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Increased contribution of DNA polymerase delta to the leading strand replication in yeast with an impaired CMG helicase complex

Michal Dmowski [a,*], Malgorzata Jedrychowska [a], Karolina Makiela-Dzbenska [a], Milena Denkiewicz-Kruk [a], Sushma Sharma [b], Andrei Chabes [b], Hiroyuki Araki [c], Iwona J. Fijalkowska [a]

[a] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5A, 02-106 Warsaw, Poland
[b] Department of Medical Biochemistry and Biophysics, Umeå University, SE-901 87 Umeå, Sweden
[c] National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan

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ABSTRACT

DNA replication is performed by replisome proteins, which are highly conserved from yeast to humans. The CMG [Cdc45-Mcm2–7-GINS(Psf1–3, Sld5)] helicase unwinds the double helix to separate the leading and lagging DNA strands, which are replicated by the specialized DNA polymerases epsilon (Pol ε) and delta (Pol δ), respectively. This division of labor was confirmed by both genetic analyses and in vitro studies. Exceptions from this rule were described mainly in cells with impaired catalytic polymerase ε subunit. The central role in the recruitment and establishment of Pol ε on the leading strand is played by the CMG complex assembled on DNA during replication initiation. In this work we analyzed the consequences of impaired functioning of the CMG complex for the division labor between DNA polymerases on the two replicating strands. We showed in vitro that the $GINS_{Psf1-1}$ complex poorly bound the Psf3 subunit. *In vivo*, we observed increased rates of L612M Pol δ-specific mutations during replication of the leading DNA strand in *psf1–1* cells. These findings indicated that defective functioning of GINS impaired leading strand replication by Pol ε and necessitated involvement of Pol δ in the synthesis on this strand with a possible impact on the distribution of mutations and genomic stability. These are the first results to imply that the division of labor between the two main replicases can be severely influenced by a defective nonpolymerase subunit of the replisome.

1. Introduction

Proper functioning of the eukaryotic genome depends on the accurate replication of genetic material, which relies on the coordinated synthesis of the leading and lagging DNA strands by replicative DNA polymerases in concert with other proteins. The temporal and physical interactions within this multiprotein complex, called the replisome, are remarkably conserved in eukaryotes. However, functions of some specific replisome components remain poorly defined. Analysis of phenotypes associated with mutations in replisome-encoding genes is important for our understanding of the role of DNA replication in genome instability and the sources of many diseases and disorders [1,2].

The three most important processes that determine a high fidelity of replication are (i) correct base selection by DNA polymerases, (ii) removal of the misinserted nucleotides by the 3′→5′ exonuclease proofreading activity of DNA polymerases, and (iii) the DNA mismatch repair system (MMR) responsible for postreplicative correction of polymerase errors [3–5]. The first two of these processes are related to the activity of replicative DNA polymerases, which are central to the high accuracy of replication. However, the contribution of other factors, e.g., noncatalytic proteins of the replisome, which may influence the fidelity of DNA replication and stability of genetic material, has recently become a specific issue of interest. Among the three major replication DNA polymerases, Pol α (alpha) is responsible for the synthesis of primers for replication[6]. It is generally accepted that Pol ε (epsilon) operates on the leading strand, while Pol δ (delta) is the major polymerase on the lagging strand [7]. Pol ε is composed of a catalytic Pol2 subunit and three auxiliary subunits, Dpb2, Dpb3 and Dpb4 [8]. Yeast Pol δ consists of three subunits: catalytic Pol3 and two noncatalytic Pol31 and Pol32 [9]. These two polymerases possess 3′→5′ proofreading exonuclease

* Corresponding author.
E-mail address: mdmowski@ibb.waw.pl (M. Dmowski).

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activity, which contributes to high-fidelity DNA synthesis. Apart from the main replicases, there are a number of other specialized DNA polymerases in eukaryotic cells. A polymerase that has a significant impact on the level of spontaneous mutagenesis is Pol ζ (zeta) [10–13]. This polymerase has no 3′→5′ proofreading exonuclease activity and, compared with the main replicative polymerases, generates more errors during DNA synthesis [14,15]. Pol ζ is composed of a catalytic subunit Rev3 and three accessory subunits – Rev7, Pol31 and Pol32, two of which (Pol31 and Pol32) are shared with Pol δ [16–18]. Pol ζ belongs to translesion synthesis polymerases (TLS); however, it also participates in the process of DNA synthesis when replicative polymerases or accessory subunits of the replisome are impaired, causing defective replisome-induced mutagenesis (DRIM) [11,19–25].

During DNA replication, highly efficient unwinding of double-stranded DNA is catalyzed by the CMG helicase (Cdc45 - Mcm2–7 - GINS) complex, which translocates along the leading strand [26]. The CMG complex is a macromolecular assembly of 11 replication factors: the Cdc45 protein, four subunits (Psf1, Psf2, Psf3 and Sld5) of the GINS complex and the heterohexameric Mcm2–7 complex, which is the helicase motor [27–31]. Additionally, CMG serves as a platform for coordinating the work of different components of the replisome [32], including Pol ε. Interactions of the CMG complex with Pol ε (together forming the CMGE complex) are both structurally and functionally important [31,33–39]. Pol ε modulates the activity of the CMG helicase when the helicase encounters barriers affecting the efficiency of the replication process, and CMG exerts a stimulating activity on Pol ε [40].

The contribution of Pol ε and Pol δ to the synthesis of a specific DNA strand has been confirmed by whole-genome mapping of errors introduced by a Pol δ variant in *S. cerevisiae* [41] or through the use of reporter genes and variants of yeast Pols α, δ and ε, Y869A Pol α, L612M Pol δ and M644G Pol ε, respectively [42–44]. Based on in vitro data, it was shown that the high affinity of Pol δ to the PCNA clamp is responsible for the preferential loading of this polymerase to the lagging strand. Participation of DNA Pol ε in leading strand replication relies on the CMG complex, which selectively recruits Pol ε to the leading strand and prevents the binding of Pol δ. Structural and biochemical data have confirmed that Pol ε interacts with CMG subunits and the interaction between the Psf1 subunit of GINS and the Dpb2 subunit of Pol ε [11,24,45].

If proper functioning of the CMG complex is important for targeting Pol ε to the leading strand, mutations in genes encoding its components may influence the participation of particular polymerases in leading or lagging strand replication. To investigate this hypothesis, we employed the *psf1–1* allele encoding the mutant version of *PSF1* with R84G substitution in the N-terminal part of the protein [46]. The presence of the *psf1–1* allele increases the level of spontaneous mutagenesis and shows a number of phenotypes indicative of impairment in the replication process [11,47]. Here, we used the yeast genetic system, in which the reporter gene *URA3* is inserted asymmetrically between two chromosomal origins of replication [48]. The distinct mutational signature of the L612M Pol δ variant allows the detection of Pol δ participation in the replication of either DNA strand in yeast cells [49].

Our results provide the first example of a mutation in GINS, a non-catalytic element of the replisome, which can affect the division of labor between the two main replicases, causing increased participation of Pol δ in leading strand replication. Our results also confirm in vivo the validity of the hypothesis proposed on the basis of in vitro results [35,36], assuming that proper functioning of the CMG complex is responsible for targeting Pol ε to the leading strand. These findings support the notion that changes in DNA polymerase usage may help to overcome replication machinery deficiencies. In light of the many reports identifying diseases associated with defects in this complex [1,2] analysis of phenotypes associated with defects in the GINS complex is particularly important. Identification of mechanisms causing dysfunctional DNA replication will allow the application of targeted therapies.

2. Results

2.1. Analysis of GINS complex formation with the Psf1-1 subunit

Our previous study showed an impaired interaction of Psf1–1 with the Psf3 subunit in the yeast two-hybrid system [11]. However, given that GINS is composed of four subunits interacting with each-other, the impact of Psf1–1 on the assembly of the complex requires verification. Therefore, we purified the GINS complex from yeast cells producing Psf1–1 and, in parallel, from cells producing wild-type Psf1 as a control. Flag-tagged variants of the Psf1 subunit were immunoprecipitated, and copurified GINS subunits were analyzed. In the control experiment with Psf1, the three other subunits, i.e., Psf2, Psf3 and Sld5, were detected (Fig. 1). However, when the Psf1–1 variant was used, Psf2 and Sld5 were observed while Psf3 was barely detectable (Fig. 1). Additionally, as a control, we analyzed another variant of the Psf1 subunit encoded by the *psf1–100* allele. The Psf1–100 subunit was modified in four highly conserved amino acids (V161A, F162A, I163A and D164A) in the B domain - the C-terminal region. In contrast to *psf1–1*, mutations in *psf1–100* had no effect on GINS complex formation – Psf2, Psf3 and Sld5 copurified with Psf1–100 (Fig. 1). This finding demonstrated that in the presence of Psf1–1 the Psf3 subunit of GINS poorly binds within the complex. It is worth mentioning that a knock-down of the *PSF3* gene in human colon carcinoma cells delayed their progression through the S-phase and inhibited their growth [50]. Therefore, defective association of Psf3 within the GINS complex in *psf1–1* cells can explain impaired cell growth and cell cycle progression as well as temperature sensitivity reported previously [46].

2.2. The genetic system for analysis of leading/lagging strand replication

Given that the CMG complex is involved in the specific recruitment of Pol ε through its interaction with GINS, our goal was to investigate whether GINS impairment due to defective complex formation could result in an increased contribution of Pol δ to leading strand replication. To achieve this goal, we analyzed the mutational signatures of L612M Pol δ alone and in combination with *psf1–1*. L612M Pol δ most

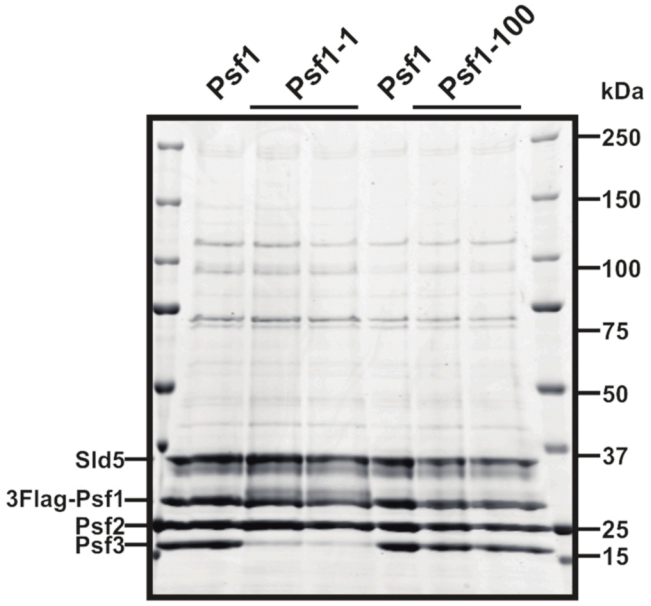

Fig. 1. Formation of the GINS complex with Psf1–1 and Psf1–100. Genes encoding the four subunits of the GINS complex were simultaneously expressed from galactose-regulated promoters. 3xFlag-tagged Psf1, Psf1–1 or Psf1–100 were immunoprecipitated with anti-Flag M2 beads. Copurified proteins were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue staining.

commonly introduces AT to GC transitions with a preference for T•dG mispairs [51] and CG to TA transition through G•dT mispairs [52] (see Fig. 2 A for details). Another class of Pol δ-specific mutations consists of GC to TA transversions formed mainly by C•dT rather than G•dA mispairs [52,53] (Fig. 2 A). We analyzed the specificity of substitutions using the yeast genetic system with the *URA3* reporter gene inserted asymmetrically between two origins of replication, ~2 kb from ARS306 and ~32 kb from ARS307, in two orientations [49]. In one orientation, the reporter gene coding sequence was replicated as the lagging strand (*URA3* orientation OR1) and in the other as the leading strand (*URA3* orientation OR2), starting from ARS306 (Fig. 2 A). To compare the specificity of base substitutions in the *URA3* coding sequence, we isolated 5-FOA-resistant mutants and sequenced the reporter gene. Such an analysis of mutagenesis spectra provides information on the frequency and specificity of mutations resulting from mispairs introduced into either DNA strand (Fig. 2 A and B).

Previous studies have shown that Pol ζ contributes to spontaneous mutagenesis in *psf1–1* cells and that errors arising in this mutant are replication errors corrected by the mismatch repair (MMR) mechanism [11]. Thus, to exclude Pol ζ-generated errors from our analysis and better visualize the replication errors, we deleted the *REV3* and *MSH6* genes in all strains used for the analysis.

A general comparison of the contribution of specific mutation classes to total mutagenesis in the six strains analyzed in this work revealed significant changes caused by the *psf1–1* allele, especially when combined with the *pol3-L612M* mutation (summarized in Fig. 3 and Table S3). In the *pol3-L612M* strain, within the reporter gene *URA3*-OR1 (where the coding sequence is replicated as the lagging strand), we observed mainly T to C and G to A substitutions (resulting from T•dG and G•dT mispairs on the lagging strand), which together accounted for over 90% of the changes, while C to T and G to T represented only 2% of the events. In *pol3-L612M URA3*-OR2 (where the coding sequence is replicated as the leading strand), T to C and G to A accounted for only 5% while over 75% of changes were C to T and G to T substitutions (resulting from G•dT and C•dT mispairs on the lagging strand, respectively). These specificities are consistent with previous studies [49,54,55], which are discussed in more detail below. In isogenic strains with the *psf1–1* allele, the contribution of C to T and G to T changes to mutagenesis in *URA3*-OR1 increased to 10%, while the T to C and G to A fraction in *URA3*-OR2 increased to over 16% ($p = 0.0463$, and 0.0396, respectively) (Fig. 3).

2.3. L612M Pol δ -specific mispairs resulting in AT to GC and CG to TA substitutions on both DNA strands in psf1-1 cells

In the *pol3-L612M* strain with the *URA3* coding sequence replicated as the lagging strand (OR1), the T to C over the A to G ratio exceeded 53, which was significantly higher than that in *POL3* cells (ratio of 12, for a detailed analysis of *POL3* strains see supplementary results) (Fig. 4A and C, upper panel; $p = 0.0007$, Table S4). This result is consistent with the preference observed in vitro for the L612M variant of Pol δ [51]. In parallel, we observed a 107-fold higher preference for G to A over C to T changes in the *pol3-L612M* strain (Fig. 4 A and C, upper panel), which was significantly higher than the 5-fold preference in *POL3* cells ($p < 0.0001$, Table S4). The T to C over A to G and G to A over C to T preferences were inversed when the *URA3* gene was cloned in the other orientation (OR2) in the *pol3-L612M* strain, with 0.1 ratios in both cases (Fig. 4C, lower panel). Importantly, these differences were significantly more pronounced than those in the *POL3* strain with *URA3* in the same orientation (ratio of 0.8 and 0.4, respectively) ($p = 0.0007$, and <0.0001, respectively, Table S4). Together, these observations demonstrated that in our experimental system, Pol δ participated mainly in lagging strand replication.

Next, to answer the question of whether a defect in CMGE led to increased participation of Pol δ in leading strand replication, we analyzed the ratios of same base substitutions in the *pol3-L612M psf1–1* mutant with the *URA3* coding sequence replicated as the leading strand (OR2) and compared them with ratios obtained in *pol3-L612M* cells. The presence of the *psf1–1* allele caused a significant increase in ratios from 0.1 to 0.8 for T to C versus A to G and from 0.1 to 0.2 for G to A versus C to T (Fig. 4C and D lower panel; $p < 0.0001$, Table S4). A similar tendency was observed in *URA3* OR2 in the *POL3 psf1–1* strain (Fig. 4A and B lower panel; for more details, see supplementary results). The effect of the *psf1–1* allele was also manifested in strains with an OR1 orientation

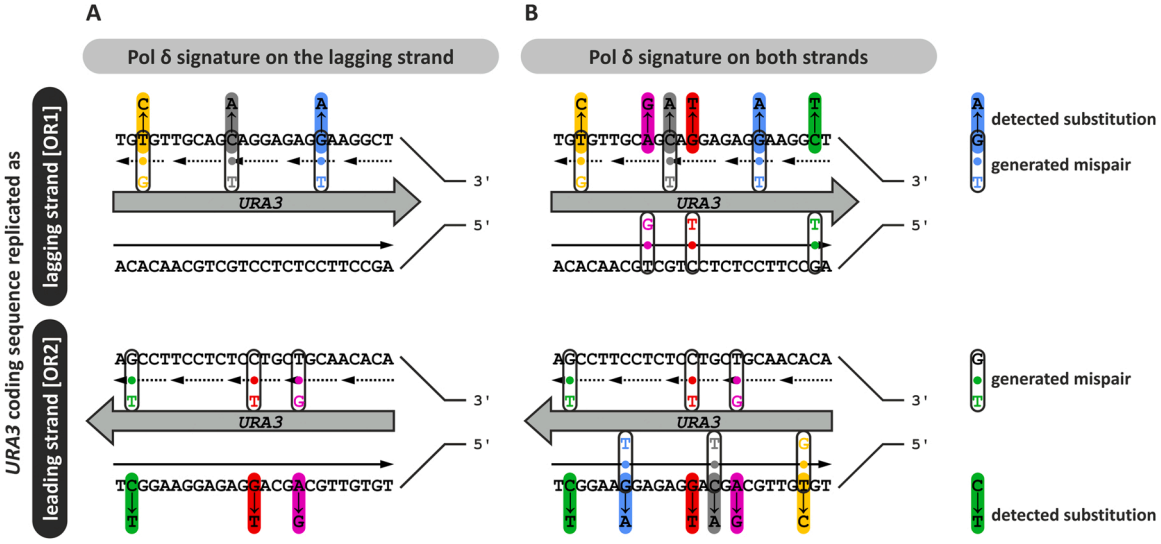

Fig. 2. Rationale for analysis of the Pol δ contribution to leading strand replication in the *psf1–1* mutant. **(A)** The *URA3* reporter gene was cloned in two orientations (OR1 and OR2) in the vicinity of ARS306. Therefore, its coding sequence was replicated as the lagging strand in OR1 and as the leading strand in OR2. As a result, L612M Pol δ–specific T•dG, C•dT and G•dT mispairs generated during lagging strand replication were detected in *URA3* OR1 as T to C, C to A, and G to A substitutions, respectively, because mispairs were generated in the coding sequence. In the *URA3* OR2, the same mispairs were generated in the complementary sequence and, as a result, were detected as A to G, G to T, and C to T substitutions. **(B)** If Pol δ additionally contributed to the replication of the leading strand, L612M Pol δ –specific mispairs would be generated in both strands. Then, in *URA3* OR1, a G•dT mispair generated on the lagging strand would be detected as a G to A substitution, while the mispair generated on the leading strand would be detected as a C to T substitution. Accordingly, in *URA3* OR2, a G•dT mispair generated on the lagging strand would be detected as a C to T substitution, while when generated on the leading strand, it would be detected as a G to A substitution.

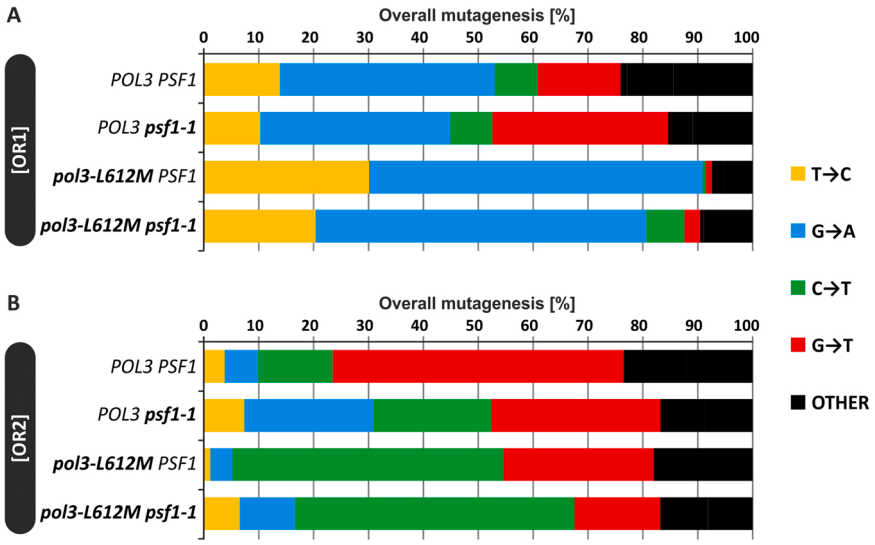

Fig. 3. Contribution of L612M Pol δ-specific types of substitutions to total mutagenesis in strains with *psf1–1* and/or *pol3-L612M* mutations in the *rev3*Δ *msh6*Δ background. The reporter gene *URA3* was cloned in two orienattions [OR1] **(A)**, and [OR2] **(B)**. Mutation spectra were analyzed in the coding sequence of *URA3* from 5-FOA-resistant yeast clones. The proportion of either substitution type found in each spectrum is shown. Detailed data are provided in Table S3.

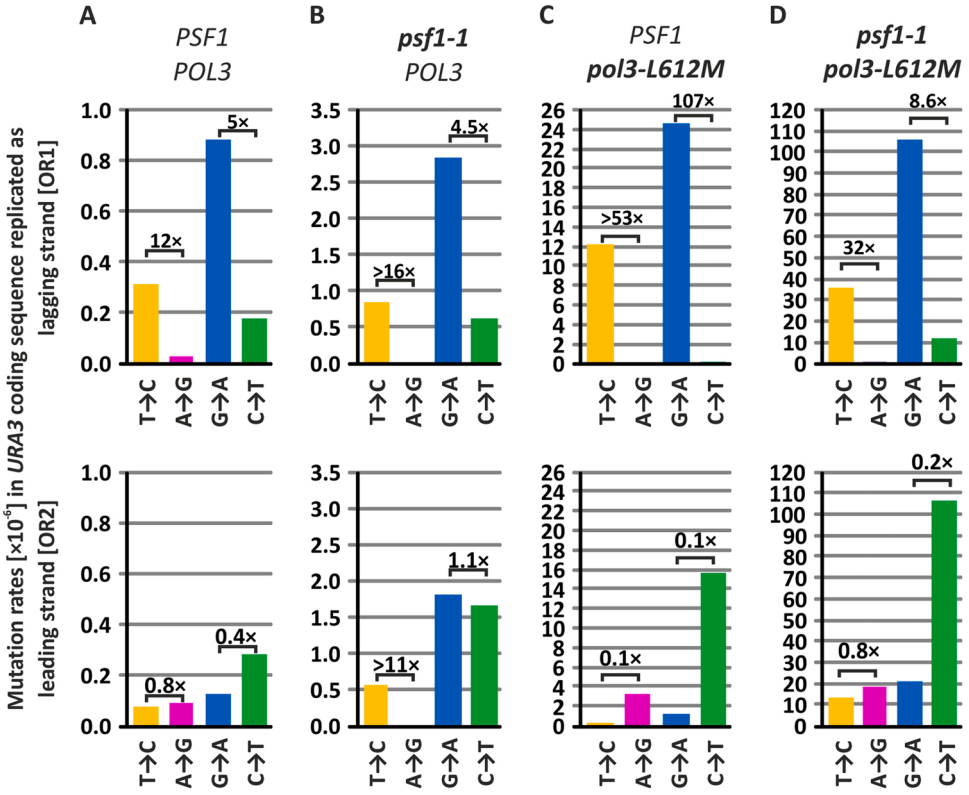

Fig. 4. Total mutation rates calculated for specific substitutions resulting in AT-GC and CG-TA substitutions found in the *URA3* sequence [5-FOA^R× 10^{-6}] in strains with *psf1–1* and/or *pol3-L612M* mutations in the *rev3*Δ *msh6*Δ background. Details of the mutation spectra are shown in Table S3. The statistical analysis is shown in Table S4.

of the reporter gene by reduced L612M Pol δ -specific ratios on the lagging DNA strand. We observed a significant decrease in the T to C versus A to G substitution ratio from > 53 to 32 ($p < 0.0001$) and G to A versus C to T substitution ratio from 107 to 8.6 ($p < 0.0001$) (Fig. 4C and D upper panel, Table S4). Additionally, the Tarone test for G to A versus C to T substitutions in OR1 and OR2 gave $p < 0.0001$. These results, together with the analysis of GC to TA substitutions (see supplementary results and Fig. S1), showed that in *psf1–1* cells, there was an increased occurrence of L612M Pol δ -specific mutations on the leading strand (OR2 results) observed as changed substitution ratios.

2.4. Mutation hotspots in pol3-L612M cells

L612M Pol δ demonstrated increased mutation rates at specific locations of the *URA3* coding strand: T to C at position 97, G to A at 764 within *URA3* in orientation OR1 (coding sequence replicated as lagging

strand), and C to T at position 310 within *URA3* in orientation OR2 (coding sequence replicated as leading strand) [49,54]. As expected from L612M Pol δ mispair specificity, we observed that in *pol3-L612M* cells, the mutation rate for T to C substitutions at position 97 was > 29-fold higher in OR1 (5.51×10^{-6}) than in OR2 ($<0.19 \times 10^{-6}$) (Fig. 5C) ($p < 0.0001$, Table S6). The rate for the same substitution at other sites was also higher (18-fold) in OR1 than in OR2 (6.65×10^{-6} and 0.37×10^{-6}, respectively, $p < 0.0001$, Table S7). We also observed

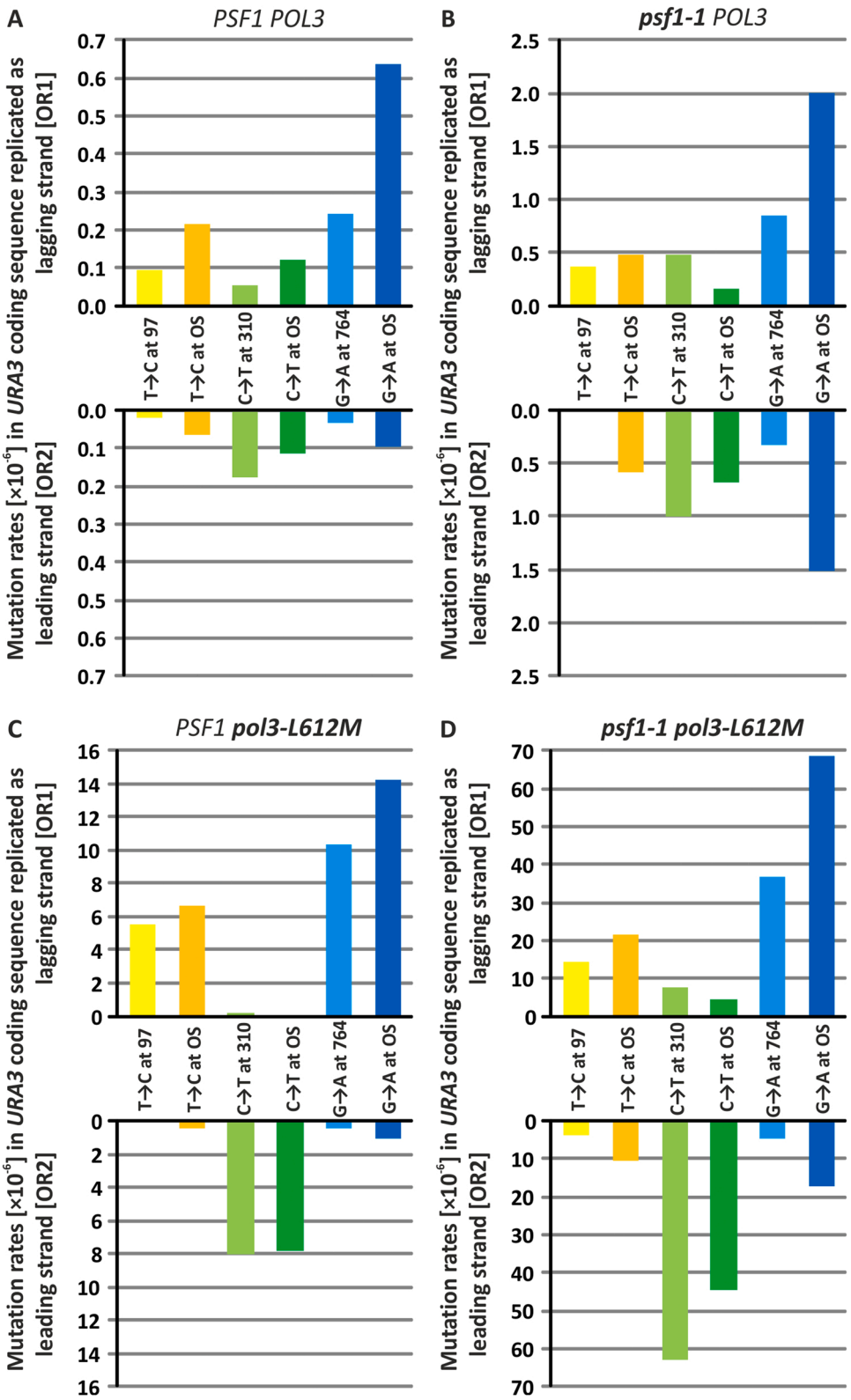

Fig. 5. Mutation rates calculated for specific substitutions at *pol3-L612M*-characteristic hotspots (97, 310 and 764) and other sites (OS) [5-FOA^R $\times 10^{-6}$] in strains with *psf1–1* and/or *pol3-L612M* mutations in the *rev3*Δ *msh6*Δ background. Details of the mutation spectra are shown in Table S3. The statistical analysis is shown in Table S6 and Table S7.

28-fold higher rates of G to A substitutions at position 764 in OR1 (10.32×10^{-6}) than in OR2 (0.37×10^{-6}) (Fig. 5C) ($p < 0.0001$, Table S6). A similar orientation-dependent effect was observed for these substitutions at other sites (14.2×10^{-6} and 0.9×10^{-6} in OR1 and OR2, respectively, $p < 0.0001$, Table S7). As expected, the C to T substitutions at position 310 occurred at a 34-fold higher rate in OR2 than in the OR1 orientation (7.95×10^{-6} and 0.23×10^{-6}, respectively, Fig. 5C, $p < 0.0001$, Table S6). The same ratio (>34) was observed for C to T substitutions at other sites (7.76×10^{-6} and $<0.23 \times 10^{-6}$, $p < 0.0001$, Table S7). These results were consistent with the mutational signature of L612M Pol δ characteristic of the lagging DNA strand.

2.5. Mutation hotspots in pol3-L612M psf1-1 cells

In *pol3-L612M psf1–1* cells compared with *pol3-L612M* cells, we observed changes in hotspots. When the reporter gene was in the OR1 orientation, there was a significant relative decrease in the contribution of T to C and G to A changes (at positions 97 and 764, respectively) to the overall mutation rate (Fig. 5C and D) ($p < 0.0001$, Table S6). In parallel, we observed a significant relative increase in C to T substitutions at position 310 (Fig. 5C and D) ($p < 0.0001$, Table S6). Similar effects were observed for the T to C and C to T substitutions in general at all sites, including hotspots (Fig. 5C and D; $p < 0.0001$, Table S7). In parallel, when we compared *pol3-L612M psf1–1* and *pol3-L612M* strains with the *URA3* reporter gene in OR2, we observed a significant relative increase in T to C changes at position 97 ($p < 0.0001$) and G to A changes at position 764 ($p = 0.0002$), which contributed to the mutagenesis rates (Fig. 5C and D, Table S6). A similar observation applied to the general contribution of T to C and G to A to the mutagenesis spectra (Fig. 5C and D; $p < 0.0001$, Table S7). Additional analysis of G to T hotspots is described in the supplementary results and Fig. S2. Together, these results clearly showed that in *psf1–1* cells the contribution of nucleotide substitutions preferentially arising in the *URA3* sequence located in a given orientation increased in the reporter gene sequence inserted in the inversed orientation. This phenomenon is often accompanied by a relative decrease in mutation rates for specific substitutions dominating the *URA3* sequence in the same orientation in *PSF1* cells. We concluded that the observed changes in mutation specificity were consistent with the increased participation of Pol δ in leading strand replication in *psf1–1* cells.

2.6. dNTP pool in psf1-1 cells

The *psf1–1* mutant, in addition to increased mutation rates (Table S3 and [11]), demonstrated slow progression through S phase, as shown through DNA content profile analysis by flow cytometry (FACS) [46] and (Fig. 6A). The perturbation of DNA replication might result in cellular stress response and modified amounts of nucleotides accessible for DNA synthesis. Therefore, to exclude the possibility that the observed changes in mutation specificity in *psf1–1* cells were caused by imbalanced amounts of the four nucleotides, we examined the dNTP pool. Although the analysis showed a 3.3-fold increase (Fig. 6B), the balance among the four dNTPs was the same as that in the wild-type cells (Fig. 6C). Although a proportional increase of dNTPs might stimulate mutagenesis [56], we assumed that it would have no significant effect on mutation specificity in *psf1–1* cells.

3. Discussion

Studies of DNA polymerase contributions to DNA replication have assigned Pol δ as the lagging strand replicase and Pol ε as the main enzyme involved in leading strand replication [49,57,58] (Fig. 7A). However, recent reports indicate that Pol δ is also involved in leading strand replication. It has been demonstrated that Pol δ participates in both the initiation and termination of leading strand replication [44,59] and that Pol δ replicates both DNA strands after homologous recombination-dependent fork restart [60] or during break-induced replication (BIR) [61]. Additionally, when cells exhibit a defect in Pol ε cathalytic subunit, or when the level of Pol ε in the cell is decreased, Pol δ takes over DNA synthesis [6,44,52,59,62,63]. Pol δ is also capable of proofreading Pol ε errors [64,65]. Participation of a defined DNA polymerase in the replication of a particular DNA strand and differences in the mechanisms of replication of leading and lagging DNA strands seem to have an impact on the distribution of mutations within the genome and to influence evolutionary flexibility, pathogenesis and genomic stability [66–69]. Therefore, the conditions and mechanisms responsible for this division of labor between DNA polymerases at the replication fork still require intensive research.

Defective assembly of $GINS_{Psf1-1}$ is pronounced by poor copurification of the Psf3 subunit (Fig. 1). Therefore, it is possible that in vivo only limited number of the GINS complexes contains all subunits. Based on two-hybrid-system analyses, the Psf3 subunit (together with Psf1) was shown to be involved in interactions with the Dpb2 essential subunit of Pol ε [11]. Therefore, it could be expected that $GINS_{Psf1-1}$ will be at least partially impaired in its interaction with Pol ε. However, our previous in vitro analysis has shown that this is not the case; $GINS_{Psf1-1}$ binds to Pol ε similarly to "wild-type" GINS [70]. However, we cannot exclude the possibility that the poor binding of Psf3 within $GINS_{Psf1-1}$ affects the functioning of Pol ε or CMG helicase stability in the replisome. Such conditions may influence the contribution of specific replicases to the leading strand replication.

Analysis of mutation spectra led to the conclusion that strand biases observed in the *pol3-L612M* mutant were similar to those found in the *POL3* strains (Fig. 5A and C, Fig. S2A and C, Table S6, and Table S7). This phenomenon was true for all four types of substitutions, with higher ratios for T to C, G to A changes in OR1 and C to T, G to T changes in OR2. However, the bias was more pronounced in *pol3-L612M* cells, demonstrating the advantage of using this Pol δ variant. In the *psf1–1 pol3-L612M* strains compared with *pol3-L612M,* T to C, G to A changes remained biased in OR1 and C to T, G to T in OR2. However, we observed an increased contribution of OR2-specific C to T substitutions (position 310) and G to T substitutions (position 679/706) to the overall mutagenesis in OR1 (Fig. 5C and D, upper panel, Fig. S2C and D, upper panel, and Table S6). In parallel, in *psf1–1 pol3-L612M* OR2 in relation to

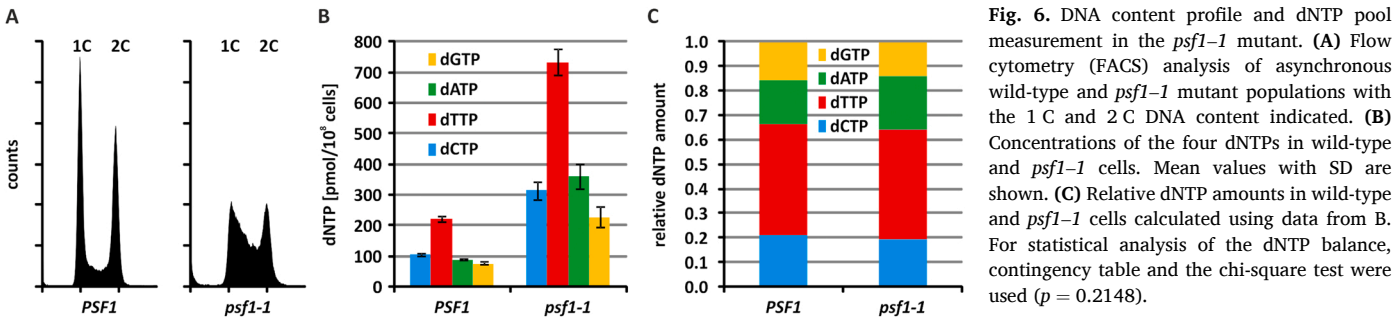

Fig. 6. DNA content profile and dNTP pool measurement in the *psf1–1* mutant. **(A)** Flow cytometry (FACS) analysis of asynchronous wild-type and *psf1–1* mutant populations with the 1 C and 2 C DNA content indicated. **(B)** Concentrations of the four dNTPs in wild-type and *psf1–1* cells. Mean values with SD are shown. **(C)** Relative dNTP amounts in wild-type and *psf1–1* cells calculated using data from B. For statistical analysis of the dNTP balance, contingency table and the chi-square test were used ($p = 0.2148$).

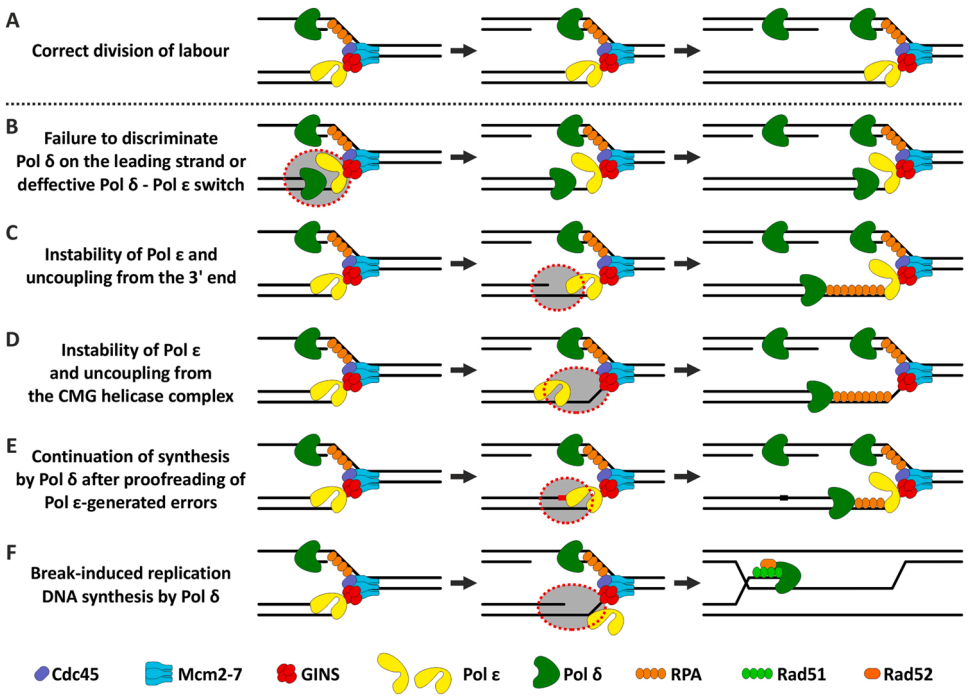

Fig. 7. Model showing possible events leading to the increased contribution of Pol δ to leading strand replication in the *psf1–1* mutant. A detailed description is provided in the text.

total mutagenesis, we observed a significant increase in OR1-specific T to C substitutions (position 97) and G to A changes (position 764) (Fig. 5C and D, lower panel and Table S6). Similar conclusions applied to the same changes at other sites in the reporter gene in OR1 and OR2 (Fig. 3 and Table S7). Therefore, this signature strongly suggested an increased participation of Pol δ in leading strand replication in *psf1–1* cells.

The apparent greater contribution of Pol δ to leading strand replication in *psf1–1* cells in vivo indicates that the GINS complex linking the CMG helicase and the leading strand replicase is important for DNA synthesis by the "correct" polymerase. The recruitment of Pol ε to the leading strand, its stability, and functioning in the replisome depend on CMG, while PCNA plays a minor role [35]. The same study has shown that Pol δ is not stabilized on the leading strand by CMG and is nearly completely dependent on PCNA. This result is supported by the finding that in contrast to Pol ε, Pol δ has a high affinity for PCNA [71]. Therefore, while Pol ε assembles faster with CMG on the leading strand, Pol δ is more efficiently loaded in the presence of PCNA and ssDNA coated with RPA on the lagging strand [35]. Later, in addition to these mechanisms of polymerase recruitment to DNA strands, mechanisms excluding the incorrect enzyme from either strand were proposed [36]. *In vitro*, the function of Pol δ on the leading strand or Pol ε on the lagging strand was suppressed even in the absence of the "correct" polymerase. The proposed mechanism excluding the incorrect polymerase on the leading strand would be CMG-dependent triggering of Pol δ ejection similar to the events at the end of an Okazaki fragment, i.e., the collision release mechanism [72]. This process would be supported by the distributive nature of Pol δ [35,36]. Therefore, the processivity of Pol δ on the leading strand would be diminished compared with Pol ε but could support DNA synthesis on this strand when CMGE functioning is compromised. It has also been proposed that Pol ε attached to CMG periodically uncouples from PCNA, allowing access for additional PCNA loading by RFC [35]. In parallel, a model of leading strand replication has been proposed where after DNA unwinding by Pol ε-bound CMG helicase and primer synthesis by Pol α, Pol δ takes over synthesis. Next, when Pol δ reaches the helicase, it dissociates, allowing Pol ε to continue DNA replication [74]. This phenomenon implies a role for Pol δ in reestablishing DNA synthesis on the leading strand after replication stress bypass. A role for Pol δ in the initiation of leading strand replication has also been proposed [59]. Finally, a recent study has demonstrated the ability of Pol δ to proofread errors made by Pol ε during replication of the leading strand [64,65].

Although Pol δ is partially involved in leading strand replication even under normal conditions, the mutation spectrum analysis and the synergistic effect of *psf1–1* and *pol3-L612M* mutations presented herein (Table S3) indicate that in *psf1–1* cells, impaired functioning of CMG significantly increases the participation of Pol δ in DNA synthesis on the leading strand. Various scenarios can be considered to explain the mechanism of these changes in the division of labor at the replisome. First, the CMG-dependent specific establishment of Pol ε at the leading strand or the discrimination of Pol δ might be impaired (Fig. 7B). Alternatively, according to the model in which Pol δ replicates the leading strand until it reaches the CMG-bound Pol ε and Pol ε takes-over synthesis, the mechanism of polymerase switch might be affected (Fig. 7B). Another possibility is that impaired functioning of Pol ε and its instability at the fork might result in the formation of ssDNA stretches covered with RPA, which together with PCNA enable more efficient loading of Pol δ on the leading strand (Fig. 7C and D). The flexible linker between the catalytic N-terminus and the C-terminus of Pol ε bound to the helicase facilitates such exchange. Importantly, more frequent formation of ssDNA in *psf1–1* cells has been shown previously [47], which supports the role of Pol δ in reestablishing leading strand synthesis when, due to impaired assembly and activity of GINS, DNA replication on the two strands becomes uncoupled. Such a scenario has also been proposed based on in vitro studies showing the involvement of Pol δ in DNA synthesis on the leading strand [62,74]. Finally, if Pol δ proofread errors made by Pol ε, under conditions of impaired CMG functioning, Pol δ synthesis on the leading strand might continue (Fig. 7E). Additionally, it cannot be excluded, that increased contribution of Pol δ results from the activation of the break-induced replication mechanism (BIR) (Fig. 7F). Previously, we proposed that repair mechanisms such as BIR are activated in *psf1–1* cells [47]. Verification of these models needs further studies. Since the GINS complex is involved in the DNA replication initiation process, in *psf1–1* cells possible reduced firing of the

origin closes to the *URA3* gene could result in marker locus replication starting from a neighboring origin. This would cause replication fork inversion what could explain the observed signature of Pol δ on the DNA strand which is now replicated as the lagging one. As a consequence, the other strand, which in normal conditions is replicated as lagging would be now replicated by Pol ε (Fig. S3A and B). To analyze this possibility, we compared *pol2-M644G* signature (pronounced by A to T substitutions, [57]) in *PSF1* and *psf1–1* strains (Fig. S3C and D, and associated Data in Brief paper by Dmowski *et al.*). We observed, that the A to T over T to A preference in OR1 and T to A over A to T in OR2 was not significantly changed in the *pol2-M644G psf1–1* mutant compared to *pol2-M644G* (Tarone test $p = 0.622$) suggesting similar involvement of Pol ε in DNA replication. This result suggests that Pol δ signature observed in *psf1–1* strains is not due to inversed orientation of the replication fork during replication of the reporter locus.

Nevertheless, our results clearly show that impaired functioning of the GINS complex in *psf1–1* cells strongly affects the replisome, and provides novel circumstances under which DNA synthesis by Pol ε, needs to be at least partially replaced by Pol δ. It is important to emphasize that these results demonstrate for the first time that defective nonpolymerase elements of the replisome can affect the division of labor between the two main polymerases. Therefore, it is important to identify the factors or mechanisms that alter the distribution of polymerase activity at the replication fork. Studies of the GINS complex in this regard are important given that a growing number of reports show a correlation of human diseases and cancer with GINS complex deficiencies [75,76] or induced expression of *GINS1* (human homolog of *PSF1*) [77,78]. Additionally, kncock-down of *PSF1* or *PSF3* inhibits growth of cancer cells [50,79].

4. Materials and methods

4.1. Yeast strains

Yeast strains used in this study are listed in Table S1. *S. cerevisiae* strains used for analyses of mutation spectra were derivatives of YTAK001, YTAK002 [57], SNM12 and SNM24 [54] strains (Table S1), kindly provided by T. A. Kunkel (NIEHS, USA). All four parental strains contained *URA3* reporter gene replacing *AGP1*. *URA3* was cloned in two orientations (OR1 and OR2) in respect to ARS306 (the nearest origin). SNM12 and SNM24 contained additionally the *pol3-L612M* allele. Disruption of *REV3* and *MSH6* was performed using PCR-amplified DNA cassettes: *rev3*Δ::*NAT1* (using primers Rev3_UPTEF and Rev3_DNTEF and pAG25 as template) and *msh6*Δ::*HPH* (using primers msh6UTEF and msh6DTEF and pAG32 as template), respectively (Table S2). The presence of the *rev3*Δ::*NAT1* cassette in nourseothricin-resistant transformants was verified by multiplex PCR with primer sets Rev3-R4 – Rev3A – nat1UO and Rev3-F4 – Rev3D – nat1DO (Table S2). The presence of the *msh6*Δ::*HPH* cassette in hygromycin-resistant transformants was verified by multiplex PCR with primers MSH6-UO – msh6up2 – HPH-UO and MSH6-DO – msh6dw2 – HPH-DO (Table S2). The *PSF1-LEU2* and *psf1–1-LEU2* alleles were introduced as described previously [11]. Additionally, *pol3-L612M rev3*Δ *psf1–1 msh6*Δ strains were obtained by tetrad dissection from heterozygous diploid strains.

4.2. Media and growth conditions

Yeast were grown at 23 or 30 °C in standard media [80]. YPD (1% Bacto-yeast extract, 2% Bacto-peptone, 2% glucose liquid or solidified with 2% Bacto-agar) supplemented when necessary with appropriate antibiotics (hygromycin B 300 μg/ml (Bioshop, Burlington, Canada), nourseothricin 100 μg/ml (Werner BioAgents, Jena, Germany). SD medium (0.67% yeast nitrogen base without amino acids, 2% glucose, liquid or solidified with 2% Bacto-agar) supplemented with appropriate amino acids and nitrogenous bases were used for nutrition selection. SD medium with 1 mg/ml 5-fluoroorotic acid (5-FOA) (US Biological, Salem, MA, USA) was used for the selection of *URA3* mutants and mutagenesis assays [81]. Yeast strains were transformed using the LiAc/ssDNA/PEG method [82]. Isolation of yeast chromosomal DNA was done using the Genomic Mini AX Yeast Spin Kit (A&A Biotechnology, Gdansk, Poland).

4.3. Analysis of mutation rates and spectra

Mutation rates were determined for, at least 8 cultures of 2 or 3 independent isolates of each strain. Yeast strains were inoculated in 2 ml of liquid SD medium supplemented with the required amino acids and nitrogenous bases, and grown at 23 °C until stationary phase. Then, appropriate dilutions were plated on nonselective and selective (supplemented with 5-FOA for selection of *URA3* mutants) media. After 4–7 days-growth at 23 °C, colonies were counted. The spontaneous mutation rates were calculated using the $\mu = f/ln(N\mu)$ equation [μ - mutation rate per round of DNA replication; f - mutant frequency (cell count from selective media divided by the cell count from nonselective media), and N - total population] [83]. Median values of mutation rates and 95% confidence intervals were calculated (GraphPad Prism software). Statistical significance of differences in the mutation rates between the respective strains (p-values) was measured using the nonparametric Mann-Whitney U test (GraphPad Prism software). The homogeneity of odds ratios was tested using the calculator downloaded from https://www.prostatservices.com/blog/calculator-for--breslow-day-and-tarone-tests-for-homogeneity-of-odds-ratios.

To define the spectrum of mutations in *URA3* reporter gene, for each strain analyzed 132–628 5-FOA-resistant colonies were analyzed. Each colony was obtained from an independent culture diluted and plated on 5-FOA-containing media. The *URA3* gene was PCR-amplified using primers URA3F393 and URA3R412 (Table S2). The same primers were used for sequencing of the PCR product. To calculate the contribution of either mutation type to overall mutagenesis, the number of specific events was divided by the total number of mutations found. Rates for specific mutations were calculated proportionally to their contribution to the mutagenesis spectrum. Statistical analyses of differences in contribution of given substitutions to overall mutagenesis was done using contingency table and chi-square statistics, while p values for differences in mutation spectra were determined using Fisher's Exact test (GraphPad Prism software).

4.4. GINS purification and analysis of complex formation in vitro

GINS subunits were purified from yeast strain BJ2168 cells expressing simultaneously *PSF1*, *PSF2*, *PSF3*, and *SLD5*. *3xFLAG-PSF1*, *3xFLAG-psf1–1*, and *3xFLAG-psf1–100*, were engineered to express protein fusions containing a 5-glycine linker and a PreScission Protease recognition site between the 3xFlag and the Psf1 N-terminus. Each *PSF1* variant, and *SLD5* were cloned in pESC-LEU (Stratagene, La Jolla, CA). *PSF2* and *PSF3* genes were cloned in pESC-URA (Stratagene, La Jolla, CA). The transformed BJ2168 cells were grown at 25ºC for *psf1–1* strain and at 30ºC for *PSF1* and *psf1–100*. Collected cells were frozen in liquid nitrogen and crushed in a freezer mill (SPEX CertiPrep 6850 Freezer/Mill). The cell powder was resuspended in lysis$^{SE+E+1\ T}$ buffer (50 mM HEPES-KOH at pH 7.6, 500 mM KCl, 2 mM EDTA, 0.1% Tween20, 1% Triton X-100%, and 10% glycerol). Next, protease inhibitor cocktails Sigma P8215 (Protease Inhibitor Cocktail for use with fungal and yeast extracts) and Roche "complete, EDTA free" were added. After sonication, cell lysates were centrifuged at 18,000 x g for 15 min, and the supernatant was collected and mixed with anti-FLAG M2 agarose beads (Sigma). The beads were washed with lysis$^{SE+E+0.1\ T}$ buffer (similar to lysis$^{SE+E+1\ T}$ buffer but containing 0.1% Triton X-100) and GINS was eluted using 200 mg/ml of the 3xFLAG peptide in 0.3 M NaCl buffer (50 mM HEPES-NaOH at pH 7.5, 0.3 M NaCl, 10% glycerol, 0.1% NP-40%, and 0.01% Tween 20). The obtained samples were run in a SDS 3–20% gradient acrylamide gel, followed by staining with CBB R-250.

4.5. Flow cytometry analysis

Yeast cells for flow cytometry analysis of *PSF1* and *psf1–1* cultured at 23 °C were prepared as described previously [84] and stained using 0.5 μM SYTOX Green (Invitrogen, Carlsbad, CA, USA). To determine the DNA content the fluorescence signal FL1 was measured using Becton Dickinson FACS Calibur and CellQuest software (BD Bioscience, San Jose, CA, United States) and analysed using Flowing Software (http://www.flowingsoftware.com).

4.6. Measurement of dNTPs concentration

The dNTP pools were determined as described previously [85]. The analysis was performed for four *PSF1* and six *psf1–1* strains (SC538 and SC539, repectively [11]) at the permissive temperature 23 °C. Contingency table and the chi-square test were used for statistical analysis of the dNTP balance.

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CRediT authorship contribution statement

Michal Dmowski: Conceptualization, Investigation, Validation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **Malgorzata Jedrychowska:** Investigation, Validation. **Karolina Makiela-Dzbenska:** Investigation. **Milena Denkiewicz-Kruk:** Investigation. **Sushma Sharma:** Investigation. **Andrei Chabes:** Validation. **Hiroyuki Araki:** Validation. **Iwona J. Fijalkowska:** Conceptualization, Validation, Funding acquisition, Writing – review & editing.

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Conflict of interest statement

The authors declare that there are no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.dnarep.2022.103272.

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