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The CysB motif of Rev3p involved in the formation of the four-subunit DNA polymerase ζ is required for defective-replisome-induced mutagenesis

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Summary

Eukaryotic DNA replication is performed by high-fidelity multi-subunit replicative B-family DNA polymerases (Pols) α, δ and ε. Those complexes are composed of catalytic and accessory subunits and organized in multicomplex machinery: the replisome. The fourth B-family member, DNA polymerase zeta (Pol ζ), is responsible for a large portion of mutagenesis in eukaryotic cells. Two forms of Pol ζ have been identified, a hetero-dimeric (Pol ζ_2) and a hetero-tetrameric (Pol ζ_4) ones and recent data have demonstrated that Pol ζ_4 is responsible for damage-induced mutagenesis. Here, using yeast Pol ζ mutant defective in the assembly of the Pol ζ four-subunit form, we show *in vivo* that [4Fe-4S] cluster in Pol ζ catalytic subunit (Rev3p) is also required for spontaneous (wild-type cells) and defective-replisome-induced mutagenesis – DRIM (*pol3-Y708A*, *pol2-1* or *psf1-100* cells), when cells are not treated with any external damaging agents.

Introduction

Faithful and efficient DNA replication is essential for cell viability and critical for an accurate inheritance of the complete genome. For proper DNA replication, a well-controlled and coordinated multiprotein machinery named the replisome is required (for review see Zhang and O'Donnell, 2016). In eukaryotic cells, four B-family DNA polymerases: alfa (Pol α), delta (Pol δ), epsilon (Pol ε) and zeta (Pol ζ), are the key components of the replisome and are assisted by a multitude of catalytic and noncatalytic accessory proteins (reviewed in Burgers and Kunkel, 2017). The Pol α/primase complex initiates DNA replication by synthesizing short primers on both leading and lagging strand, which are subsequently elongated by the two major DNA polymerases: Pol ε and Pol δ (Burgers and Kunkel, 2017). In contrast to the well-defined role of Pol α, specific roles of Pol ε and Pol δ in DNA replication have been much debated for years. However, recent evidence strongly supports a model of the replication fork wherein the leading- and the lagging-strand templates are primarily copied by Pol ε and Pol δ respectively (for review see Kunkel and Burgers, 2017).

DNA polymerase ζ plays a predominant role in genomic integrity maintenance by protecting a cell from the consequences of endogenous and exogenous DNA damage, albeit at the cost of increased emergence of mutations (reviewed in Vaisman and Woodgate, 2017). In *Saccharomyces cerevisiae*, Pol ζ is responsible for the majority of DNA damage-induced mutagenesis and 50–70% of spontaneous mutations (Lemontt, 1971; Quah *et al.*, 1980; Kochenova *et al.*, 2015). Pol ζ is a translesion (TLS) polymerase which, due to more flexible active site and lack of the 3′→5′ exonuclease proofreading activity, has the ability to bypass DNA lesions that cannot be bypassed by the major replicative polymerases (Vaisman and Woodgate, 2017). Pol ζ mainly acts as an 'extender' from a nucleotide incorporated across a lesion site by an 'inserter' DNA polymerase (reviewed in Zhao and Washington, 2017). However, Pol ζ may introduce errors not only during mutagenic lesion bypass but also while replicating undamaged DNA (Northam *et al.*, 2010; Kraszewska *et al.*, 2012; Grabowska *et al.*, 2014; Northam *et al.*, 2014). Pol ζ may then perform the extension step from poorly matched primer termini, thus, contributing to the fixation of the mutation (Johnson *et al.*, 2000) or, due to its lower intrinsic fidelity (Zhong *et al.*, 2006), incorrect nucleotide may be inserted by Pol ζ itself. Indeed, it has been shown that in particular case of yeast mutant strains with defective replisome, 80–90% of arising

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mutations (defective-replisome-induced mutagenesis [DRIM]) is attributed to Pol ζ replicating undamaged DNA (Northam *et al*., 2006; Aksenova *et al*., 2010; Northam *et al*., 2010; Becker *et al*., 2014; Grabowska *et al*., 2014; Garbacz *et al*., 2015).

Pol ζ was first described as a hetero-dimer (Pol ζ_2) composed of the catalytic subunit Rev3p and the auxiliary subunit Rev7p (Nelson *et al*., 1996). In 2012, combined *in vivo* and *in vitro* approaches revealed Pol ζ as a hetero-tetramer with two additional subunits: Pol31p and Pol32p (Baranovskiy *et al*., 2012; Johnson *et al*., 2012; Makarova *et al*., 2012). A Pol ζ structure composed of four subunits, Rev3p, Rev7p, Pol31p and Pol32p (Pol ζ_4), was then confirmed by electron microscopy (Gómez-Llorente *et al*., 2013). Interestingly, these two newly recognized subunits, Pol31p and Pol32p, are shared with the major replicative lagging-strand polymerase, Pol δ (Pol3p, Pol31p, Pol32p) (Baranovskiy *et al*., 2012; Johnson *et al*., 2012; Makarova *et al*., 2012). The C-terminal domains (CTD) of the two catalytic subunits of Pol δ and Pol ζ, Pol3p and Rev3p, respectively, show strong sequence homology in two conserved cysteine-rich metal-binding motifs, CysA and CysB, which are responsible for the interaction with their common subunit Pol31p (Garcia *et al*., 2004; Baranovskiy *et al*., 2012). In contrast to Pol δ, where both intact motifs are required for proper DNA replication (Netz *et al*., 2012), in Pol ζ only CysB motif of Rev3p is required for the proper interactions between Rev3p and Pol31p and, thus, for formation of a proficient four-subunit Pol ζ (Baranovskiy *et al*., 2012; Johnson *et al*., 2012; Makarova *et al*., 2012).

Biochemical studies have shown that the TLS activity of Pol ζ_4 is much more efficient compared to that of Pol ζ_2; likewise, physiological assays have shown that yeast strains in which Pol ζ is composed of only two subunits are defective for UV-induced mutagenesis (Baranovskiy *et al*., 2012; Makarova *et al*., 2012). The TLS requires switching from more faithful DNA replicase to an error-prone TLS polymerase, and two models of Pol ζ_4 recruitment to a damage site have been proposed (Siebler *et al*., 2014). The observation that Pol δ and Pol ζ share the Pol31p and Pol32p subunits has led to a model of a Pol δ/Pol ζ switch occurring through the exchange of the respective catalytic subunits, Pol3p and Rev3p, while Pol31p and Pol32p remain associated with DNA (Baranovskiy *et al*., 2012). The observed proteasomal degradation of the released Pol3p by Def1p protease supports this scenario (Daraba *et al*., 2014). In contrast, in another proposed model, the Pol δ hetero-trimer is replaced *en masse* by a hetero-tetrameric Pol ζ_4, which in turn is supported by the observation that the Pol ζ_4 complex is stable throughout the cell cycle (Makarova *et al*., 2012). However, the subunit composition of Pol ζ participating in the replication of undamaged DNA, rather than in lesion bypass, has not been studied. Moreover, the proposed models describe only the Pol δ/Pol ζ switch, whereas when the replication is defective, not only a Pol δ/Pol ζ switch, but also, a Pol ε/Pol ζ switch may occur. Although the shared subunits between Pol δ and Pol ζ suggest that tetrameric Pol ζ is most likely involved when Pol δ is impaired, lack of such shared subunits between Pol ε and Pol ζ poses the question whether Pol ζ_2 or Pol ζ_4 is involved when Pol ε is defective.

To genetically investigate if Pol ζ_2 or Pol ζ_4 is involved when the lagging- or the leading-strand DNA polymerase is impaired, we used yeast mutant strains carrying *pol3-Y708A* or *pol2-1* mutations, which, respectively, affect the catalytic subunits of the two major replicative polymerases, Pol δ and Pol ε (Morrison *et al*., 1990; Pavlov *et al*., 2001). Mutagenesis in these mutant strains has been described as predominantly Pol ζ-dependent and demonstrated to be a consequence of the Pol ζ involvement in the replication of undamaged DNA (DRIM) (Northam *et al*., 2010). Additionally, a third mutant strain was analyzed carrying the *psf1-100* mutation affecting the Psf1p, one of the GINS subunits (Grabowska *et al*., 2014). Most importantly, the increased mutagenesis in the *psf1-100* mutant strain was found to be almost fully Pol ζ-dependent (Grabowska *et al*., 2014). To establish which Pol ζ form is involved when the leading- or the lagging-strand DNA polymerases are defective, the above three defective replisome mutant strains were analyzed in combination with *REV3* deletion (*rev3*Δ) abolishing Pol ζ (both dimeric and tetrameric) activity or with the *rev3-cysB* allele selectively compromising only Pol ζ_4 formation (Makarova *et al*., 2012). The mutational spectra analysis indicated that the four-subunit Pol ζ complex plays an important role both in spontaneous mutagenesis in the wild-type strain and in mutant strains in which the participation of Pol ζ in the replication of undamaged DNA is increased.

Results

Characterization of Can^R mutations in rev3-cysB *strain*

Specific interaction between Rev3p and Pol31p requires a functional iron-sulfur [4Fe-4S] cluster at the CysB motif of Rev3p (Baranovskiy *et al*., 2012; Johnson *et al*., 2012; Makarova *et al*., 2012). *In S. cerevisiae*, substitution of the four cysteines coordinating the [4Fe-4S] cluster with alanines (as in Baranovskiy *et al*. 2012) or of two of these cysteines by serines [as in Makarova *et al*. (2012)] abrogates Fe-S binding and prevents Pol ζ_4

formation. Accordingly, in the *rev3-CC*1449,1473*SS* mutant strain (named *rev3-cysB*) only a two-subunit Pol ζ complex (Pol ζ_2) was detected by biochemical methods (Makarova *et al*., 2012). *In vitro* studies have shown that Pol ζ_2 is active, although its catalytic efficiency for DNA extension is much lower compared to Pol ζ_4 (Johnson *et al*., 2012; Makarova *et al*., 2012; Lee *et al*., 2014). Interestingly, strains carrying mutations in the CysB motif are almost completely defective for UV-induced mutagenesis, similarly to strains with catalytically inactive Rev3p or with *rev3*Δ (Baranovskiy *et al*., 2012; Makarova *et al*., 2012; Siebler *et al*., 2014). Moreover, mutations in the CysB motif do not change substantially the level of mutated Rev3p form compared to wild-type Rev3p (Supporting Information Fig. S1), as reported before (Baranovskiy *et al*., 2012; Siebler *et al*., 2014). These results suggest that Rev3p binding to Pol31p, and therefore, formation of Pol ζ_4, is indispensable for the Pol ζ participation in damage-induced mutagenesis.

To investigate if the CysB motif is relevant for mutagenesis, when cells are not treated with any external damaging agents, we used the mutant strain carrying the *rev3-cysB* allele, described in Makarova *et al*. (2012). As *rev3-cysB* mutation prevents Rev3p-Pol31p interaction and in turn Pol ζ_4 formation, based on *in vitro* data (Makarova *et al*., 2012), we refer to Pol ζ form in *rev3-cysB* mutant strain as Pol ζ_2. In the earlier report, the *rev3-cysB* allele was expressed from a plasmid under an inducible promoter (Makarova *et al*., 2012). Here, to study the Pol ζ activity with native-like expression of all its potential constituents, the *rev3-cysB* allele was integrated into the chromosome under the control of its native promoter. Then, rates of spontaneous mutagenesis in strains derived from the ΔI(-2)I-7B-YUNI300 strain (Pavlov *et al*., 2002) carrying either the *rev3*Δ or the *rev3-cysB* allele were determined using the *CAN1* reporter gene, which enables simultaneous detection of a wide spectrum of mutational events (Chen and Kolodner, 1999). Any mutation that inactivates the arginine permease encoded by the *CAN1* gene prevents the uptake of canavanine, a toxic analog of arginine, and results in resistance to canavanine (Can^R) (Whelan *et al*., 1979).

The *REV3* deletion has been described to have an antimutator effect and the spontaneous mutation rate at the *CAN1 locus* is 50% lower in the *rev3*Δ strain compared to the wild-type (Cassier *et al*., 1980; Quah *et al*., 1980; Roche *et al*., 1994; Lawrence, 2002; Sabbioneda *et al*., 2005; Northam *et al*., 2010; Kraszewska *et al*., 2012; Grabowska *et al*., 2014; Garbacz *et al*., 2015). Our results confirmed that half of spontaneous mutations is due to the Pol ζ activity (30×10^{-8} in the *rev3*Δ strain compared to 60×10^{-8} in the wild-type *REV3* strain) (Table 1). The mutation in CysB motif also resulted in slight but statistically significant decrease in the mutagenesis rate in *rev3-cysB* strain (47×10^{-8}) compared to the wild-type strain (47×10^{-8}), although the decrease was not as pronounced as in the strain lacking *REV3*.

Table 1. Spontaneous mutation rates for wild-type, *pol3-Y708A*, *pol2-1* and *psf1–100* strains and their *rev3*Δ and *rev3-cysB* derivatives.

Relevant genotype	Can^R ($\times 10^{-8}$)	Relative rate[a]
wild-type	60 (56–64)[b]	1
*rev3*Δ	30 (26–34)	0.50
rev3-cysB	47 (44–51)	0.78
wild-type	60 (56–64)	1
pol3-Y708A	480 (390–640)	8.0
*pol3-Y708A rev3*Δ	92 (86–100)	1.5
pol3-Y708A rev3-cysB	130 (120–140)	2.2
wild-type	60 (56–64)	1
pol2-1	250 (190–310)	4.2
*pol2-1 rev3*Δ	99 (86–120)	1.7
pol2-1 rev3-cysB	96 (73–100)	1.6
PSF1	86 (77–90)	1
psf1–100	170 (150–220)	2.0
*psf1–100 rev3*Δ	45 (40–76)	0.52
psf1–100 rev3-cysB	56 (48–66)	0.65

a. Relative rate is the rate of mutagenesis of the respective mutant strain divided by the corresponding mutagenesis rate of the wild-type strain (or *PSF1* strain in the case of *psf1–100* strain and its derivatives).
b. Ninety five percent confidence intervals are shown in parentheses; *p* values between corresponding strains were calculated using nonparametric Mann–Whitney *U* test (data shown in Supporting Information Tables S1–S4).

To further analyze the effect of mutation in CysB motif on spontaneous mutagenesis, the *CAN1* mutagenesis spectrum in the *rev3-cysB* mutant strain was determined and compared with the spectra of isogenic wild-type and *rev3*Δ strains (Grabowska *et al*., 2014; Garbacz *et al*., 2015). Such analysis of mutation specificity should reveal whether the elimination of the Rev3p-Pol31p interaction, thus lack of Pol ζ_4, leads to the disappearance of some features attributable to Pol ζ. As shown by a recent *in vitro* study, base substitutions, most frequently G→T, predominate in Pol ζ_4 spectra and also a noticeable number of complex mutations is observed (Kochenova *et al*., 2017). Additionally, a significant increase in the rates of X-dCTP mispairs is observed in spectra of Pol ζ_5 (stoichiometric Rev3p, Rev7p, Pol31p, Pol32p and Rev1p complex), apparently as a result of Rev1p deoxycytidyl-transferase activity (Kochenova *et al*., 2017). These results are consistent with previous *in vivo* studies in which complex mutations and GC→CG transversions were considered as hallmarks of Pol ζ-dependent mutagenesis (Harfe and Jinks-Robertson, 2000; Kraszewska *et al*., 2012; Grabowska *et al*., 2014; Northam *et al*., 2014).

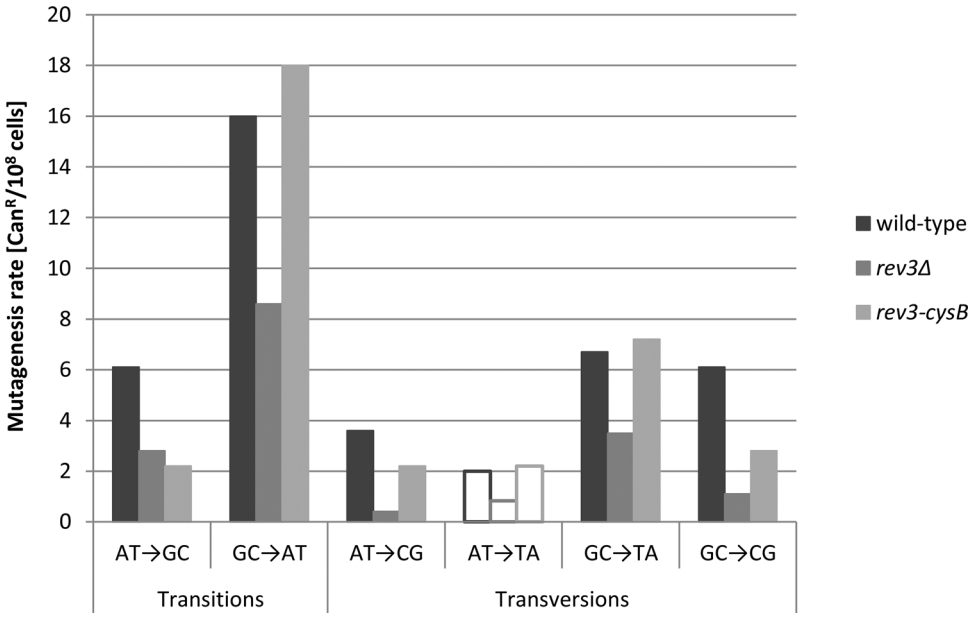

Fig. 1. Rates of individual base substitutions types in *rev3Δ* and *rev3-cysB* strains. Rates, number of mutations and percentage are presented in Supporting Information Tables S3. If the number of events is insufficient for comparison, the rates are indicated as open bars.

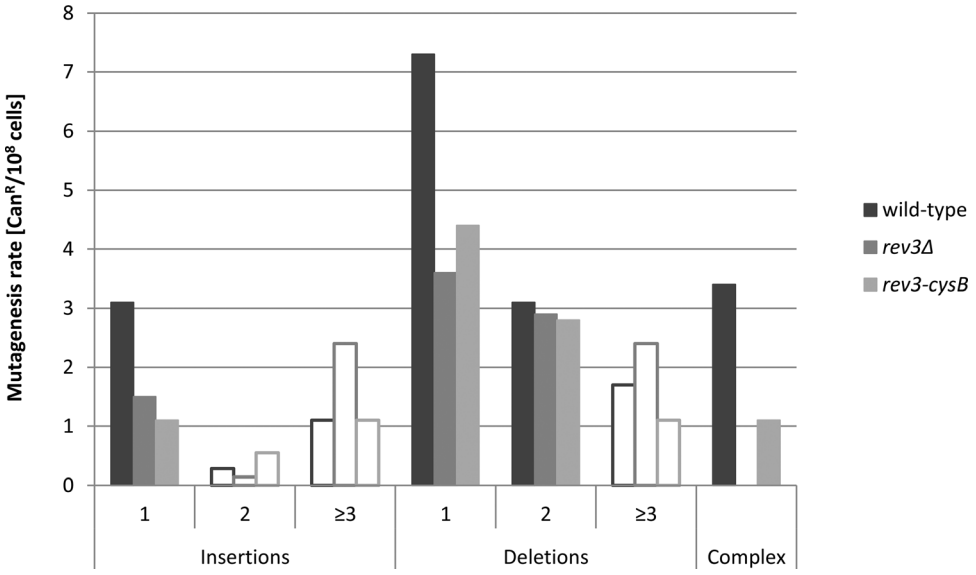

Fig. 2. Rates of individual insertion and deletion types and complex mutations in *rev3Δ* and *rev3-cysB* strains. Rates, number of mutations and percentage are presented in Supporting Information Tables S3. If the number of events is insufficient for comparison, the rates are indicated as open bars.

The *CAN1* mutation data obtained previously in our laboratory were combined with the results of this work for both the wild-type and *rev3Δ* strains. In total, 215 and 217 independent Can^R mutants were analyzed, in the wild-type and *rev3Δ* strains, respectively, whereas 85 independent Can^R mutants were examined for the *rev3-cysB* strain. The number of events, percentage and mutation rates were calculated for each type of mutation to simplify the comparison of the mutagenesis spectra. The distribution of particular mutations is presented in Figs 1 and 2 and Supporting Information Table S3, locations of the mutations in the *CAN1* sequence is in Supporting Information Fig. S2. As aforementioned, the overall rate of Can^R mutations was 50% lower in the *rev3Δ* strain than in the wild-type strain, mainly due to a decreased rate of base substitutions (2.4-fold, from 40 to 17 $\times$ 10^{-8}), especially transversions (3.1-fold, from 18 to 5.8 $\times$ 10^{-8}). The characteristic for Pol ζ complex mutations were eliminated in the *rev3Δ* spectrum. Moreover, the rate of GC→CG transversions was decreased (5.5-fold, from 6.1 to 1.1 $\times$ 10^{-8}). Although a major fraction of these mutations is a result of 'C' incorporation by Rev1p, the presence of active Rev3p is apparently required for mutation fixation and, thus, GC→CG transversions could also be considered as characteristic for Pol ζ. These results are in accordance with previous

reports on Pol ζ-dependent mutagenesis (Roche *et al.*, 1994; Endo *et al.*, 2007; Abdulovic *et al.*, 2008; Kraszewska *et al.*, 2012; Grabowska *et al.*, 2014; Garbacz *et al.*, 2015). The difference in the spontaneous mutagenesis rate between *rev3*Δ and *rev3-cysB* strains (Table 1) could also be noticed between corresponding *CAN1* mutational spectra (Supporting Information Table S7). However, we did not observe any statistically significant difference between any particular classes of mutations, including those characteristic for Pol ζ (Supporting Information Tables S8–S11). These results may suggest that Pol ζ_4 is indeed involved, not only in damage-induced, but also in spontaneous mutagenesis. To further study the effect of mutation in CysB motif, several mutant strains were analyzed in which, due to defective lagging- or leading-strand polymerase, the Pol ζ access to undamaged DNA is increased.

Characterization of Can^R mutations in pol3-Y708A rev3-cysB *strain*

The sharing of subunits between Pol δ and Pol ζ may suggest that it is Pol ζ_4, not Pol ζ_2, that is involved in replication of undamaged DNA when the lagging-strand polymerase is defective, although until now no *in vivo* evidence has been provided. To find out which Pol ζ form is required, the Pol δ mutant strain *pol3-Y708A* was chosen. In this mutant strain, tyrosine 708 in the active site of Pol3p, the catalytic subunit of Pol δ, is substituted with alanine (Pavlov *et al.*, 2001), which leads to a strong DRIM phenotype, largely Pol ζ-dependent (Northam *et al.*, 2010). Previous studies have shown that DRIM is a consequence of defective replisome stalling at small hairpin structures, which can be proficiently bypassed by Pol ζ complex with Rev1p (Northam *et al.*, 2014). Therefore, the observed DRIM phenotype rather results from error-prone Pol ζ replication of undamaged DNA than from lesion bypass (Northam *et al.*, 2010, 2014).

The mutagenesis rates at *CAN1 locus* were analyzed in the *pol3-Y708A* mutant strain additionally carrying the *rev3*Δ or the *rev3-cysB* allele (Table 1). The mutation rate in the *pol3-Y708A* mutant strain was elevated eight-fold compared to the wild-type (480 vs. 60 × 10^{-8}) and Pol ζ was responsible for about 80% of the observed mutator effect (92 × 10^{-8} in *pol3-Y708A rev3*Δ), in accordance with previously published data (Pavlov *et al.*, 2001; Northam *et al.*, 2006, 2010). The mutagenesis rate in *pol3-Y708A rev3-cysB* was also decreased (130 × 10^{-8}). A slight, but statistically significant difference was noted between the mutation rates in the *pol3-Y708A rev3*Δ and *pol3-Y708A rev3-cysB* strains (Supporting Information Table S1). Nonetheless, our results indicates that Pol ζ_4 is responsible for the mutator effect observed in the *pol3-Y708A* mutant strain.

To compare mutational specificities of the two defective Pol ζ alleles, the spectra of Can^R mutations were determined in relevant strains, as before. For each strains 91 to 94 independent Can^R mutants were analyzed (Figs 3 and 4 and Supporting Information Table S4 and Fig. S2). In the *pol3-Y708A* mutant strain the rates of all types of mutations were increased significantly compared to the wild-type strain. Base substitutions increased 9.3-fold (from 40 to 370 × 10^{-8}), in particular transversions (18-fold, from 18 to 320 × 10^{-8}). Of those, the strongest increase was for GC→CG transversions (32-fold, from 6.1 to 200 × 10^{-8}). The rate of complex mutations rose 12-fold (from 3.4 to 42 × 10^{-8}). Deletion of the *REV3* gene decreased the rates of base substitutions (8.8-fold, from 377 to 42 × 10^{-8}), especially transversions (20-fold, from 320 to 16 × 10^{-8}); the GC→CG transversions were virtually eliminated and no complex mutations, which are also characteristic for Pol ζ activity, were found in *pol3-Y708A rev3*Δ. These data are in agreement with an earlier report (Northam *et al.*, 2010).

Also in the *pol3-Y708A rev3-cysB* double mutant strain a decrease in similar classes of mutations was observed, compared to the spectrum of the *pol3-Y708A* single mutant strain (Figs 3 and 4 and Supporting Information Table S4 and Fig. S2). Base substitutions, particularly transversions fell significantly (respectively, 5.4-fold, from 370 to 68 × 10^{-8} and 10-fold, from 320 to 32 × 10^{-8}). The most pronounced decrease was observed for GC→CG transversions (29-fold, from 200 to 6.9 × 10^{-8}), result of Pol ζ activity preceded by the 'C' insertion by Rev1p. Complex mutations characteristic for Pol ζ were also severely decreased (15-fold, from 42 to 2.8 × 10^{-8}). No statistically significant differences between *pol3-Y708A rev3*Δ and *pol3-Y708A rev3-cysB CAN1* mutational spectra (Supporting Information Tables S7–S11) confirms that the intact CysB motif is required for mutagenesis in *pol3-Y708A* strain. These results may thus suggest that in *pol3-Y708A*, mutant strain with defective Pol δ, primarily Pol ζ_4 participates in the replication of undamaged DNA.

Characterization of Can^R mutations in pol2-1 rev3-cysB *strain*

To determine which Pol ζ form is required in the situation of impaired Pol ε, the *pol2-1* mutant strain was studied. In this strain, the *URA3* gene is inserted in the midpoint of *POL2* gene encoding Pol2p, the catalytic subunit of Pol ε (Morrison *et al.*, 1990). Most importantly, this insertion leads to a strong DRIM phenotype,

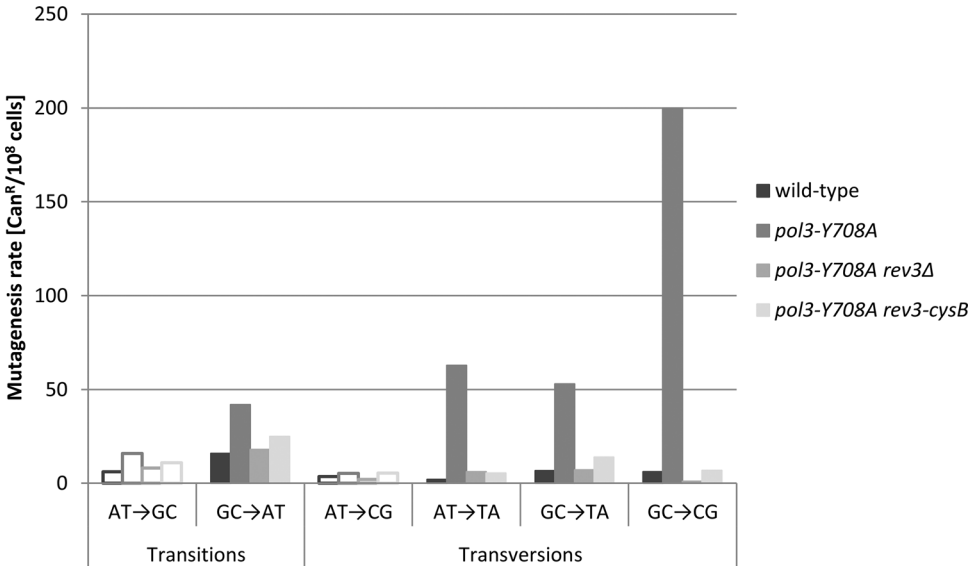

Fig. 3. Rates of individual base substitutions types in *pol3-Y708A* strain and its *rev3Δ* and *rev3-cysB* derivatives.
Rates, number of mutations and percentage are presented in Supporting Information Tables S4. If the number of events is insufficient for comparison, the rates are indicated as open bars.

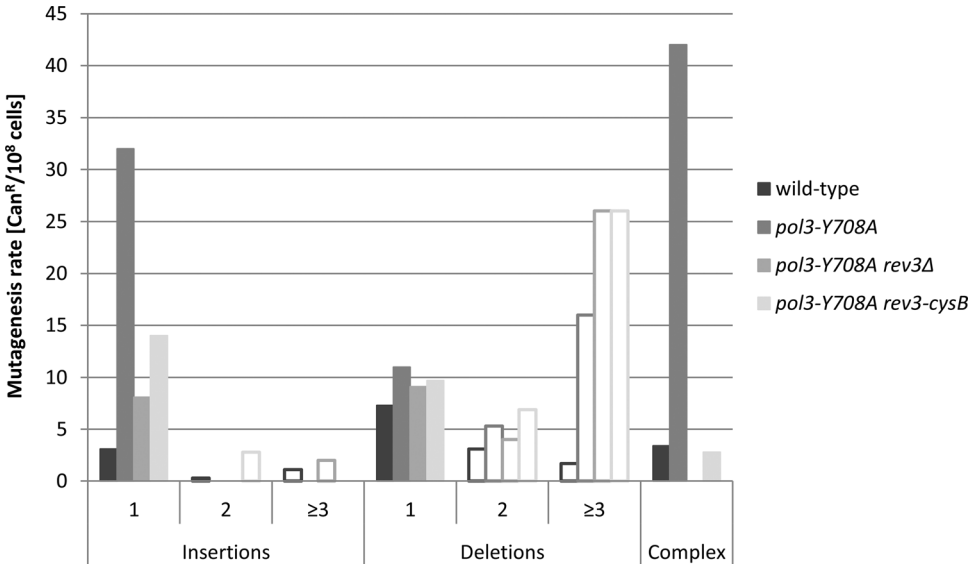

Fig. 4. Rates of individual insertion and deletion types and complex mutations in *pol3-Y708A* strain and its *rev3Δ* and *rev3-cysB* derivatives.
Rates, number of mutations and percentage are presented in Supporting Information Tables S4. If the number of events is insufficient for comparison, the rates are indicated as open bars.

largely Pol ζ-dependent (Shcherbakova *et al.*, 1996; Northam *et al.*, 2006, 2010).

Mutagenesis rates at the *CAN1 locus* were analyzed in the *pol2-1* mutant strain additionally carrying the *rev3Δ* or the *rev3-cysB* allele (Table 1). The mutation rate in *pol2-1* was elevated 4.2-fold compared to the wild-type strain (250 and 60 $\times$ 10^{-8} respectively) and about 60% of mutations were mediated by Pol ζ (250 $\times$ 10^{-8} in *pol2-1* and 99 $\times$ 10^{-8} in *pol2-1 rev3Δ*). In our genetic background, the level of mutagenesis in *pol2-1* mutant strain was lower than previously reported (Shcherbakova *et al.*, 1996; Northam *et al.*, 2006, 2010). Nevertheless, like in all genetic backgrounds, the observed mutator effect was largely Pol ζ-dependent. The *rev3-cysB* mutation had exactly the same antimutator effect (96 $\times$ 10^{-8} in the *pol2-1 rev3-cysB* strain) as the complete elimination of Pol ζ activity (*rev3Δ*). Thus, in this particular situation, when Pol ε is defective, the intact CysB motif of Rev3p is required for mutagenesis.

To determine the mutational specificity of the two defective Pol ζ alleles, the mutational spectra of relevant strains were determined as before, with 82 to 99 independent Can^R mutants analyzed per strain (Figs 5 and 6 and Supporting Information Table S5 and Fig. S2). In the *pol2-1* strain, the rates of base substitutions, but not of complex mutations, were increased as compared to the wild-type strain. Base substitutions increased 5.3-

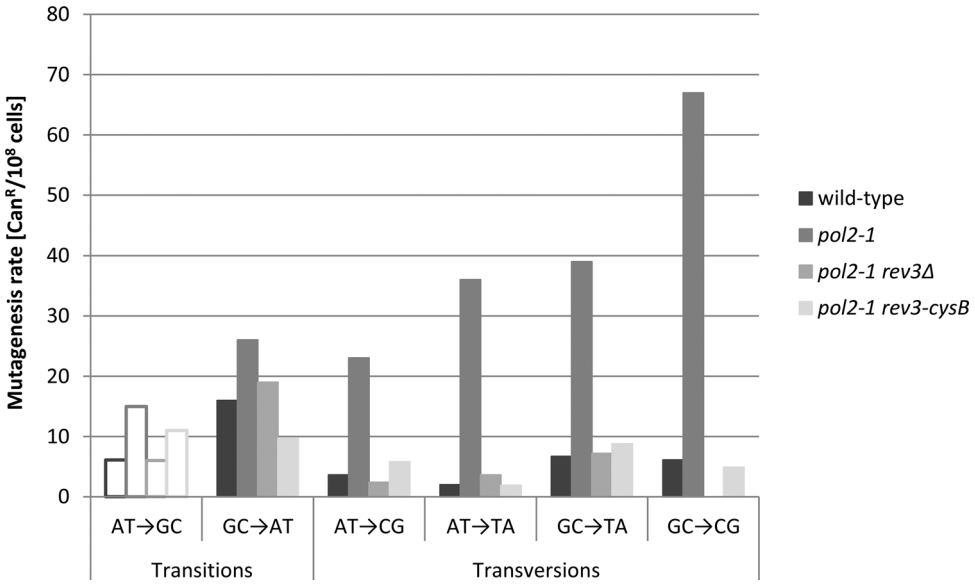

Fig. 5. Rates of individual base substitutions types in *pol2-1* strain and its *rev3Δ* and *rev3-cysB* derivatives. Rates, number of mutations and percentage are presented in Supporting Information Tables S5. If the number of events is insufficient for comparison, the rates are indicated as open bars.

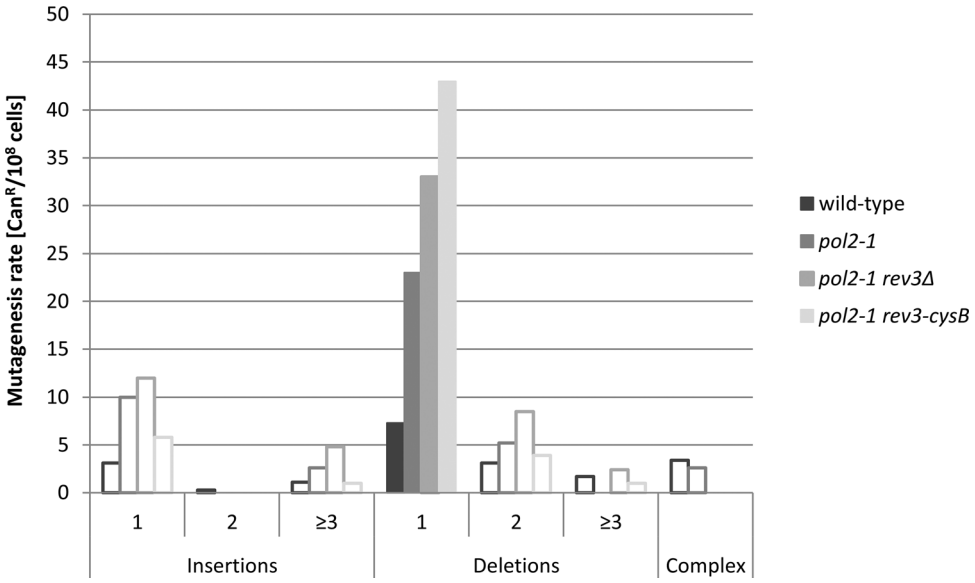

Fig. 6. Rates of individual insertion and deletion types and complex mutations in *pol2-1* strain and its *rev3Δ* and *rev3-cysB* derivatives. Rates, number of mutations and percentage are presented in Supporting Information Tables S5. If the number of events is insufficient for comparison, the rates are indicated as open bars.

fold (from 40 to 210 $\times$ 10^{-8}) and most of this increase was due to transversions (8.9-fold, from 18 to 160 $\times$ 10^{-8}), mainly GC→CG (11-fold, from 6.1 to 67 $\times$ 10^{-8}). Compared to *pol2-1*, the *pol2-1 rev3Δ* spectrum showed a significantly decreased rate of base substitutions (5.4-fold, from 210 to 39 $\times$ 10^{-8}), mainly transversions (12-fold, from 160 to 13 $\times$ 10^{-8}). No GC→CG transversions, one of the hallmarks of Pol ζ activity, were found in the *pol2-1 rev3Δ* strain. In the absence of Pol ζ, frameshifts leading to deletion errors frequently occur in the *pol2-1* strain as deletions represent 44% of all the mutations arising in the *pol2-1 rev3Δ* strain. This is in accordance with the observation that frameshifts are rarely generated through Pol ζ action (Northam *et al.*, 2006; Zhong *et al.*, 2006).

The *rev3-cysB* allele had a similar effect on the mutation spectrum of the *pol2-1* mutant strain as *REV3* deletion (Figs 5 and 6 and Supporting Information Table S5 and Fig. S2). Base substitutions were decreased fivefold (from 210 to 42 $\times$ 10^{-8}), particularly transversions (7.6-fold, from 160 to 21 $\times$ 10^{-8}). Again, GC→CG transversions were severely decreased (14-fold, from 67 to 4.9 $\times$ 10^{-8}). As for *rev3Δ*, 50% of all the mutations occurring in *pol2-1 rev3-cysB* are represented by deletions. Thus, we observed the similarity of the mutational spectra of the *pol2-1 rev3Δ* and *pol2-1 rev3-cysB* double

mutant strains, especially in mutations characteristic for Pol ζ. These results strongly suggest that in the mutant strain with the defective Pol ε, *pol2-1*, Pol ζ_4 seems to be the only Pol ζ form engaged in replication of undamaged DNA.

Characterization of Can^R mutations in psf1-100 rev3-cysB *strain*

Similarly to defective catalytic subunits of the major replicases, also defects in noncatalytic components of the replisome may lead to defective DNA replication and increased Pol ζ involvement (Kraszewska *et al.*, 2012; Grabowska *et al.*, 2014; Garbacz *et al.*, 2015). One such strain compromised in a noncatalytic component of the replisome, therefore, experiencing conditions of DRIM, is *psf1-100*, isolated and described in our laboratory (Grabowska *et al.*, 2014). Psf1p is a GINS subunit required for its functional interaction with the leading-strand DNA polymerase Pol ε (MacNeill, 2010; Hogg and Johansson, 2012). Substitution of four amino acids (V161A, F162A, I163A and D164A) in the C-terminal region of Psf1p impairs the interaction between Psf1p and the Dpb2p subunit of Pol ε (Grabowska *et al.*,

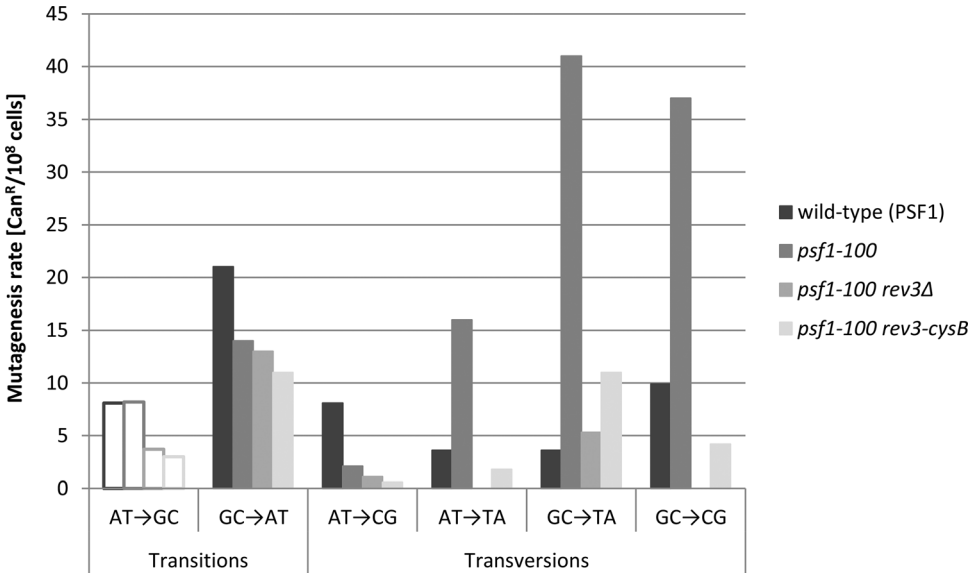

Fig. 7. Rates of individual base substitutions types in *psf1–100* strain and its *rev3Δ* and *rev3-cysB* derivatives. Rates, number of mutations and percentage are presented in Supporting Information Tables S6. If the number of events is insufficient for comparison, the rates are indicated as open bars.

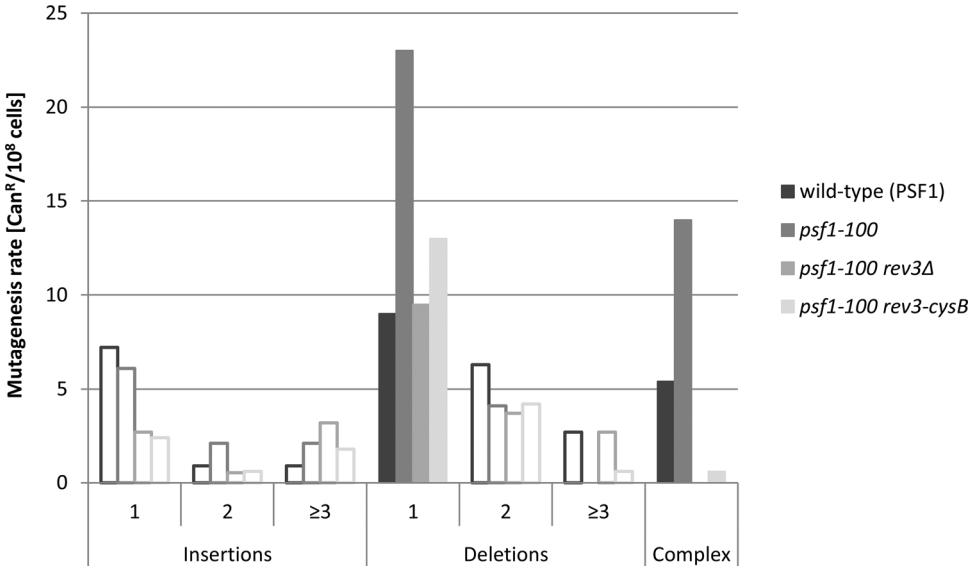

Fig. 8. Rates of individual insertion and deletion types and complex mutations in *psf1–100* strain and its *rev3Δ* and *rev3-cysB* derivatives. Rates, number of mutations and percentage are presented in Supporting Information Tables S6. If the number of mutations is insufficient for comparison, the rates are indicated as open bars.

2014). As a result, the *psf1-100* strain displays a moderate, but almost entirely Pol ζ-dependent mutator phenotype (Grabowska *et al*., 2014). Therefore, the *psf1-100* mutant strain is another appropriate candidate to study which Pol ζ form is involved in the replication mediated by a defective replisome.

Mutagenesis rates at the *CAN1 locus* were determined in the *psf1-100* mutant strain additionally carrying the *rev3*Δ or the *rev3-cysB* allele (Table 1). In agreement with the previous report (Grabowska *et al*., 2014) the mutation rate was increased about twofold in *psf1-100* compared to the wild-type strain (170 to 86 $\times$ 10^{-8}) and inactivation of Pol ζ (*rev3*Δ) fully eliminated this increase (45 $\times$ 10^{-8} in *psf1-100 rev3*Δ). Notably, the *rev3-cysB* allele had almost the same antimutator effect (56 $\times$ 10^{-8} in *psf1-100 rev3-cysB*) as *REV3* deletion. These results indicate that Pol ζ_4 is the major form of Pol ζ responsible for the increase of mutagenesis due to the compromised GINS-Pol ε interaction in the *psf1-100* mutant strain.

For the mutation spectra analysis, we used the data published earlier for the wild-type (*PSF1*), *psf1-100* and *psf1-100 rev3*Δ strains (Grabowska *et al*., 2014) and 93 independent Can^R mutants isolated for the *psf1-100 rev3-cysB* strain in the present study (Figs 7 and 8 and Supporting Information Table S6 and Fig. S2). Deletion of *REV3* gene decreased the rate of base substitutions (5.2-fold, from 120 to 23 $\times$ 10^{-8}), in particular transversions (15-fold, from 96 to 6.4 $\times$ 10^{-8}). No GC→CG transversions and no complex mutations, characteristic for Pol ζ activity, were observed in the *psf1-100 rev3*Δ strain (Grabowska *et al*., 2014). Likewise, in the *psf1-100 rev3-cysB* strain a decrease in the rates of similar types of mutations was observed, especially base substitution (3.6-fold, from 122 to 33 $\times$ 10^{-8}). GC→CG transversions fall 8.8-fold (from 37 to 4.2 $\times$ 10^{-8}) and complex mutations 23-fold (from 14 to 0.6 $\times$ 10^{-8}). In general, the mutation spectra of the *psf1-100 rev3*Δ and *psf1-100 rev3-cysB* strains were highly similar, with mutations characteristic for Pol ζ severely reduced relative to their levels in the single *psf1-100* mutant strain. This analysis confirmed that mutations in the CysB motif preventing Pol ζ_4 complex formation decrease the mutagenesis rate in the *psf1-100* strain in the same manner as the *REV3* gene deletion does. This indicates that Pol ζ_4, not Pol ζ_2, is responsible for the defective-replisome-induced mutator phenotype of the *psf1-100* strain.

Discussion

Recent *in vitro* data (Baranovskiy *et al*., 2012; Johnson *et al*., 2012; Makarova *et al*., 2012) indicate that Pol ζ is a hetero-tetramer composed of Rev3p, Rev7p, Pol31p and Pol32p and not the Rev3p-Rev7p hetero-dimer, as it was previously proposed. In line with the data, *in vivo* studies have also revealed that the four-subunit Pol ζ is indispensable for damage-induced mutagenesis (Baranovskiy *et al*., 2012; Makarova *et al*., 2012). In this report, we have asked which of the two described forms of Pol ζ, Pol ζ_2 or Pol ζ_4, is required for mutagenesis when cells are not treated with any external damaging agents and replication is proceeded by defective replisome.

To discriminate between the two Pol ζ forms we took advantage of the *rev3-cysB* mutation (Makarova *et al*., 2012) eliminating the interaction between the Rev3p and Pol31p subunits and therefore excluding solely the Pol ζ_4 form. Thus, by comparing rates and spectra of spontaneous *CAN1* mutations in three strains: wild-type (*REV3*), Pol ζ null (*rev3*Δ) and *rev3-cysB*, both Pol ζ forms, Pol ζ_2 and Pol ζ_4, could be distinguished. Under conditions of unperturbed DNA replication, modest, but statistically significant decrease of spontaneous mutagenesis rate was observed in both mutant strains compared to the wild-type strain, although in *rev3-cysB* strain not to the same extent as in *rev3*Δ strain (Table 1 and Supporting Information Table S1) and this difference was also observed in other genetic backgrounds (Supporting Information Tables S17–S19). However, the analysis of *rev3*Δ and *rev3-cysB* spectra of mutagenesis did not reveal any statistically significant differences between particular mutation classes, including those characteristic for Pol ζ (Supporting Information Tables S7–S16). These results may thus suggest that Pol ζ_4 is also required, at least in part, for spontaneous mutagenesis in the wild-type strain.

To study which of Pol ζ forms is involved in perturbed replication when replisome is impaired, mutant strains with increased Pol ζ participation in DRIM were studied and analyzed in combination with *rev3*Δ or *rev3-cysB* allele (Makarova *et al*., 2012). Two mutant strains in the catalytic subunits of the major replicative polymerases were used: *pol3-Y708A* (Pol δ) and *pol2-1* (Pol ε), in which mutagenesis was described as 80–90% Pol ζ-dependent (Northam *et al*., 2006). Additionally, almost fully Pol ζ-dependent defective replisome mutant strain, *psf1-100,* was studied (Grabowska *et al*., 2014). Since the increased mutagenesis in those strains is mostly Pol ζ-dependent, large decreases in the mutagenesis are observed when Pol ζ is compromised. Due to this clear difference in the mutagenesis rates between the respective single and double mutant strains, the chosen strains are convenient tools to differentiate between Pol ζ_2 and Pol ζ_4 action during defective replication. The data obtained in this work confirmed the Pol ζ-dependent mutator phenotypes of the three mutant strains. A comparable weakening of the mutator phenotype was observed in all those strains upon *rev3*Δ and *rev3-cysB* mutation (Table 1). The analysis of the

mutagenesis spectra showed that in all the mutant strains studied, both the *REV3* deletion and mutation in CysB motif led to similar decreases in the rates of GC→CG transversions and complex mutations, both hallmarks of Pol ζ mutagenesis (Figs 3–8 and Supporting Information Tables S4–S6). Therefore, our *in vivo* analysis of the mutagenesis rates suggests that, when the replisome is defective, Pol ζ_4 is predominantly required for mutagenesis.

The common subunit composition of Pol δ and Pol ζ (Pol31p and Pol32p subunits) has led to the postulation of the switching hypothesis in which the catalytic subunits of Pol δ and Pol ζ could be exchanged when the replication fork stalls (Baranovskiy *et al*., 2012). Therefore, Pol ζ composed of four subunits might be expected to be involved in the defective lagging-strand replication. However, no data are available to indicate if Pol ζ_4 also participates in the replication of the leading DNA strand, since Pol ε and Pol ζ do not share any common subunits. All of the mutants with DRIM phenotypes studied in this report destabilize the replisome, although it has not been shown if they selectively impair the lagging or the leading strand synthesis. However, it may be assumed that if the lagging-strand polymerase is impaired, like in the *pol3-Y708A* mutant strain, it is the lagging-strand replication that is defective. Thus, our data may suggest that the four-subunit Pol ζ is required during defective replication on the lagging strand. Similarly, assuming that in the *pol2-1* mutant strain the leading-strand replication is deficient, our data may indicate that the four-subunit Pol ζ is also required during defective replication of the leading strand. However, a more complicated scenario cannot be excluded in which, prior to Pol ζ recruitment, a switch between Pol ε and Pol δ occurs, or even, due to presumably more global replication impediment caused by the structural defect of Pol2p, Pol δ could be responsible for replication of the most part of the leading strand in *pol2-1* cells (reviewed in Pavlov and Shcherbakova, 2010; Stillman, 2015).

Both the *REV3* deletion and the *rev3-cysB* mutation resulted in a significant decrease in the mutagenesis rates in all the studied strains. Despite that, slight but statistically significant, differences between the respective *rev3*Δ and *rev3-cysB* mutant strains were noted in the wild-type and *pol3-Y708A* mutant strain (Table 1) and between the rates of some mutation types in *pol2-1* and *psf1-100* (Supporting Information Tables S5–S6). In contrast to strains with *rev3*Δ, residual Pol ζ activity can be observed in strains carrying the *rev3-cysB* mutation as the Rev3-cysB protein is not catalytically deficient but only its interaction with Pol31p is impaired (Makarova *et al*., 2012). It was also shown that *rev3*Δ*C* mutant strain, which lacks the entire C-terminal domain of Rev3p required for Pol31p binding, is partially proficient in Pol32p-dependent UV-induced mutagenesis (Siebler *et al*., 2014). Thus, when Pol ζ is required, some fraction of Pol ζ_4 may still be reconstituted in strains with mutation in CysB motif, through interaction between other subunits within the Pol ζ holoenzyme. Since Pol31p interacts with Pol32p and Rev3p with Rev7p, and an additional interaction between Pol32p and Rev7p has been recently proposed (Gómez-Llorente *et al*., 2013), it may be supposed that in the absence of the Rev3p-Pol31p binding the Pol ζ_4 hetero-tetramer could be stabilized through this Rev7p-Pol32p interaction (Gómez-Llorente *et al*., 2013). Moreover, Rev1p, indispensable for Pol ζ action *in vivo* (Acharya *et al*., 2006), interacts with two Pol ζ_4 subunits: Rev7p (Acharya *et al*., 2005) and Pol32p (Acharya *et al*., 2009; Pustovalova *et al*., 2016) and thus may serve as a connector to support the Pol ζ_4 assembly in the *rev3-cysB* mutant strain. Another possibility is that Pol ζ_2 could be responsible *in vivo* for some fraction of the mutagenesis, as Pol ζ_2 activity has been showed by biochemical methods, although it was much weaker than the activity of Pol ζ_4 (Makarova *et al*., 2012). An *in vitro* analysis has revealed that the lack of the Pol31p and Pol32p subunits prevents the direct Pol ζ interaction with PCNA (proliferating cell nuclear antigen) (Makarova *et al*., 2012), which would certainly limit Pol ζ_2 role during DNA replication. However, Pol ζ_2 efficiency of extension from mispaired primer template could also be enhanced by its indirect interaction with monoubiquitinated PCNA *via* Rev1p (Acharya *et al*., 2006), thus Pol ζ_2 could as well be hypothetically recruited *via* this pathway.

The differences in the mutagenesis rates between strains carrying the *rev3*Δ and the *rev3-cysB* mutations are more pronounced in the wild-type and *pol3-Y708A* contexts than in the strains with the defective leading-strand polymerase. This difference could be due to common Pol31p and Pol32p subunits of Pol ζ and Pol δ (Baranovskiy *et al*., 2012; Johnson *et al*., 2012; Makarova *et al*., 2012). For instance, whenever Pol δ dissociates and is replaced by Pol ζ, if Pol31p-Pol32p remain attached to DNA (Siebler *et al*., 2014), Pol ζ_4 could be partially reconstituted in the *rev3-cysB* strain via additional protein-protein interactions described above. Residual Pol ζ_4 formation and activity could thus be much more pronounced in the Pol δ strain than those in the Pol ε mutant strain. In addition to DNA replication, Pol ζ-dependent spontaneous mutations could arise also in the wild-type strain through various DNA repair processes (Giot *et al*., 1997; Halas *et al*., 2009; Brocas *et al*., 2010; Skoneczna *et al*., 2015) and the difference in mutagenesis between the *rev3*Δ and the *rev3-cysB* mutation might be due to a more frequent Pol δ participation in DNA repair compared to Pol ε (Sparks *et al*., 2012; Ganai *et al*., 2016) and consequently more

frequent Pol δ-Pol ζ exchange. Nevertheless, an involvement of Pol ζ_2 in DNA repair cannot be excluded either.

The findings described here show that a vast majority of mutations caused by defective replisome requires the action of the four-subunit Pol ζ form. While the common Pol δ and Pol ζ subunit composition and the proposed switching hypothesis could explain the Pol ζ_4 involvement when the lagging-strand DNA polymerase is impaired (Makarova and Burgers, 2015), further studies are required to determine the mode of possible Pol ζ_4 recruitment during undamaged DNA replication on the leading strand. Our *in vivo* results indicate that Pol ζ_4 is by far more functional in spontaneous mutagenesis than is Pol ζ_2, but the exact Pol ζ subunit composition in various physiological conditions should also be further investigated. Thorough understanding of Pol ζ composition, recruitment and functioning is of great importance, since misregulation of Pol ζ activity may lead to genomic instability and cancer (Knobel and Marti, 2011; Lange *et al.*, 2011, 2012, 2013, 2016; Sale, 2013; van Loon *et al.*, 2015; Tomida *et al.*, 2015; Suzuki *et al.*, 2016; Tumini *et al.*, 2016). Since human Pol ζ has also been described as a four-subunit complex (Baranovskiy *et al.*, 2012; Lee *et al.*, 2014), our observations in yeast could also be relevant to human Pol ζ.

Experimental procedures

Strains and media

Yeast strains used in this study were constructed in SC765 background (Grabowska *et al.*, 2014), derivative of ΔI(-2)I-7B-YUNI300 (Pavlov *et al.*, 2002). Yeast and bacterial and strains are listed in Supporting Information Table S20. Bacteria were grown at 37°C in standard media and yeast at 30°C in standard media (Amberg *et al.*, 2005) LB (1% tryptone, 1% NaCl and 0.5% yeast extract) supplemented with appropriate antibiotics were used for bacterial transformants. Nonselective yeast complete medium (YPD) (1% yeast extract, 1% peptone and 2% glucose) and minimal medium (SD; 0.67% yeast nitrogen base without amino acids and 2% glucose, supplemented with required amino acids and nitrogenous bases), were used for yeast transformants, mutagenesis assays and 5-FOA selection. SD medium supplemented with L-canavanine (60 mg l^{-1}) was used to determine the frequency of spontaneous mutations at the *CAN1 locus*. SD medium supplemented with 5-fluoroorotic acid (5-FOA) (1 g l^{-1}) was used for selection against *URA3* marker.

Plasmid construction for introduction of rev3-cysB *mutations into chromosome*

Integrative plasmid was constructed as follows: *REV3* sequence with 500 bp of 5′- and 500 bp of 3′-flanking regions was PCR-amplified with primers 5′-GGTACCT CCCTTCATTCACTTGATCATTTG-3′ (KpnI digestion site) and 5′-ACTAGTGAACCCAATCGCTTATGGAAAC-3′ (SpeI digestion site) and cloned into pJET1.2 vector. Using pJET 1.2-*REV3* plasmid as a template, mutations in CysB motif (*rev3-CC1449,1473SS* (Makarova *et al.*, 2012)) were introduced sequentially by site-directed mutagenesis using two pairs of primers for PCR: 5′- CCGTGTGCAGGACGTCCA GTTATCGTTACAC-3′ and 5′- GTGTAACGATAACTGGA CGTCCTGCACACGG-3′ to introduce the G4346C mutation, and 5′- GTAAATGCAATTCATATGACAGTCCAGTA TTTTACTCTCG −3′ and 5′-CGAGAGTAAAATACTGGA CTGTCATATGAATTGCATTTAC-3′ to introduce the T4417A mutation. Correctness of sequences was verified by sequencing. Next, the 2920 bp fragment containing 3′-part of *rev3-cysB* was cut from pJET1.2-*rev3-cysB* with HindIII and SpeI and ligated into the integrative plasmid pRS306 carrying *URA3* marker, cut with HindIII and SpeI.

Integration of rev3-cysB *allele into* REV3 *locus*

Substitution of chromosomal wild-type *REV3* with the point-mutated *rev3-cysB*, *REV3* was performed by two-step gene replacement. SC765 strain was transformed with pRS306-*rev3-cysB* linearized with KfII and transformants growing on plates depleted of Uracil were selected. To remove the *URA3* marker from the *REV3* locus, 5-FOA selection was carried out. The presence of *rev3-cysB* mutation was verified by sequencing of PCR-amplified fragment (primers: 5′-AAAGGGCGAGCACAACTACTAC-3′ and 5′- CTTAGAGGA TACGAAGATTC-3′).

Disruption of REV3 *gene*

The *rev3*::*LEU2 cassette* described in Kraszewska *et al.* (2012) was used for *REV3* disruption in SC765 strain. Deletion of the *REV3* open reading frame was confirmed by PCR using primers flanking the *REV3* locus (5′- GATAAGTATT-CACTAACACC-3′ and 5′- CTTAGAGGATACGAAGATTC-3′).

Integration of psf1-100 *cassette*

The *psf1-100* cassette was integrated into chromosomal *PSF1* locus in strains carrying the *rev3-cysB* mutation. The presence of the *psf1-100* (*CaURA3*) allele was confirmed as described in Grabowska *et al.* (2014).

Construction of pol3-Y708A *strain and its derivatives*

The *pol3-Y708A* allele was integrated into the *POL3* locus of the SC765 strain and its *rev3*Δ and *rev3-cysB* derivatives by two-step gene replacement, as described in Pavlov *et al.* (2001). The presence of the *pol3-Y708A* mutation was verified by sequencing of PCR-amplified fragment (primers: 5′-GTGCCTGGAGATTGATACTGTG-3′ and 5′-CGGAATCA GTGTCACCGTAAAC-3′).

Construction of *pol2-1* strain and its derivatives

The *pol2-1* cassette was integrated into *POL2* locus of the SC765 strain and its *rev3*Δ and *rev3-cysB* derivatives, as

described in (Morrison *et al*., 1990; Shcherbakova *et al*., 1996). The presence of the *pol2-1*(*URA3*) allele was confirmed by PCR using primers flanking the *POL2 locus* (5′-GGCTCTCGTTGGTATTCC-3′ and 5′-GTTAACTAGATCACTGCCTTC-3′).

Determination of spontaneous mutation rates

The mutation rates at the *CAN1 locus* were determined in 9–40 cultures of 2 or 3 independent isolates of each strain. Stationary-phase cultures of each strain grown at 30°C under agitation were diluted as requested and plated on selective and nonselective media. Colonies were counted after 3–5 days of incubation at 30°C. Each experiment was repeated at least three times.

To determine mutant frequency, the respective mutant count was divided by the total cell count. To calculate mutation rates, the following equation was used: $\mu = f/\ln(N{\cdot}\mu)$, where f is the mutant frequency, N is the total population size and μ is the mutation rate per replication (Drake, 1991). To calculate the median values of the mutation rates and 95% confidence intervals STATISTICA 6.0 was used. To determine the *p*-values of the differences between the mutation rates of the respective strains, nonparametric Mann–Whitney *U*-test was used.

Can^R mutation spectra

Single Can^R colonies were selected randomly from plates supplemented with canavanine used to determine spontaneous mutation frequencies at the *CAN1 locus* (see above) and chromosomal DNA was isolated from each colony (Amberg *et al*., 2005). The *CAN1 locus* was PCR-amplified and sequenced using primers described in Kraszewska *et al*. (2012). Sequence alignment and identification of mutations were performed using Clone Manager 9.

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