The CysB motif of Rev3p involved in the formation of the four-subunit DNA polymerase \( \zeta \) 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 ) \( \alpha, \delta \) and \( \varepsilon \). 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 \( \zeta \) ), is responsible for a large portion of mutagenesis in eukaryotic cells. Two forms of Pol \( \zeta \) have been identified, a hetero-dimeric ( Pol \( \zeta_2 \) ) and a hetero-tetrameric ( Pol \( \zeta_4 \) ) ones and recent data have demonstrated that Pol \( \zeta_4 \) is responsible for damage-induced mutagenesis. Here, using yeast Pol \( \zeta \) mutant defective in the assembly of the Pol \( \zeta \) four-subunit form, we show in vivo that [4Fe-4S] cluster in Pol \( \zeta \) 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 \( \alpha \) ), delta ( Pol \( \delta \) ), epsilon ( Pol \( \varepsilon \) ) and zeta ( Pol \( \zeta \) ), 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 \( \alpha \)/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 \( \varepsilon \) and Pol \( \delta \) ( Burgers and Kunkel, 2017 ). In contrast to the well-defined role of Pol \( \alpha \), specific roles of Pol \( \varepsilon \) and Pol \( \delta \) 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 \( \varepsilon \) and Pol \( \delta \) respectively (for review see Kunkel and Burgers, 2017).

DNA polymerase \( \zeta \) 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 \( \zeta \) 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 \( \zeta \) is a translesion ( TLS ) polymerase which, due to more flexible active site and lack of the 3’→5’ exonuclease proof-reading activity, has the ability to bypass DNA lesions that cannot be bypassed by the major replicative polymerases ( Vaisman and Woodgate, 2017 ). Pol \( \zeta \) 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 \( \zeta \) 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 \( \zeta \) 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 \( \zeta \) itself. Indeed, it has been shown that in particular case of yeast mutant strains with defective replisome, 80–90% of arising...
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 ζδ) 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 ζ4), 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 Pol31p (Baranovskiy et al., 2012; Johnson et al., 2012; Makarova et al., 2012).

Biochemical studies have shown that the TLS activity of Pol ζ4 is much more efficient compared to that of Pol ζδ; 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 replicate to an erron-prone TLS polymerase, and two models of Pol ζ4 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 proteosomal degradation of the released Pol3p by Def1p protease supports this scenario (Daraba et al., 2014). In contrast, in another proposed model, the Pol δ heterotrimer is replaced en masse by a hetero-tetrameric Pol ζ4, which in turn is supported by the observation that the Pol ζ4 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 ζδ or Pol ζ4 is involved when Pol ε is defective.

To genetically investigate if Pol ζδ or Pol ζ4 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 ζδ-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 psfl-100 mutation affecting the Psf1p, one of the GINS subunits (Grabowska et al., 2014). Most importantly, the increased mutagenesis in the psfl-100 mutant strain was found to be almost fully Pol ζδ-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 (rev3Δ) abolishing Pol ζ (both dimeric and tetrameric) activity or with the rev3-cysB allele selectively compromising only Pol ζ4 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 CanR 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 ζ4
formation. Accordingly, in the rev3-CC1449,1473SS mutant strain (named rev3-cysB) only a two-subunit Pol ζ complex (Pol ζ2) was detected by biochemical methods (Makarova et al., 2012). In vitro studies have shown that Pol ζ2 is active, although its catalytic efficiency for DNA extension is much lower compared to Pol ζ4 (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 rev3Δ (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 ζ4, 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 ζ4 formation, based on in vitro data (Makarova et al., 2012), we refer to Pol ζ form in rev3-cysB mutant strain as Pol ζ2. 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 Δ(k-2)-7B-YUNI300 strain (Pavlov et al., 2002) carrying either the rev3Δ 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 (CanR) (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 rev3Δ 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 rev3Δ 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.

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 rev3Δ 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 ζ4, 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 ζ4 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 ζ5 (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).

### Table 1. Spontaneous mutation rates for wild-type, pol3-Y708A, pol2-1 and psf1–100 strains and their rev3Δ and rev3-cysB derivatives.

| Relevant genotype | CanR (× 10^{-8}) | Relative rate |
|-------------------|------------------|---------------|
| wild-type         | 60 (56–64)^b     | 1             |
| rev3Δ            | 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 rev3Δ | 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 rev3Δ     | 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 rev3Δ   | 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).

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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 CanR mutants were analyzed, in the wild-type and rev3Δ strains, respectively, whereas 85 independent CanR 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 CanR 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 rev3A 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 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 CanR mutations in pol3-Y708A rev3-cysB strain

The sharing of subunits between Pol δ and Pol ζ may suggest that it is Pol ζ, not Pol ζ2, 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 ζ 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 rev3Δ or the rev3-cysB allele (Table 1). The mutation rate in the pol3-Y708A mutant strain was elevated eightfold 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 rev3A), 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 rev3Δ and pol3-Y708A rev3-cysB strains (Supporting Information Table S1). Nonetheless, our results indicates that Pol ζ4 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 CanR mutations were determined in relevant strains, as before. For each strains 91 to 94 independent CanR 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 rev3Δ. 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 rev3Δ 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 ζ4 participates in the replication of undamaged DNA.

Characterization of CanR 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,
largely Pol \( \zeta \)-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 rev3\( \Delta \) 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 \( \zeta \) (250 \( \times \) \( 10^{-8} \) in \( pol2-1 \) and 99 \( \times \) \( 10^{-8} \) in \( pol2-1 \ rev3\( \Delta \)). 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 \( \zeta \)-dependent. The rev3-cysB mutation had exactly the same antimutator effect (96 \( \times \) \( 10^{-8} \) in the \( pol2-1 \ rev3\( \Delta \) strain) as the complete elimination of Pol \( \zeta \) activity (rev3\( \Delta \)). Thus, in this particular situation, when Pol \( \epsilon \) is defective, the intact CysB motif of Rev3p is required for mutagenesis.

To determine the mutational specificity of the two defective Pol \( \zeta \) alleles, the mutational spectra of relevant strains were determined as before, with 82 to 99 independent Can\( \text{R} \) 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-
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

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mutant strains, especially in mutations characteristic for Pol \( \zeta \). These results strongly suggest that in the mutant strain with the defective Pol \( \epsilon \), pol2-1, Pol \( \zeta_4 \) seems to be the only Pol \( \zeta \) form engaged in replication of undamaged DNA.

**Characterization of CanR 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 \( \zeta \) involvement (Kraszew ska 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 \( \epsilon \) (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 \( \epsilon \) (Grabowska et al.,

**Fig. 7.** Rates of individual base substitutions types in psf1-100 strain and its rev3\( \Delta \) 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 rev3\( \Delta \) 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.
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 rev3Δ 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 × 10⁻⁸) and inactivation of Pol ζ (rev3Δ) fully eliminated this increase (45 × 10⁻⁸ in psf1-100 rev3Δ). Notably, the rev3-cysB allele had almost the same antimutator effect (56 × 10⁻⁸ in psf1-100 rev3-cysB) as REV3 deletion. These results indicate that Pol ζ is the major form of Pol ζ responsible for the increase of mutagenesis due to the compromised GINS-Pol ζ 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 rev3Δ strains (Grabowska et al., 2014) and 93 independent CanR 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 × 10⁻⁸), in particular transversions (15-fold, from 96 to 6.4 × 10⁻⁸). No GC–CG transversions and no complex mutations, characteristic for Pol ζ activity, were observed in the psf1-100 rev3Δ 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 × 10⁻⁸), GC–CG transversions fall 8.8-fold (from 37 to 4.2 × 10⁻⁸) and complex mutations 23-fold (from 14 to 0.6 × 10⁻⁸). In general, the mutation spectra of the psf1-100 rev3Δ 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 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 ζΔ, not Pol ζΔ, 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 dispensable 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 ζΔ or Pol ζΔ, 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 ζΔ form. Thus, by comparing rates and spectra of spontaneous CAN1 mutations in three strains: wild-type (REV3), Pol ζ null (rev3Δ) and rev3-cysB, both Pol ζ forms, Pol ζΔ and Pol ζΔ, 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 rev3Δ 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 rev3Δ 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 ζΔ 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 rev3Δ or rev3-cysB allele (Makarova et al., 2012). Two mutant strains in the catalytic subunits of the major replicative polymerases were used: pol3-7078A (Pol 5) and pol2-1 (Pol c), in which mutagenesis was described as 80–90% Pol 5-dependent (Northam et al., 2006). Additionally, almost fully Pol 5-dependent defective replisome mutant strain, psf1-100, was studied (Grabowska et al., 2014). Since the increased mutagenesis in those strains is mostly Pol 5-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 ζΔ and Pol ζΔ 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 rev3Δ 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 ε 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 ε 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 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 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 rev3Δ 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 rev3Δ, 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 rev3ΔC 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 ζ4 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 ζ4 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 ζ4 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 ζ4 assembly in the rev3-cysB mutant strain. Another possibility is that Pol ζ2 could be responsible in vivo for some fraction of the mutagenesis, as Pol ζ2 activity has been showed by biochemical methods, although it was much weaker than the activity of Pol ζ4 (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 ζ2 role during DNA replication. However, Pol ζ2 efficiency of extension from mis-paired primer template could also be enhanced by its indirect interaction with monoubiquitinated PCNA via Rev1p (Acharya et al., 2006), thus Pol ζ2 could as well be hypothetically recruited via this pathway.

The differences in the mutagenesis rates between strains carrying the rev3Δ 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 ζ4 could be partially reconstituted in the rev3-cysB strain via additional protein-protein interactions described above. Residual Pol ζ4 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 rev3Δ 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 ζ2 in DNA repair cannot be excluded either.

The findings described here show that a vast majority of mutations caused by defective replisomes 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 involvement when the lagging-strand DNA polymerase is impaired (Makarova and Burgers, 2015), further studies are required to determine the role of possible Pol ζ4 recruitment during undamaged DNA replication on the leading strand. Our in vivo results indicate that Pol ζ4 is by far more functional in spontaneous mutagenesis than is Pol ζ2, 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 (Baranovsky 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 Δl-(2)-7B-YUNI300 (Pavlov et al., 2002). Yeast and bacterial strains are listed in Supporting Information T able 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, 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 1-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 CGCTTCATCTTACCACTTG-3' (KpnI digestion site) and 5'-ACTAGTGACCCATCGTATCGGAAC-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'-CGGTGTGAGAGCTCCTGACCCATCG-3' and 5'-GGTAAAGATGAACTCCTGACCCATCG-3'. The presence of the SC765 strain and its derivatives pol3-Y708A and pol3-CC1449,1473SS (Makarova et al., 2012)) were introduced sequentially by site-directed mutagenesis using two pairs of primers for PCR: 5'-GGTAAAGATGAACTCCTGACCCATCG-3' and 5'-GGTAAAGATGAACTCCTGACCCATCG-3'. The presence of the SC765 strain and its derivatives pol3-Y708A and pol3-CC1449,1473SS (Makarova et al., 2012)) were introduced sequentially by site-directed mutagenesis using two pairs of primers for PCR: 5'-GGTAAAGATGAACTCCTGACCCATCG-3' and 5'-GGTAAAGATGAACTCCTGACCCATCG-3'. The presence of the SC765 strain and its derivatives pol3-Y708A and pol3-CC1449,1473SS (Makarova et al., 2012)) were introduced sequentially by site-directed mutagenesis using two pairs of primers for PCR: 5'-GGTAAAGATGAACTCCTGACCCATCG-3' and 5'-GGTAAAGATGAACTCCTGACCCATCG-3'. The presence of the SC765 strain and its derivatives pol3-Y708A and pol3-CC1449,1473SS (Makarova et al., 2012)) were introduced sequentially by site-directed mutagenesis using two pairs of primers for PCR: 5'-GGTAAAGATGAACTCCTGACCCATCG-3' and 5'-GGTAAAGATGAACTCCTGACCCATCG-3'. The presence of the SC765 strain and its derivatives pol3-Y708A and pol3-CC1449,1473SS (Makarova et al., 2012)) were introduced sequentially by site-directed mutagenesis using two pairs of primers for PCR: 5'-GGTAAAGATGAACTCCTGACCCATCG-3' and 5'-GGTAAAGATGAACTCCTGACCCATCG-3'. The presence of the SC765 strain and its derivatives pol3-Y708A and pol3-CC1449,1473SS (Makarova et al., 2012)) were introduced sequentially by site-directed mutagenesis using two pairs of primers for PCR: 5'-GGTAAAGATGAACTCCTGACCCATCG-3' and 5'-GGTAAAGATGAACTCCTGACCCATCG-3'. The presence of the SC765 strain and its derivatives pol3-Y708A and pol3-CC1449,1473SS (Makarova et al., 2012)) were introduced sequentially by site-directed mutagenesis using two pairs of primers for PCR: 5'-GGTAAAGATGAACTCCTGACCCATCG-3' and 5'-GGTAAAGATGAACTCCTGACCCATCG-3'. The presence of the SC765 strain and its derivatives pol3-Y708A and pol3-CC1449,1473SS (Makarova et al., 2012)) were introduced sequentially by site-directed mutagenesis using two pairs of primers for PCR: 5'-GGTAAAGATGAACTCCTGACCCATCG-3' and 5'-GGTAAAGATGAACTCCTGACCCATCG-3'.

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 KflI and transfectants 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'-AAAGGGCCGACACACTA-3' and 5'-TTTAGAGGATGATTC-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'- GTAATGCATGTGCACGCA-3' and 5'-TTTAGAGGATGATTC-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 rev3Δ 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'-GTTGCGGAGATGATGATGATG-3' and 5'-CGGAAATCA GTGTCACCAGAAC-3').

Construction of pol2-1 strain and its derivatives

The pol2-1 cassette was integrated into POL2 locus of the SC765 strain and its rev3Δ 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’-GGCTCTCGTTGGTATCC-3’ and 5’-GTTAACTAGATCAC TGCCCTC-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: \( \mu = f/Nl \), where \( f \) is the mutant frequency, \( N \) is the total population size and \( l \) 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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Supporting information

Additional supporting information may be found in the online version of this article at the publisher’s web-site.