A.J. Green, T.R. Dafforn, G.C. Fox, L. Blanco, A.J. Doherty, Structure of a NHEJ polymerase-mediated DNA synaptic complex, Science 318 (2007) 456–459.
[36] J. Budman, G. Chu, Processing of DNA for nonhomologous end-joining by cell-free extract, EMBO J. 24 (2005) 849–860.
[37] S.J. Boulton, S.P. Jackson, Components of the Ku-dependent non-homologous end-joining pathway are involved in telomeric length maintenance and telomeric silencing, EMBO J. 17 (1998) 1819–1828.
[38] D. Pang, S. Yoo, W.S. Dynan, M. Jung, A. Dritschilo, Ku proteins join DNA fragments as shown by atomic force microscopy, Cancer Res. 57 (1997) 1412–1415.
[39] A.J. Doherty, S.P. Jackson, DNA repair: how Ku makes ends meet, Curr. Biol. 11 (2001) R920–924.
[40] R. Jessberger, P. Schar, P. Robins, E. Ferrari, B. Riwar, U. Hubscher, Regulation of DNA metabolic enzymes upon induction of preB cell development and V(D)J recombination: up-regulation of DNA polymerase delta, Nucleic Acids Res. 25 (1997) 289–296.
[41] P.A. Jeggo, G.E. Taccioli, S.P. Jackson, Menage a trois: double strand break repair, V(D)J recombination and DNA-PK, Bioessays 17 (1995) 949–957.