Agnieszka Halas*, Agnieszka Podlaska*, Joanna Derkacz, Justyna McIntyre, Adrianna Skoneczna and Ewa Sledziewska-Gojska
Institute of Biochemistry and Biophysics Polish Academy of Sciences, 02-106 Warsaw, Poland

*These authors contributed equally to this work

The roles of PCNA-SUMOylation, Mms2-Ubc13 and Rad5 in translesion DNA synthesis in *Saccharomyces cerevisiae*.

Correspondence: Ewa Sledziewska-Gojska, Institute of Biochemistry and Biophysics Polish Academy of Sciences, ul. Pawinskiego 5A, 02-106 Warsaw, Poland
Tel: 48 22 658 47 34
Fax: 48 39 12 16 23
Email: esg@ibb.waw.pl

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Abstract

Mms2, in concert with Ubc13 and Rad5, is responsible for polyubiquitination of replication processivity factor PCNA. This modification activates recombination-like DNA damage-avoidance mechanisms, which function in an error-free manner. Cells deprived of Mms2, Ubc13 or Rad5 exhibit mutator phenotypes due to the channeling of premutational DNA lesions to often error-prone translesion DNA synthesis (TLS). Here we show that Siz1-mediated PCNA SUMOylation is required for the stimulation of this TLS, despite the presence of PCNA monoubiquitination. The stimulation of spontaneous mutagenesis by Siz1 in cells carrying *rad5* and/or *mms2* mutations is connected with the known role of PCNA SUMOylation in the inhibition of Rad52-mediated recombination. However, following UV irradiation, Siz1 is engaged in additional, as yet undefined, mechanisms controlling genetic stability at the replication fork. We also demonstrate that in the absence of PCNA SUMOylation, Mms2-Ubc13 and Rad5 may, independently of each other, function in the stimulation of TLS. Based on this finding and on an analysis of the epistatic relationships between *SIZ1*, *MMS2* and *RAD5*, with respect to UV sensitivity, we conclude that PCNA SUMOylation is responsible for the functional differences between the Mms2 and Rad5 homologs of *S. cerevisiae* and *S. pombe.*

Introduction

Despite the continuous action of many DNA repair mechanisms, not all DNA lesions induced by the environment and reactive species produced by cellular metabolism can be removed before the onset of DNA replication. Stalling of the replication complex on such template lesions represents a critical threat to cell viability. DNA damage-tolerance mechanisms permit the completion of DNA replication without the removal of template DNA lesions blocking the replication complex (Lawrence, 1994; 2007; Barbour and Xiao, 2003). One mechanism of damage tolerance is translesion DNA synthesis (TLS) employing specialized DNA polymerases. These polymerases are able to carry out nucleotide insertion opposite a damaged base in the DNA template and elongate this non-canonical primer:template base pair by inserting a few further nucleotides until DNA synthesis is taken over by the regular DNA replicases. Budding yeast possesses three TLS polymerases: Pol eta, Pol zeta and Rev1. Another mechanism active in DNA damage tolerance is the recombination-like DNA damage-avoidance pathway (DDA), mediated by Rad5-Ubc13-Mms2. In this pathway, the replicating polymerase avoids synthesis opposite damaged DNA by transient switching of the template, from the damaged DNA strand to the intact newly synthesized strand of the sister chromatid

or another homologous DNA sequence, if available. The selection of which tolerance mechanism is employed during a fork stalling event depends on the modification status of the DNA replication processivity factor, PCNA (Hoege *et al*., 2002). A trimer of PCNA forms a sliding clamp on the DNA that binds DNA replicases and ensures their processive action during DNA synthesis. Besides binding DNA polymerases, PCNA also coordinates the functions of enzymes on the lagging DNA strand that are involved in the maturation of Okazaki fragments (Kao and Bambara, 2003; Garg and Burgers, 2005). In response to DNA damage, the Rad6/Rad18 complex monoubiquitinates Lys^{164} of PCNA. It has been established that TLS polymerases Pol eta and Rev1 have ubiquitin binding domains that recognize ubiquinated Lys^{164} of PCNA (Bienko *et al.*, 2005). The attachment to monoubiquitinated PCNA is required for TLS, and presumably positions the TLS polymerases in the replication fork (Kannouche *et al*., 2004; Friedberg *et al*., 2005). In support of this presumption, it has been reported that the binding of Rev1 to monoubiquitinated PCNA may influence the processivity and specificity of DNA synthesis by this TLS polymerase (Wood *et al*., 2007). It has also recently been shown, using an *in vitro* reconstitution system, that monoubiquitination of PCNA is required for efficient replacement of stalled replicative polymerase delta by Pol eta (Zhuang *et al*., 2008). These *in vitro* experiments provide a mechanistic basis for the results of numerous genetic experiments indicating that monoubiquitination of PCNA promotes TLS.

Monoubiquitinated PCNA can be further modified by the addition of a Lys^{63}-linked polyubiquitin chain (Hofmann and Pickart, 1999). This polyubiquitination is mediated by a Mms2-Ubc13 ubiquitin-conjugating complex, which functions in concert with RING E3 ubiquitin ligase Rad5. Rad5 is a multi-functional protein that interacts with Ubc13 and also with Rad18 and PCNA, and carries DNA helicase activity (Blastyak *et al*., 2007). Rad5-Ubc13-Mms2-mediated polyubiquitination of PCNA turns on the recombination-like DDA pathway. Although the precise mechanism of this pathway is yet to be defined, it is likely to involve a copy choice-type of DNA synthesis using the daughter strand of the sister duplex as the template for bypassing the lesion (Higgins *et al*., 1976; Haracska *et al*., 2004). It has been shown that the ATP-dependent helicase activity of Rad5 is important in the process of transient template switching (Blastyak *et al*., 2007). Rad5-Ubc13-Mms2-controlled DDA functions in an error-free manner and defect in any component of the polyubiquitination complex causes a mutator phenotype, which reflects the interception of processing of template damage by TLS performed by error-prone Pol zeta (Broomfield *et al*., 1998; Brusky *et al*., 2000).

In *S. cerevisiae*, the Lys^{164} moiety of PCNA can not only be mono- and polyubiquitinated, it may also be SUMOylated. In contrast to ubiquitination, SUMOylation of PCNA occurs at the onset of S phase, independently of DNA damage, and is directed by the SUMO-conjugating enzyme Ubc9 in concert with specific SUMO ligase, Siz1. In the spontaneous mutagenesis observed in *rad18* mutants, in which Lys^{164} of PCNA cannot be ubiquitinated, the mutagenic DNA damage bypass mediated by Pol zeta is dependent on Siz1 (Stelter and Ulrich, 2003). The mechanisms underlying this mutagenic pathway are poorly understood. On the other hand, it has been demonstrated that PCNA SUMOylation inhibits homologous recombination (HR) *via* stimulation of the recruitment of antirecombinogenic helicase Srs2 (Papouli *et al.*, 2005; Pfander *et al*., 2005). This mechanism may aid replication by preventing unwanted recombination. However, when Rad6-directed mechanisms of DNA lesion bypass are inactivated, Rad52-dependent recombinational repair may provide a salvage pathway. Although, consistently with this notion, deletion of *SRS2* and/or *SIZ1* has been shown to suppress the lethal effects of DNA damaging treatments in *rad6* and *rad18* mutants (Friedl *et al*., 2001; Broomfield and Xiao, 2002; Pfander *et al*., 2005), the nature of the interplay between Rad52-dependent recombination repair and TLS is not clear.

In this study, we further examine the role of Siz1, the factor responsible for PCNA SUMOylation, in TLS. We show that Siz1 promotes both spontaneous and UV-induced mutator phenotypes in yeast strains proficient in PCNA monoubiquitination but defective in Mms2, Ubc13 and/or Rad5. Surprisingly, our results also indicate that Mms2-Ubc13 and Rad5 may, independently of one another, stimulate TLS in yeast cells devoid of Siz1. Finally, presented results point to the functioning of Siz1 in a novel mechanism, which contributes to the maintenance of genome stability during replication.

Results

SUMOylation of PCNA stimulates the mutator phenotype caused by Mms2 deficiency.

The mutator phenotype caused by disruption of the *MMS2* gene is explained by the channeling of premutational DNA lesions to the often error-prone TLS pathway. Consistent with this interpretation, the spontaneous mutator effect caused by the *mms2* mutation is entirely dependent on error-prone TLS polymerase Pol zeta (Broomfield *et al*., 1998; Xiao *et al*., 1999; Brusky *et al*., 2000). Since it has been shown that Pol zeta-dependent mutagenesis

can be stimulated by Siz1-mediated PCNA SUMOylation (Stelter and Ulrich, 2003), we examined whether this PCNA modification also stimulates the mutagenesis caused by Mms2 deficiency. The effect of disruption of *SIZ1* on the rate of *trp1-1* (*amber*) reversions and on forward mutations leading to canavanine resistance (Can^R) was analyzed. In Mms2-deficient yeast strains, the absence of PCNA SUMOylation caused decreases of 70% and 50% in *trp1-1* reversions and Can^R forward mutations, respectively, (Fig.1 AB). The role of Siz1 in Pol zeta-dependent mutagenesis in yeast cells devoid of PCNA monoubiquitination was demonstrated by Stelter and Ulrich (2003). Yeast strains deficient in Mms2 lack PCNA polyubiquitination, although monoubiquitination of PCNA is still detected (Hoege *et al*., 2002). Therefore, our results indicate that PCNA SUMOylation plays an important role in mutagenesis occurring in yeast cells proficient in PCNA monoubiquitination.

The dependence of the mutator effect caused by Mms2 deficiency on both Pol zeta and PCNA SUMOylation strongly suggests that this PCNA modification plays a role in the stimulation of TLS. However, as it has been proposed that spontaneous lesion bypass (SLB) and TLS induced by UV (or other exogenous mutagenic agents) may employ different mechanisms (Minesinger and Jinks-Robertson, 2005), we examined the effect of *siz1* deletion on UV-induced mutagenesis in Mms2-deficient strains. The absence of PCNA SUMOylation resulted in a significant decrease in the levels of UV-induced *trp1-1* reversions and Can^R mutations in the *mms2* strain (Fig.1 CD), confirming that despite the presence of PCNA monoubiquitination, at least some TLS requires PCNA SUMOylation to proceed.

Mms2-Ubc13 and Rad5 independently promote TLS in PCNA SUMOylation-deficient cells

To determine whether Siz1 activity is involved in mutagenesis in yeast cells specifically lacking Mms2, or in those generally defective in PCNA polyubiquitination and DDA activity, we examined the Siz1-dependence of the mutator effects connected with deficiencies of Rad5 and Ubc13. Both *ubc13*- and *rad5*-mediated spontaneous and UV-induced mutator effects appeared to be significantly reduced by deletion of *SIZ1* (Table 1). This suggests that the processing of DNA lesions by TLS in DDA-deficient strains is at least partially dependent on PCNA SUMOylation. To further investigate the suggestion that DDA deficiency leads to a mutator phenotype stimulated by PCNA SUMOylation, we examined the role of Siz1 in spontaneous and UV-induced mutagenesis in three double mutants: *mms2ubc13*, *mms2rad5* and *rad5ubc13*. Surprisingly, we found that both the spontaneous and the UV-induced mutator phenotypes of *mms2rad5* or *ubc13rad5* double mutants were entirely alleviated in

cells lacking Siz1 (Fig.2 AB and S1 AB). The levels of UV-induced mutations in the triple mutants *siz1mms2rad5* and *siz1ubc13rad5* were in fact even lower than those in yeast proficient in DDA. The suppression effect of Siz1 deficiency on the mutator phenotype conferred by the *mms2ubc13* double mutation was comparable with that established for *mms2* and *ubc13* single mutants (Fig.2 C and S1 C). Therefore, on the one hand, we confirmed the role of Siz1 in the stimulation of TLS when DDA is defective, but, on the other, we found that Mms2, Ubc13 and Rad5 can stimulate TLS in PCNA SUMOylation-deficient strains. Mms2 and Ubc13 act in concert in this process, whereas Rad5 functions independently. Since each of the investigated single mutations (*mms2, ubc13* and *rad5*) is sufficient to prevent polyubiquitination of PCNA and in consequence inactivate DDA, the additivity of the antimutator effects observed in *mms2rad5siz1* or *ubc13rad5siz1* triple mutants indicates that other activities of Mms2-Ubc13 and Rad5, independent of PCNA ubiquitination, are involved in the stimulation of TLS in yeast cells deficient in PCNA SUMOylation. To establish if enzymatic activities of Rad5 are involved in stimulation of TLS, we compared the effects of plasmids encoding wild type Rad5 and its variants Rad5-DE681,682AA (ATPase deficient) or Rad5-CC914,917AA (defective in ubiquitin ligase activity) on frequencies of *trp1-1* reversion in *ubc13rad5siz1* and *mms2rad5siz1* triple disruptants (Fig.3). The plasmid born Rad5 defective in ATPase, as well as Rad5 defective in ubiquitin ligase activity, caused a mutation frequency increase comparable to that resulting from activity of intact Rad5. These results extend our previous conclusion, that Rad5 stimulates TLS independently of its role in PCNA polyubiquitination, indicating that both Rad5 activities engaged in DDA are dispensable in Rad5-mediated stimulation of TLS.

Siz1 either stimulates or suppresses UV-induced mutagenesis depending on the activity of Rad5, Mms2 and Rad52

The most well characterized function of Siz1-mediated SUMOylation of PCNA is the inhibition of homologous recombination (HR) during the S phase, *via* the recruitment of the Srs2 helicase (Papouli *et al*., 2005; Pfander *et al*., 2005). Srs2 disrupts the Rad51 nucleoprotein filament, which is a crucial early recombinogenic intermediate (Krejci *et al*., 2003; Veaute *et al*., 2003). Inactivation of the Siz1/Srs2 antirecombinogenic mechanism suppresses the UV sensitivity of yeast cells deficient in Rad6, in a Rad52-dependent manner. The role of Rad52 indicates that HR may provide a salvage pathway for stalled replication forks in cells deficient in Siz1-mediated PCNA SUMOylation (Schiestl *et al*., 1990). By analogy, it may be hypothesized that in absence of PCNA SUMOylation (*siz1*), some portion

of DNA lesions may be processed by HR in an error-free manner, whereas in Siz1-proficient cells, the lesions are channeled to mutagenic TLS due to the inhibition of HR. To test this hypothesis, we examined the role of Siz1 in modulating the level of error-prone TLS by analyzing the dependence of spontaneous and UV-induced mutagenesis on Rad52 in yeast cells carrying deletions of the genes involved in PCNA polyubiquitination, accompanied by Siz1 deficiency. As anticipated, deletion of *RAD52* increased the levels of spontaneous mutagenesis in *mms2siz1*, and *rad5siz1* double mutants as well as in the *mms2rad5siz1* triple mutant (Fig.4 ACEG). These results confirmed that Siz1-mediated inhibition of the Rad52 salvage pathway is an important factor in the mutator phenotype in cells defective in DDA (the interplay between DDA, HR and TLS is summarized in Fig. S2). However, the effects on UV-induced mutagenesis were more complicated. Although the deletion of *RAD52* in strains deficient in DDA (*mms2* and/or *rad5* mutants) and devoid of Siz1, caused an increase in mutation frequency, the extent of these increases was surprisingly high (Fig.4 BDFH). The induced mutation frequencies in s*iz1mms2rad52* or *siz1rad5rad52* triple mutants significantly exceeded those observed in the initial mutator strains deficient in DDA (*mms2* or/and *rad5*), Rad52, or both (*mms2rad52*, *rad5rad52*). Since the mutator phenotypes of cells deficient in HR and/or DDA completely depend on activity of Pol zeta, we checked whether Rev3, the catalytic subunit of this TLS polymerase, is also required for hyper-mutator phenotypes of s*iz1mms2rad52* and *siz1rad5rad52* triple mutants. Deletion of the *REV3* gene in both *siz1mms2rad52* and *siz1rad5rad52* triple mutants eliminated over 90% of spontaneous and UV induced mutations. Namely, the frequencies of spontaneous mutations leading to TRP prototrophy in *siz1mms2rad52rev3* and *siz1rad5rad52rev3* were 0.29×10^{-7} and 0.11×10^{-7}, respectively. The frequencies of UV induced mutations were 2.4×10^{-7} and 0.8×10^{-7} for *siz1mms2rad52rev3* and *siz1rad5rad52rev3*, respectively. These results indicate that Pol zeta activity is responsible for the hyper-mutator phenotype occurring in DDA and HR deficient strains in response to Siz1 deficiency. This hyper-mutator phenotype cannot be explained by switching off the antirecombinogenic activity of Siz1. We conclude that besides its function in the inhibition of HR, Siz1 plays an additional unknown role in controlling genetic stability in response to UV radiation. To further characterize this additional role of Siz1, we investigated the effects of Siz1 and/or Rad52 deficiency on the UV sensitivity of cells carrying *RAD5* and/or *MMS2* deletions (Fig.5). In agreement with the findings of a previous study suggesting specific suppression of UV sensitivity, caused by DDA pathway defects, by dysfunction of a Siz1/Srs2 anti-recombinogenic mechanism (Broomfield and Xiao, 2002), we found that deletion of *SIZ1* largely suppressed the UV sensitivity of the *rad5* mutant.

Surprisingly, this suppression was not seen in cells defective in Mms2, and under the experimental conditions employed, the *mms2siz1* double mutant was slightly but consistently more sensitive than the *mms2* single mutant. This result indicates that *siz1*-mediated suppression of UV sensitivity, caused by *rad5* mutation, is not related to the DDA deficiency conferred by this mutation (since both *mms2* and *rad5* mutations result in DDA deficiency). Intriguingly, we noticed that in the *siz1* background, UV sensitivity due to Rad5 deficiency was similar to that caused by the absence of Mms2. This similarity, existing only in Siz1-deficient cells of *S. cerevisiae*, resembles the situation in *S. pombe*, where PCNA is normally not SUMOylated and *mms2* and *rad5* cause similar UV sensitivity (Frampton *et al*., 2006).

Disruption of *RAD52* appeared to neutralize the suppression of *rad5*-mediated UV sensitivity by *siz1*, in a manner similar to that seen in *siz1*-mediated suppression of UV sensitivity in *rad18* mutants (Pfander *et al*., 2005). However, the *siz1rad5rad52* and *siz1mms2rad52* triple mutants and the *siz1mms2rad5rad52* quadruple mutant were significantly more sensitive to UV radiation than *rad5rad52*, *mms2rad52* and *mms2rad5rad52* mutants, respectively (Fig.5). These results are consistent with those of the mutagenesis experiments, and support the notion that, besides its function in the anti-recombinogenic pathway, Siz1 plays an additional role in the defense against lesions blocking the replication fork.

Discussion

This genetic analysis of the role of Siz1 and proteins involved in homology-dependent mechanisms of DNA damage tolerance (DDA and HR) in the modulation of Pol zeta-dependent mutagenesis (spontaneous and UV-induced), challenges several aspects of the current view of the roles of PCNA modifications in the stimulation of TLS.

First, our results postulate roles for both Rad5 and Mms2-Ubc13 in stimulating TLS in the yeast *S. cerevisiae* (Fig.2 and S1). Although the well established function of Rad5 is connected with error-free DDA, and deficiency of this protein increases the frequency of spontaneous and UV-induced mutagenesis, an antimutator phenotype triggered by deletion of *RAD5* has been reported for selected markers (Lawrence and Christensen, 1978; Johnson *et al*., 1992; Minesinger and Jinks-Robertson, 2005; Gangavarapu *et al*., 2006). For one of these markers, *arg4-17*, it appears that stimulation of UV-induced mutagenesis by Rad5 is independent of its ubiquitin ligase and helicase activities as well as of the activity of Mms2-Ubc13 (Gangavarapu *et al*., 2006). It has also recently been shown that Rad5 is required for DNA synthesis through the site specifically inserted AP site, cis-syn TT dimer, (6-4) TT

photoproduct and AAF adduct in a plasmid system (Pagès *et al*., 2008). These findings strongly suggest that Rad5 may promote TLS under certain physiological conditions. However, for the majority of chromosomal mutagenesis markers investigated to date, the TLS-stimulating activity of Rad5 could not be detected. The results of the present study point to PCNA SUMOylation as a factor that suppresses the TLS-associated activity of Rad5. We have shown that the antimutagenic activity connected with defective Rad5 can be detected in *siz1* mutants deprived of PCNA SUMOylation.

The results of this study also indicate that besides Rad5, also Mms2 and Ubc13 are involved in the stimulation of TLS in cells deprived of Siz1 SUMO ligase. The role of Mms2 in the stimulation of TLS in *S. cerevisiae* was previously unrecognized (Gangavarapu *et al*., 2006; Pagès *et al*., 2008). However, while this paper was in preparation, the results of a study on TLS employing site specifically inserted DNA lesions in a plasmid system in *S. pombe*, have been published, which indicate that both Mms2 and Ubc13 are required for Pol zeta-dependent TLS in fission yeast (Coulon *et al*., 2010). This finding is consistent with the results of our experiments with *S. cerevisiae* deprived of Siz1. Intriguingly, one important difference between budding and fission yeasts is that PCNA is not SUMOylated in the latter (Ulrich, 2009). This supports our conclusion that PCNA SUMOylation suppresses the TLS-stimulating activity of Rad5 and Mms2-Ubc13.

The findings of previous studies on *S. pombe* (Coulon *et al*., 2010) and those of the present study indicate that all three proteins involved in polyubiquitination of PCNA (Rad5, Mms2 and Ubc13) are engaged in the stimulation of TLS. The similar effects of *Δrad5*, *Δmms2* and *Δubc13* on the efficiency of synthesis through the lesions site specifically inserted into plasmid DNA, prompted Coulon *et al*. to suggest that whole polyubiquitinating complex Rad5-Ubc13-Mms2 is required for TLS stimulation and, in consequence, that the PCNA polyubiquitination plays stimulatory role in TLS. However, according to our epistatic analysis, whereas Mms2 and Ubc13 function together (Fig.2 C and S1C), which strongly suggests that the E2 activity of the Mms2/Ubc13 complex is important in stimulation of TLS, Rad5 affects TLS independently of Mms2 and Ubc13 (Fig.2 AB and S1AB). Since stimulation of TLS by Mms2-Ubc13 occurs in Rad5-deficient cells in which PCNA cannot be polyubiquitinated, it may be assumed that Mms2-Ubc13-dependent polyubiquitination of other than PCNA, currently unknown target favors Pol zeta-dependent TLS. Analogously, it may be also assumed that separate function of Rad5, other than that involved in DDA, is engaged in TLS stimulation (Fig.2 AB and Fig.3). The presence of Rad5 in stalled replication forks, performing a role that is independent of its activity in DDA, may be due to its

interaction with both PCNA and Rad18 (Ulrich and Jentsch, 2000). A recent report indicates that Rad5 also physically interacts with Rev1, which subsequently interacts with both Pol zeta and monoubiquitinated PCNA (Pagès *et al*., 2008). These interactions of Rad5 may stimulate the recruitment of Pol zeta to stalled replication forks and reinforce Pol zeta-dependent TLS.

Since in our analysis, the new TLS stimulating activities of Rad5 and Ubc13-Mms2 have been shown in the context of DDA inactivation, resulting from the absence of Rad5-Mms2-Ubc13 heterotrimer and PCNA polyubiquitination, we cannot formally exclude the possibility, that Rad5-Mms2-Ubc13 heterotrimer and PCNA polyubiquitination, besides the obvious roles in initiation of DDA, have also additional structural roles in modulation of TLS. However, alternative possibility is, that Rad5-Mms2-Ubc13 heterotrimer and/or PCNA polyubiquitination do not play any extra roles in TLS modulation and that occurrence of unprocessed DNA lesions, due to elimination of DDA, is a sufficient condition to reveal the new activities of Rad5 and Mms2-Ubc13 in stimulation of TLS.

On the other hand, since Rad5 (predominantly) and Mms2-Ubc13 (exclusively) become important for TLS in *S. cerevisiae* lacking Siz1, it may be assumed that Siz1-dependent stimulation of TLS somehow suppresses the requirement for Rad5 and Mms2-Ubc13 in this process. This raises questions about the mechanism responsible for the Siz1-mediated stimulation of TLS. It is well established that Siz1-dependent PCNA SUMOylation causes inhibition of HR during S phase, *via* recruitment of the Srs2 helicase (Pfander *et al*., 2005). Deletion of either *SRS2* or *SIZ1* suppresses the UV sensitivity of cells deficient in Rad6 or Rad18 in a Rad52-dependent manner (Schiestl *et al*., 1990; Pfander *et al*., 2005). The suppression effect of *siz1* on Pol zeta-dependent TLS analyzed in our experiments was also found to be neutralized by deletion of *RAD52* (Fig.4 A). These data may be interpreted in a manner analogous to that employed to explain previous results concerning UV sensitivity (Schiestl et *al*., 1990; Pfander *et al*., 2005), i.e. in the absence of DDA activity, DNA lesions are processed by error prone Pol zeta-dependent TLS in Siz1-proficient cells, while in Siz1-deficient cells these lesions are channeled to error-free processing by HR, which results in a decrease in mutation frequency. This scenario assumes competition between TLS and HR for the substrate (Fig. S2). Following this assumption, the positive effect of Rad5 and/or Mms2-Ubc13 on TLS may reflect either stimulation of TLS *per se* or some indirect effect *via* inhibition of HR. The possibility that Rad5 and/or Mms2-Ubc13 could somehow be involved in an HR inhibition, in redundancy to the Siz1-Srs2 pathway, seems especially puzzling in light of the absence of Siz1 and Srs2 activities in the *S. pombe* and the human replication

forks (Ulrich, 2009). Some important questions about the mechanisms controlling HR activity in replication forks in cells of these organisms remain unanswered.

Our results on the UV sensitivity of the analyzed mutants further support the notion that the observed differences between the functions of Mms2 and Rad5 homologs in *S. cerevisiae* and *S. pombe* result from the presence or absence, respectively, of PCNA SUMOylation in these organisms. Surprisingly, we found that while Siz1 deficiency suppresses the lethal effects of UV radiation in *rad5* mutants (Fig.5 B), it does not suppress the UV sensitivity of *mms2* mutants (Fig.5 A). This finding challenges the idea that suppression of genetic defects within the *RAD6* pathway, due to the absence of Srs2-mediated HR inhibition, is related specifically to DDA. In previous investigations (Ulrich, 2001; Broomfield and Xiao, 2002), the suppression of DNA damage-induced lethal effects by *srs2* was obvious in *rad5* mutants, but the results were less clear cut for *mms2* mutants. Besides, since Srs2 plays multiple functions in HR (Marini and Krejci, 2010), it is likely that, in relation to events occurring at the DNA replication fork, the consequences of a lack of Siz1 are more specific than those resulting from Srs2 deficiency. In our experimental conditions, in agreement to previous results (Gangavarapu *et al*., 2006), UV sensitivity caused by *rad5* was considerably more pronounced than that conferred by the *mms2* mutation in PCNA SUMOylation-proficient cells of *S. cerevisiae*. However, the UV sensitivity of *siz1rad5* and *siz1mms2* double mutants was similar (Fig.5 AB). This situation recalls the relationship between Rad5 and Mms2 homologs in *S. pombe* cells , where PCNA is normally not SUMOylated and the sensitivities of *mms2* and *rad5* mutants to UV radiation are similar (Frampton *et al*., 2006). Consistent with the finding that Mms2-Ubc13 and Rad5 can stimulate Pol zeta-dependent TLS in *S. cerevisiae* defective in Siz1, these results further support the conclusion that the observed differences in the functions of Mms2 and Rad5 between budding and fission yeasts are due to the presence or absence of PCNA SUMOylation in these organisms.

Finally, the results of the present study show that Siz1 protects against hyper-sensitivity to both the lethal and mutagenic effects of UV radiation when deficiencies in Mms2 and Rad5 are accompanied by *rad52*. This finding supports an earlier suggestion (Pfander *et al*., 2005), that besides its participation in antirecombinogenic mechanisms, Siz1 plays an additional, currently undefined, role in the replication fork of *S. cerevisiae*.

Experimental procedures

Yeast strains and plasmids

The haploid *S. cerevisiae* strains used in mutagenesis and survival experiments are derivatives of *S. cerevisiae* C10-15a (McDonald *et al*., 1997) or WCG4a (Heinemeyer *et al*., 1997) and are listed in Table 2. Targeted gene disruptions were performed *via* direct transformation of yeast cells with PCR-amplified disruption cassettes. The desired integrants were verified by PCR and subsequent analysis of the respective DNA repair phenotypes. The *mms2::kanMX4* cassette was prepared as described previously (McIntyre *et al*., 2007). The strain YJM67 carrying *mms2::HIS3* was constructed using *in vivo* cassette replacement, by transformation of YAS98 carrying *mms2::kanMX4* with plasmid M4754 (Voth *et al*., 2003) encoding *kanMX::HIS3*, and selection for histidine prototrophs sensitive to G418. The *mms2::HIS3* cassette used in further constructions was amplified by PCR from DNA isolated from YJM67, using previously described primers (McIntyre *et al*., 2007). The *rad5::HIS3* cassette was amplified by PCR from DNA isolated from C22-16Aa, using primers $^{5'}$TGAAAAGAAGTTGAGTGAAA$^{3'}$ and $^{5'}$CTGAGGATAAAAGTGAA$^{3'}$. The s*iz1::kanMX4* cassette was produced as described by McIntyre et al. (2006). To produce the *siz1::URA3* cassette, YAS305 was constructed with the "marker-swap" plasmid M4758 (Voth *et al*., 2003), with selection for uracil prototrophs sensitive to G418. DNA of this strain was used in PCR with the same primers employed for the amplification of s*iz1::kanMX4* to produce the *siz1::URA3* cassette. The *rad52::LEU2* cassette was constructed using 70-mer primers representing sequences complementary to the 5' and 3' ends of the *RAD52* ORF (corresponding to positions -48–+2 for the upper primer, and +1414–+1463, for the lower primer) fused to sequences complementary to DNA flanking the *LEU2* marker in vector pRS315 (Sikorski and Hieter, 1989). These primers were used in a PCR with DNA of pRS315 as the template. The *Ubc13::kanMX4* cassette was produced by PCR with DNA isolated from BY4741 *ubc13* (Euroscarf), using primers recommended by Euroscarf. *rev3::natMX4* cassette was produced as previously described (Halas *et al*., 2009). Centromeric plasmids carrying *Rad5*, *rad5-DE681,682AA* or *rad5- CC914,917AA* were kindly provided by L. Prakash (Gangavarapu *et al.* 2006).

Spontaneous mutagenesis assay

To determine the *trp1-1* reversion frequency, yeast strains were cultured at 30^{o}C to logarithmic growth phase (OD_{600} 0.8) in minimal YNBG media supplemented according to their nutritional requirements, as described previously (Mieczkowski *et al.*, 2000). The number of mutant cells was estimated by plating 100 μl of the undiluted cultures on minimal plates supplemented with all nutritional requirements except tryptophan. For the detection of Can^{R} forward mutants, cultures were plated on complete minimal medium (lacking arginine)

containing 30 μg/ml L-canavanine sulfate (Sigma). Mutant colonies were counted following incubation of the plates for 4–5 days at 30°C. To estimate the number of colony forming units, serial dilutions of the cultures were plated on fully supplemented minimal plates and incubated at 30°C for 2–3 days. The frequency of mutations in each culture was calculated as the ratio of the TRP revertant or Can^R mutant count to the viable cell number. Data from at least 30 independent cultures in 3–6 independent experiments were used for each rate determination. At least two independently isolated strains of each genotype were used in the assays. *P*-values for statistical differences of mutant frequencies between analyzed strains were determined using the Mann–Whitney criterion (Sokal and Rolf, 1981).

Determination of UV radiation sensitivity and UV-induced mutagenesis.

To determine the UV sensitivity of yeast strains they were cultured to logarithmic growth phase in supplemented minimal medium at 30°C, as described above. The cells were then plated in triplicate, at different dilutions, onto supplemented minimal YNBG plates and immediately exposed to specified doses of UV radiation (254 nm) using a UV crosslinker (UVP model CL-1000). The plates were incubated in the dark for 2–3 days at 30°C and then colony forming units were counted. To determine the frequency of UV-induced mutagenesis, the assay was performed in a similar manner except that undiluted cell cultures were plated, in triplicate, onto plates containing all requirements but tryptophan or supplemented with 30 μg/ml canavanine sulfate. The mutant frequency was calculated by dividing the TRP^+ or Can^R mutant count by the viable cell number. The results represent the arithmetic means (for Trp^+ reversion) or medians (for Can^R mutation) from 3–6 separate experiments, with 8–10 independent cultures of each genotype in each experiment.

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Table 1. Mutator phenotypes conferred by Rad5 or Ubc13 deficiency are stimulated by Siz1.

		Trp^+		Can^R	
relevant genotype	UV dose (J/m^2)	mutations[a] x 10^{-7}	fold induction	mutations[b] x 10^{-6}	fold induction
wt	-	0.33 (0.04)	1.0	1.12	1.0
	5	2.89 (0.66)	1.0	8.62	1.0
siz1	-	0.46 (0.06)	1.4	1.06	0.9
	5	3.44 (0.6)	1.2	8.63	1.1
rad5	-	1.67 (0.16)	5.0	6.38	5.7
	5	12.67 (2.04)	4.4	20.00	2.3
rad5siz1	-	0.55 (0.08)	1.7	3.78	3.3
	5	3.78 (1.06)	1.3	11.64	1.4
ubc13	-	2.08 (0.56)	6.3	3.86	3.4
	5	5.93 (0.67)	2.1	15.50	1.8
ubc13siz1	-	0.86 (0.16)	2.6	1.63	1.5
	5	2.32 (0.52)	0.8	8.90	1.0

[a] Mean value of three to five independent experiments (SD).

[b] Median value of 20–25 independent cultures in at least three separate experiments. *P*-values for statistical differences of mutant frequencies between *rad5* and *rad5siz1* as well as *ubc13* and *ubc13siz1* were < 0.05

Table 2. *Saccharomyces cerevisiae* strains

Strain	Relevant genotype	Source
C10-15a	*MAT*a *ade2-1 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 RAD5*	McDonald *et al.* (2000)
YAS305	C10-15a *siz1::URA3*	This study
YAS98	C10-15a *siz1::kanMX4*	McIntyre *et al.* (2006)
C22-16Aa	C10-15a *rad5::HIS3*	McDonald *et al.* (2000)
YAS43	C10-15a *mms2::kanMX4*	This study
YJM67	C10-15a *mms2::HIS3*	This study
YAS40	C10-15a *ubc13::kanMX4*	This study
YAH76	C10-15a *rad52::LEU2*	This study
YAS285	C10-15a *mms2::kanMX4 rad5::HIS3*	This study
YAH51	C10-15a *mms2::HIS3 ubc13::kanMX4*	This study
YAS308	C10-15a *ubc13::kanMX4 rad5::HIS3*	This study
YAS100	C10-15a *siz1::kanMX4 rad5::HIS3*	This study
YJD24	C10-15a *siz1::URA3 rad5::HIS3*	This study
YJM72	C10-15a *mms2::HIS3 siz1::URA3*	This study
YAS295	C10-15a *mms2::kanMX4 siz1::URA3*	This study
YAH41	C10-15a *siz1::URA3 ubc13::KanMX4*	This study
YAH75	C10-15a *siz1::URA3 rad52::LEU2*	This study
YAH70	C10-15a *rad5::HIS3 rad52::LEU2*	This study
YAH66	C10-15a *mms2::kanMX4 rad52::LEU2*	This study
YAS297	C10-15a *mms2::kanMX4 rad5::HIS3 siz1::URA3*	This study
YAS309	C10-15a *ubc13::kanMX4 siz1::URA3 rad5::HIS3*	This study
YAH52	C10-15a *mms2::HIS3 siz1::URA3 ubc13::kanMX4*	This study
YAH61	C10-15a *mms2::kanMX4 siz1::URA3 rad52::LEU2*	This study
YMP3	C10-15a *mms2::kanMX4 siz1::URA3 rad52::LEU2 rev3::NatMX4*	This study
YAH67	C10-15a *mms2::kanMX4 rad5::HIS3 rad52::LEU2*	This study
YAH82	C10-15a *rad5::HIS3 siz1::URA3 rad52::LEU2*	This study
YZG6	C10-15a *rad5::HIS3 siz1::URA3 rad52::LEU2 rev3::NatMX4*	This study
YAH80	C10-15a *mms2::kanMX4 rad5::HIS3 siz1::URA3*	This study

		rad52::LEU2	
	WCG4a	*MAT*a *ura3 leu2-3, 112 his3-11,*	Heinemayer *et al.* (1997)
		15 rad5-535 GAL2	
	YAH7	WCG4a *siz1::kanMX4*	McIntyre *et al.* (2006)
	YJM40	WCG4a *siz1::URA3*	This study
	YJM26	WCG4a *rad5::HIS3*	This study
	YJM56	WCG4a *mms2::kanMX4*	McIntyre *et al.* (2007)
	YJD23	WCG4a *mms2::kanMX4 rad5::HIS3*	This study
	YAH59	WCG4a *mms2::HIS3*	This study
	YAP11	WCG4a *mms2::HIS3 siz1::URA3*	This study
	YAH57	WCG4a *ubc13::kanMX4*	This study
	YAH60	WCG4a *ubc13::kanMX4 mms2::HIS3*	This study
	YAH54	WCG4a *ubc13::kanMX4 mms2::HIS3 siz1::URA3*	This study
	YAH42	WCG4a *ubc13::kanMX4 rad5::HIS3*	This study
	YAH46	WCG4a *ubc13::kanMX4 rad5::HIS3 siz1::URA3*	This study
	YAS118	WCG4a *rad5::HIS3 siz1::kanMX4*	This study
	YJD22	WCG4a *rad5::HIS3 siz1::URA3*	This study
	YAH53	WCG4a *ubc13::kanMX4 siz1::URA3*	This study
	YJD21	WCG4a *rad5::HIS3 mms2::KanMX4 siz1::URA3*	This study
	YJD13	WCG4a *mms2::KanMX4 siz1::URA3*	This study

Figures Legends

Figure 1. The mutator phenotype of Mms2-deficient *S. cerevisiae* is modulated by Siz1. Spontaneous (AB) and UV-induced (CD) mutagenesis in *mms2* derivatives of C10-15a (YAS43) (AC) and WCG4a (YJM56) (BD). *trp1-1* (*amber*) reversions (AC) are mean values ± SD (error bars) from 3-5 independent experiments. Forward mutations leading to canavanine resistance (BD) are median values from 20-30 independent cultures of each strain in three separate experiments. *P*-values for statistical differences of mutant frequencies between investigated strains (with the exception of difference between *WT* and *siz1*) were < 0.05

Figure 2. The effect of *siz1* on spontaneous and UV-induced reversion of *trp1-1*in yeast strains carrying double-deletions in genes involved in polyubiquitination of PCNA. Results of 4-6 separate experiments. Bars indicate standard deviations from the mean value.

Figure 3. Analysis of the roles of ATPase and ubiquitin ligase activities of Rad5 in stimulation of TLS. Frequency of spontaneous and UV-induced reversion of *trp1-1* in *rad5ubc13siz1*(A) and *rad5mms2siz1*(B) triple disruptants transformed with derivatives of YCplac111 encoding Rad5 (pR5-28), Rad5-DE681,682AA defective in ATPase activity (pR5-30) or Rad5-CC914,917AA defective in ubiquitin ligase activity (pR5-19). Results of 3 separate experiments. Bars indicate standard deviations from the mean value.

Figure 4. Deletion of *RAD52* neutralizes the antimutagenic effect of *siz1* in cells deficient in DDA. Frequency of spontaneous (ACEG) and UV-induced (BDFH) mutagenesis. Results of 4-6 separate experiments. Bars indicate standard deviations from the mean value.

Figure 5. Rad52 and Siz1 modulate the UV sensitivity of strains deficient in DDA: *mms2* (A), *rad5* (B) and *mms2rad5* (C) derivatives of C10-15a. Results are the means from three to five experiments with bars indicating standard deviations.