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Brief Communication

Ribonucleotide incorporation by yeast DNA polymerase ζ

Alena V. Makarova[a], Stephanie A. Nick McElhinny[b], Brian E. Watts[b], Thomas A. Kunkel[b], Peter M. Burgers[a,*]

[a] Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO 63110, USA
[b] Laboratory of Molecular Genetics and Laboratory of Structural Biology, National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services, Research Triangle Park, NC 27709, USA

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ABSTRACT

During replication in yeast, the three B family DNA replicases frequently incorporate ribonucleotides (rNMPs) into DNA, and their presence in the nuclear genome can affect genome stability. This prompted us to examine ribonucleotide incorporation by the fourth B family member, Pol ζ, the enzyme responsible for the majority of damage-induced mutagenesis in eukaryotes. We first show that Pol ζ inserts rNMPs into DNA and can extend primer termini containing 3′-ribonucleotides. We then measure rNMP incorporation by Pol ζ in the presence of its cofactors, RPA, RFC and PCNA and at normal cellular dNTP and rNTP concentrations that exist under unstressed conditions. Under these conditions, Pol ζ stably incorporates one rNMP for every 200–300 dNMPs incorporated, a frequency that is slightly higher than for the high fidelity replicative DNA polymerases. Under damage-induced conditions wherein cellular dNTP concentrations are elevated 5-fold, Pol ζ only incorporates one rNMP per 1300 dNMPs. Functional interaction of Pol ζ with the mutasome assembly factor Rev1 gives comparable rNMP incorporation frequencies. These results suggest that ribonucleotide incorporation into DNA during Pol ζ-mediated mutagenesis *in vivo* may be rare.

1. Introduction

Due to the strict geometry of their active sites and their intrinsic 3′-5′-proofreading activities, replicative DNA polymerases (replicases) rarely misincorporate incorrect deoxynucleotides into DNA. While replicative polymerases also efficiently discriminate against insertion of rNTPs into DNA (reviewed in [1]), rNTPs are present in cellular nucleotide pools at much higher concentrations than dNTPs [2,3], thereby significantly increasing the probability of ribonucleotide incorporation during DNA replication. Moreover, unlike base–base mismatches, newly inserted ribonucleotides (rNMPs) are poorly proofread by DNA polymerases (Pols) δ and ε [4,5]. As a consequence, rNMPs are incorporated during DNA synthesis *in vitro*, as well as during DNA replication *in vivo*, with consequences that can be both beneficial, when used as a signal for mismatch repair, but also deleterious when not properly repaired [6–8].

In studies using physiological rNTP and dNTP concentrations, the frequency of rNMP incorporation by replicative polymerases *in vitro* varies depending on the DNA polymerase, the identity of the ribonucleotide and the local DNA sequence context, as well as on the assay used. In experiments using oligonucleotide DNA substrates, average rNMP incorporation frequencies by Pol δ, Pol ε and Pol α are 1 in 5000; 1 in 1250 and 1 in 625, respectively, with site-to-site variations of more than 10-fold [3]. In an assay that copies a single-stranded plasmid DNA template that more closely resembles condition under which DNA replication occurs, rNMP incorporation frequencies by Pol δ and Pol ε are 1 in 720 and 1 in 640, respectively. These frequencies are not substantially affected by the presence of the accessory factors, i.e. the single-stranded binding protein RPA, the replication clamp PCNA, and its loader RFC [9]. Differences in average ribonucleotide incorporation frequencies between the two assays may reflect sequence context effects and/or potential hotspots for rNMP incorporation.

Pol ζ is a B-family DNA polymerase that has a major role in translesion DNA synthesis (TLS) in eukaryotes and is responsible for the bulk of mutations induced by DNA damage (reviewed in [10]). About half of the spontaneously arising mutations can be ascribed to the participation of Pol ζ during synthesis at stalled DNA replication forks [11]. Pol ζ may also be involved in the repair of DNA double strand breaks [12,13]. Pol ζ-dependent mutagenesis *in vivo* is consistent with its lack of proofreading activity and its ability to efficiently extend mismatched primer termini. Moreover, yeast

* Corresponding author. Tel.: +1 314 362 3872; fax: +1 314 362 7183l.
E-mail address: burgers@biochem.wustl.edu (P.M. Burgers).

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Table 1
dNTP:rNTP discrimination factors for exonuclease-deficient yeast Pols ζ, α and δ.

Nucleotide	Pol ζ	Pol α [b]	Pol δ-exo⁻ [c]
dA: rA	4700	10,000	31,000
dT: rU	1600	8000	20,000
dG: rG	690	3000	4800
dC: rC	470	3800	3000
BPS Error Rate [a]	130×10^{-4}	9.6×10^{-4}	13×10^{-4}

[a] From [14]. BPS, base pair substitution.
[b] From [3].
[c] Determined as describe in [3], but for exonuclease-deficient Pol δ.

Pol ζ generates single base–base mismatches during DNA synthesis *in vitro* at rates that are about ten-fold higher than for naturally proofreading-deficient Pol α or proofreading-deficient variants of Pol δ and Pol ε (Table 1) [14]. This fact indicates that Pol ζ discrimination against insertion of incorrect bases is lower than its B-family siblings. We therefore reasoned that it may also discriminate poorly against rNMPs, and thereby contribute to incorporation in cells, particularly in response to DNA damage when Pol ζ is actively engaged. Indeed, TLS in *Escherichia coli* is accompanied by frequent ribonucleotide incorporation, and if the dNMP/rNMP discrimination capacity of the machinery is reduced, genome stability is negatively affected [15].

Here we have measured the ability of Pol ζ to misincorporate rNMPs into DNA using different types of assay and replication conditions. Surprisingly, our data indicate that Pol ζ discrimination against ribonucleotide incorporation is only slightly lower than for the replicative DNA polymerases. Under TLS conditions, rNMP incorporation is even lower than that occurring during normal DNA replication. Given that Pol ζ likely performs much less DNA synthesis in cells than do the replicases, these results suggest that ribonucleotide incorporation into DNA by Pol ζ may be rare *in vivo*.

2. Materials and methods

2.1. Enzymes

RPA, RFC and PCNA were expressed and purified from *E. coli* [16,17]. The two- and four-subunit forms of *Saccharomyces cerevisiae* Pol ζ were purified as described [18]. Rev1 and Rev1 mutants were produced in *S. cerevisiae* and purified from 50 to 100 g of yeast cells, using a modification of protocols described for Pol ζ [18]. Briefly, proteins from the cell lysate were precipitated with 0.3 g/ml ammonium sulfate and purified by glutathione affinity chromatography, followed by GST-tag removal with prescision protease. After additional heparin-sepharose purification, Rev1, and its mutant forms, were dialyzed overnight against storage buffer: 30 mM Hepes (pH 7.4), 200 mM NaCl, 16% glycerol, 0.05% ampholytes, 1 mM DTT. All buffers except for the storage buffer were supplemented with 1 mM EDTA.

2.2. Measuring discrimination against insertion of an rNTP

The assay was performed as described earlier [3]. Briefly, insertion of dA/rA and dG/rG were analyzed using a substrate made by annealing a ^{32}P-labeled primer strand (5′-CTGCAGCTGATGCGC) to a template strand (5′-GTACCCGGGGATCCGTAC(T/C)GCGCATCAGCTGCAG) that either contained a T or a C at the templating position for the incoming nucleotide. Insertion of dC/rC and dT/rU were analyzed using a substrate made by annealing a 5′-^{32}P-labeled primer strand (5′-CTGCAGCTGATGCGA) to a template strand (5′-GTACCCGGGGATCCGTAC(G/A)TCGCATCAGCTGCAG) that contained a G or an A at the templating position for the incoming nucleotide. Assays contained a single dNTP or rNTP at its measured cellular concentration in yeast under normal (unstressed) growth conditions. Reactions were initiated by adding polymerase, and incubation was at 30 °C. In these experiments, we used the two-subunit (Rev3–Rev7) form of yeast Pol ζ. Polymerase concentrations varied from 0.2 to 10 nM and incubation times varied from 1 to 20 min, in all cases resulting in extension of less than 20% of the initial primer. Reactions were analyzed on a denaturing 15% polyacrylamide gel. Products were detected and quantified using a PhosphorImager and ImageQuaNT software.

2.3. Analysis of rNMP incorporation in primed plasmid DNA

rNMP incorporation during DNA synthesis was performed by a protocol described previously with modifications [9]. The 3 kb single-stranded pSKII plasmid DNA was annealed to primer 682 (5′-TATCGATAAGCTTGATATCGAATTCCT) and used as DNA template for rNMP incorporation assays. Where indicated, the 682 primer was 5′-labeled with [γ-^{32}P]ATP. [α-^{32}P] dATP was added to the reactions as radioactive tracer if non-labeled primer was used. Standard reactions contained 40 mM Tris–HCl, pH 7.8, 0.2 mg/ml BSA, 3% glycerol, 80 mM NaCl, 4 nM primed pSKII DNA, 400 nM RPA, 3 nM RFC, 30 nM PCNA, 10 nM of wild type Rev1 or the indicated mutant form of Rev1, and Pol ζ as indicated in legends to figures. All pSKII replication assays contained the 4-subunit form of Pol ζ. Reactions with only dNTPs contain 8 mM Mg-acetate and 50 μM ATP, to ensure efficient PCNA loading by RFC. Reactions with dNTPs and rNTPs contain 13 mM Mg-acetate. Normal levels of rNTPs and dNTPs were: 16 μM dATP, 14 μM dCTP, 12 μM dGTP, 30 μM dTTP, 3000 μM ATP, 500 μM CTP, 700 μM GTP, and 1700 μM UTP [3]. Under TLS conditions, the dNTP concentrations were increased five-fold [2]. Reactions were assembled on ice and preheated at 30 °C for 30 s. The assay was started by adding Pol ζ alone, or an equimolar mixture of Pol ζ and Rev1 (or a mutant form of Rev1) that had been preincubated on ice for 10 min. Reactions were incubated at 30 °C for 15–120 min, stopped with 0.5% SDS and 20 mM EDTA and heated at 55 °C for 10 min. Where indicated, the reaction was treated with 0.3 M NaOH for 2 h at 65 °C in order to hydrolyze the replicated DNA at rNMP positions, and precipitated with ethanol as described [9]. The samples were analyzed by electrophoresis on a 1–3% alkaline denaturating agarose gels at 1.1 V/cm, at 4 °C for 20 h. Under these conditions, denaturation of dsDNA was complete, but hydrolysis at ribonucleotide positions was negligible. Dried gels were quantified using a Typhoon phosphoimager and ImageJ software. Radioactivity distributions as a function of DNA length were determined. In order to convert the radioactivity distribution into product length distribution, the radioactivity distribution was divided by the length distribution in kb. From this normalized distribution, the median length of alkali-stable DNA was calculated. From this, we calculated the incorporation frequency = $(L^-/L^+ - 1)/L^-$, where L^- and L^+ are median product lengths before and after NaOH treatment, respectively. Alternatively, acid-precipitable radioactivity from incorporated [^{32}P]-dAMP was detected by scintillation counting [19].

2.4. Extension of ribonucleotide-containing primer termini

Standard assays were as described above, except that the SKII 682 primer was 5′-Cy3-labelled 5′-TATCGATAAGCTTGATATCGAATTCCTX-3′ with X either dG or rG, or 5′-TATCGATAAGCTTGATATCGAATTCCX-3′ with X either dT or rU. Reactions were incubated at 30 °C for the indicated times and analyzed on a 14% polyacrylamide-7M urea gel. Quantification was performed using fluorescence imaging on a Typhoon system.

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3. Results

3.1. dNTP:rNTP discrimination by Pol ζ

Stable incorporation of a ribonucleotide into DNA first requires insertion of a rNTP opposite a DNA template base. We began this study by determining discrimination against rNTP insertion opposite a single template base in an oligonucleotide, in primer extension reactions containing only one correct dNTP or rNTP present at the concentrations estimated to be present in unstressed yeast cells, which here we define as the normal concentration [3]. As previously described [3], discrimination factors were determined by quantifying the band intensities of extension products, dividing the percentage of dNMP product by the percentage of rNMP product, and then multiplying by the ratios of nucleotide concentrations and by the differences in enzyme concentrations and reaction times used to extend no more than 20% of the starting substrate. The results (Table 1) indicate that Pol ζ strongly prefers to incorporate dNMPs rather than rNMPs, by factors ranging from 470 to 4700, with discrimination against rATP and rUTP being higher than against rCTP and rGTP. The selectivity of Pol ζ against rNMP insertion is 5- to 10-fold lower than that of Pol α or exonuclease-deficient Pol δ. This fact correlates with a similar difference in base substitution error rates that depend on misinsertion of incorrect dNTPs (Table 1, from [14]).

3.2. Stable incorporation of ribonucleotides by Pol ζ

Next we measured the frequency of rNMP incorporation by Pol ζ during copying of a circular three kb single-stranded DNA template. The 4-subunit form of Pol ζ was used in these assays, because only this form shows the functional interactions with the replication clamp PCNA that are required for processive replication of this long template. PCNA was loaded by RFC onto the primed template, which was coated with the single-stranded DNA binding protein RPA. Synthesis was initiated by adding Pol ζ (Fig. 1A). One set of experiments used concentrations of dNTPs and rNTPs estimated to be present in yeast cells under normal physiological conditions (normal, Fig. 1B). Replication products were treated with alkali to hydrolyze the DNA backbone at rNMP positions, and the products were separated on a denaturing agarose gel. The reaction products generated by Pol ζ were sensitive to alkali, but only if rNTPs were present, indicating that Pol ζ stably incorporated rNMPs into DNA (Fig. 1B). The radioactivity profile was divided by the length distribution, which generated a normalized product length distribution (Fig. 1C). The median length of products synthesized by Pol ζ in the presence of normal dNTPs and rNTPs was 0.68 kb. After treatment with alkali, this median distribution decreased substantially, to 0.21 kb. Therefore, the average rNMP ribonucleotide incorporation frequency by Pol ζ is ~1/300 (Section 2). Among several experiments, this frequency varied from 1/200 to 1/300. When the dNTP concentrations were increased 5-fold (TLS conditions)

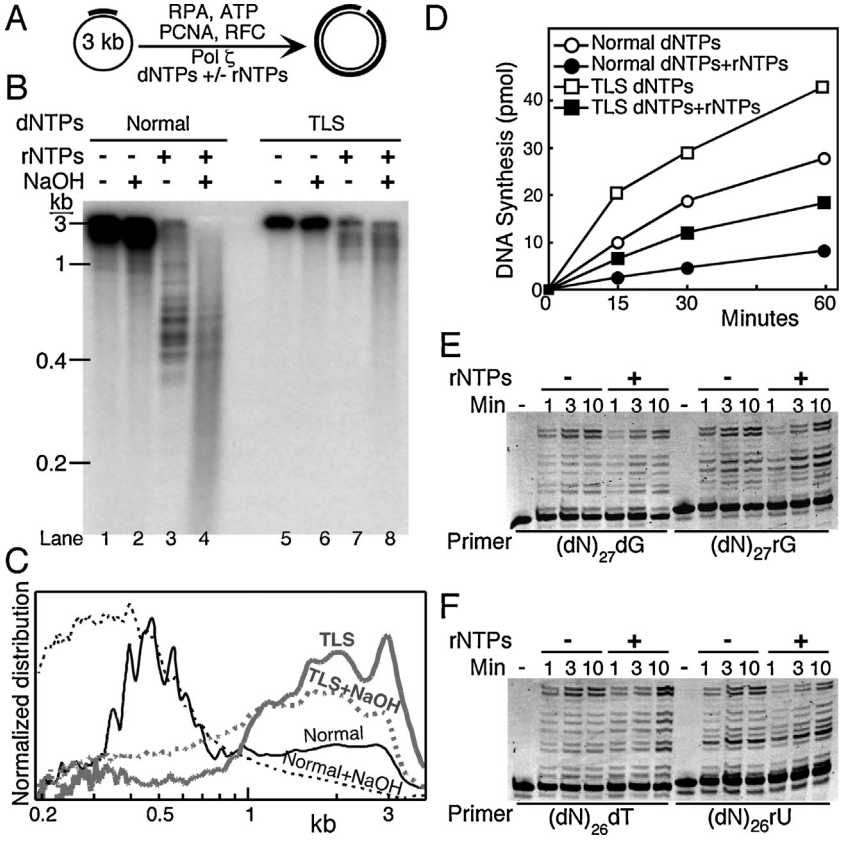

Fig. 1. rNMPs incorporation by Pol ζ during DNA synthesis at physiologically relevant rNTP/dNTP concentrations. (A) Scheme of the DNA replication assay. DNA polymerization was carried out on primed 3 kb ss pSKII plasmid DNA in the presence of RPA, RFC and PCNA with rNTP and dNTP concentrations estimated to be present in yeast under normal physiological conditions ("normal") or under replication stress ("TLS") conditions (Section 2). (B) Pol ζ misincorporates rNMPs into DNA. [α-^{32}P] dATP was the radioactive tracer. Where indicated, replication products were treated with NaOH to cleave at the ribonucleotide positions. Products were separated on a denaturing 3% metaphor agarose gel. (C) Normalized length of replication products before and after alkali treatment. (D) The presence of rNTPs inhibits Pol ζ replication activity. Acid-precipitable radioactivity was determined by scintillation counting (Section 2). (E and F) Efficient extension of ribonucleotide primer termini by Pol ζ. DNA replication was carried out at normal dNTPs with or without rNTPs. The 3 kb ss pSKII plasmid DNA was primed with a 5′-Cy3-labeled primer containing either dT, rU, dG or rG at the indicated positions. Replication products were separated on 14% urea-PAGE.

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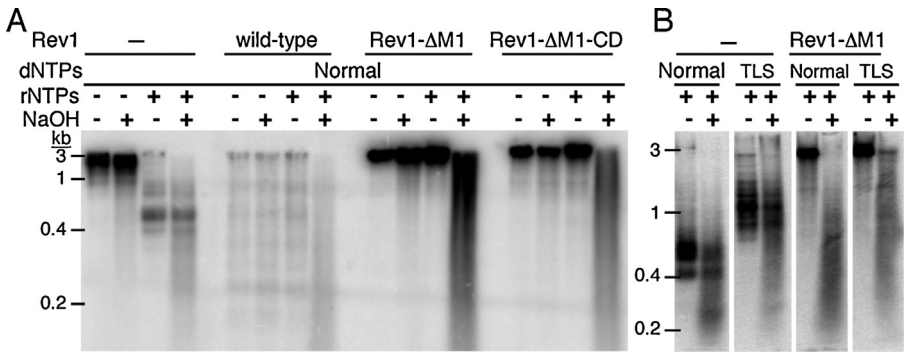

Fig. 2. Misincorporation of rNMPs by the Pol ζ-Rev1 complex. (A) Replication reactions were as described in Section 2 and the legend to Fig. 1. Three forms of Rev1 were used in these experiments: wild type Rev1 (Rev1), an activated form of Rev1 (Rev1-ΔM1), and the catalytically inactive form of Rev1-ΔM1 (Rev1-ΔM1-cd). [α-^{32}P] dATP was the radioactive tracer. Non-treated and alkali-treated replication products were separated on a denaturing 3% metaphor agarose gel. (B) DNA replication was carried out on ss pSKII DNA primed with a 5′-[^{32}P]-labeled primer. In these assays, the concentration of plasmid DNA was increased to 10 nM and that of RPA to 600 nM. Products were separated on a denaturing 2% metaphor agarose gel.

to mimic cellular dNTP concentrations induced by DNA damage [2,3], the median lengths before and after alkali treatment were 1.4 and 0.67 kb, respectively, which calculates to a rNMP incorporation frequency of about 1/1300 (Fig. 1B, right panel).

3.2.1. *rNTPs inhibit polymerization by Pol ζ*

These experiments further revealed that the presence of rNTPs significantly inhibited the DNA polymerase activity of yeast Pol ζ (Fig. 1B, compare lane 1 with lane 3 and lane 5 with lane 7). Pol ζ activity was decreased ~3–4-fold when dNTPs were present at normal physiological concentrations and it was decreased ~2.5–3-fold under damage-induced (TLS) conditions (Fig. 1D). This inhibition of activity may either be due to competition by rNTPs for binding into the polymerase active site, or to the inability of Pol ζ to efficiently extend the ribonucleotide-containing primer produced upon rNMP incorporation. We tested the latter possibility for two different primer termini, and found that Pol ζ extended primer termini containing 3′-ribonucleotides with almost the same efficiency as the analogous deoxy termini (Fig. 1E and F). Thus, inhibition of Pol ζ activity is likely caused by direct competition between rNTPs and dNTPs for binding into the active site of Pol ζ. This type of inhibition has also been observed for other DNA polymerases, such as Pol δ, and *E. coli* DNA polymerase III holoenzyme [9,20].

3.3. *Ribonucleotide incorporation by Pol ζ in a complex with activated Rev1*

In addition to Pol ζ, Rev1 is absolutely required for mutagenesis in eukaryotes [10]. Rev1 is a deoxycytidyl transferase that forms a complex with Pol ζ [21,22]. Rev1 shows fairly low sugar discrimination, compared to the B-family DNA polymerases [23]. We have recently identified a mutant form of Rev1, designated Rev1-ΔM1 (Rev1-(Δ135–150)), that activates translesion synthesis and processive replication by Pol ζ by several fold (Makarova et al., manuscript in preparation). We hypothesize that this Rev1 mutant, which deletes the highly conserved M1 motif in Rev1, phenocopies a required posttranslational modification of Rev1, which activates the Rev1-Pol ζ complex for TLS. Furthermore, the catalytic activity of Rev1 is dispensable for this *in vitro* activation of TLS, and it is also dispensable for damage-induced mutagenesis in response to most forms of DNA damage in yeast [24,25].

We investigated whether the presence of Rev1 or its activated form, i.e. Rev1-ΔM1, affected rNMP incorporation by Pol ζ. These studies were carried out at normal cellular dNTP and rNTP concentrations. Under these conditions (Fig. 2A), replication of the three kb DNA by Pol ζ alone did not proceed to completion. After alkali treatment, the major normalized product distribution ranged from 150 to 300 nucleotides, consistent with an incorporation frequency of 1/200–1/300. Addition of wild-type Rev1 did not stimulate Pol ζ activity; in fact it caused inhibition. However, addition of the activated mutant form Rev1-ΔM1 stimulated Pol ζ, and complete replication was observed. The median size lengths of products before and after alkali treatment were 1.6 kb and 0.29 kb, respectively, which calculates to a rNMP incorporation frequency of 1/350. The same size distributions were observed with the catalytically inactive form of Rev1-ΔM1, indicating that the catalytic activity of Rev1 does not contribute significantly to rNMP incorporation during TLS.

Finally, we repeated these studies with a primed plasmid template, but with the primer 5′-labeled (Fig. 2B). A hotspot for rNMP incorporation on the three kb template would be detected as the alkali-dependent appearance of a unique band, whereas uniform and stochastic incorporation of rNMPs would result in a continuous distribution of products after treatment with alkali. The latter was indeed observed. In addition, incorporation frequencies deduced from the product length distribution were comparable to those calculated from the studies using internal labeling of replication products.

4. Conclusions

- Pol ζ is a low fidelity B-family DNA polymerase that is required for TLS in response to DNA damage. We measured rNMP incorporation at normal physiological levels of dNTPs and rNTPs that prevail when the cell is not subjected to DNA damaging agents. Under those conditions, our rNMP insertion studies with oligonucleotides show that the rNMP insertion by Pol ζ is 5–10 fold higher for the yeast replicative DNA polymerases (Table 1). When the stable incorporation frequency was measured on a three kb plasmid that samples many sequence contexts, rNMP incorporation frequencies were 1/200–1/300, which is 2–4-fold higher than that of replicative DNA polymerases.
- Under conditions of DNA damage, cellular dNTP levels are increased ~5 fold. Accordingly, with increased dNTP levels, we measured a decreased incorporation frequency by Pol ζ, about 1/1300.
- Mutagenic replication *in vivo* is performed by an activated complex of Rev1 and Pol ζ. This complex showed a rNMP incorporation frequency of 1/350, compared to 1/200–1/300 for Pol ζ alone. The distributions of replication products obtained after alkaline digestion that reveals incorporated rNMPs is broad, precluding any conclusion as to whether the frequencies with Pol ζ

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alone or with the activated Rev1-Pol ζ mutasome are significantly different. The catalytic activity of Rev1 does not contribute significantly to rNMP incorporation.
- Taking into consideration that Pol ζ is a highly specialized DNA polymerase with limited and tightly controlled access to replication forks in cells, we believe that Pol ζ-mediated TLS is unlikely to make a significant contribution to rNTP incorporation *in vivo*.

Conflict of interest

The authors declare that they have no conflict of interest with this work.

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