

Role of DNA damage-induced replication checkpoint in promoting lesion bypass by translesion synthesis in yeast

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Unrepaired DNA lesions in the template strand block the replication fork. In yeast, Mec1 protein kinase-mediated replication checkpoint prevents the breakdown of replication forks and maintains viability in DNA-damaged cells going through the S phase. By ensuring that the replisome does not dissociate from the fork stalled at the lesion site, the replication checkpoint presumably coordinates the action of lesion bypass processes with the replisome. However, it has remained unclear as to which of the lesion bypass processes—translesion synthesis (TLS) and/or template switching—depend on the activation of the replication checkpoint. Here we determine if the Mec1 kinase and the subunits of the checkpoint clamp and the clamp loader are required for TLS. We show that proficient TLS can occur in the absence of these checkpoint proteins in nucleotide excision repair (NER)-proficient cells; however, in the absence of NER, checkpoint protein-mediated Rev1 phosphorylation contributes to increasing the proficiency of DNA polymerase ζ -dependent TLS.

[*Keywords:* DNA damage; translesion synthesis; replication checkpoint; lesion bypass; Mec1 kinase Rev1 phosphorylation]

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Unrepaired DNA lesions in the template strand block synthesis by replicative DNA polymerases. In eukaryotes, the Rad6–Rad18 ubiquitin-conjugating enzyme complex regulates replication through DNA lesions via translesion synthesis (TLS) by specialized DNA polymerases (Pols) and by means that use template switching (Prakash et al. 2005). Genetic studies in the yeast *Saccharomyces cerevisiae* have indicated that Rad6–Rad18-dependent replication through UV-induced DNA lesions can occur by Pol η - or Pol ζ -mediated TLS (Nelson et al. 1996b; Johnson et al. 1999, 2000a; Bresson and Fuchs 2002; Prakash et al. 2005), or by a Rad5–Mms2–Ubc13 pathway that promotes lesion bypass by template switching (Torres-Ramos et al. 2002; Gangavarapu et al. 2006; Blastyak et al. 2007). A Rad6–Rad18-independent template-switching pathway dependent on Rad51, Rad52, and Rad54 proteins can also restore the continuity of newly synthesized DNA from UV-damaged DNA templates (Prakash 1981; Gangavarapu et al. 2007).

When progression of the replication fork is halted by a DNA lesion or by inhibitors of DNA synthesis, eukaryotic cells activate an intra-S-phase checkpoint pathway,

also known as a replication checkpoint (Paulsen and Cimprich 2007). The activated replication checkpoint plays a crucial role in the stabilization of replication forks by maintaining the association of the replisome with the fork, thereby enabling resumption of replication from the stalled fork after the lesion has been bypassed (Tercero and Diffley 2001; Tercero et al. 2003; Paulsen and Cimprich 2007). In the absence of a replication checkpoint, the replisome dissociates and the stalled replication forks degenerate, generating large sections of ssDNA and the accumulation of reversed forks (Sogo et al. 2002; Cobb et al. 2003, 2005; Paulsen and Cimprich 2007). The replication checkpoint also slows S-phase progression, allowing time for DNA repair or lesion bypass to occur, and down-regulates late origin firing (Tercero et al. 2003; Paulsen and Cimprich 2007).

In *S. cerevisiae*, Mec1 kinase is a key component of the replication checkpoint, and it initiates the checkpoint pathway by phosphorylating the downstream effector kinase Rad53 (Nyberg et al. 2002; Paulsen and Cimprich 2007). Checkpoint activation by Mec1 additionally may require the checkpoint clamp–clamp loader complex, in which the checkpoint clamp is a heterotrimer comprised of Rad17, Mec3 (we find that *MEC3* and *RAD15* represent the same gene), and Ddc1 subunits, and the clamp loader Rad24–Rfc is comprised of the four small subunits of Rfc

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(Rfc2–5) together with the Rad24 subunit. In vitro studies with purified checkpoint proteins have shown that the checkpoint clamp loaded onto partial duplex DNA activates the kinase activity of Mec1 and the activated Mec1, then phosphorylates Rad53 (Majka and Burgers 2003).

Although experimental evidence clearly points to the requirement of a Mec1-controlled replication checkpoint for the stabilization of replication forks in DNA-damaged yeast cells, it remains unclear as to which of the lesion bypass processes—TLS or template switching—requires activation of the replication checkpoint. For example, one could envisage a situation in which a lesion bypass process operates in such tight coordination with the DNA replication ensemble that progression of the replication fork is halted minimally. In that case, the absence of multiple stalled replication forks might fail to generate the threshold level of signal necessary for checkpoint activation to occur (Tercero et al. 2003).

The most pertinent information relating to the requirement of checkpoint proteins in lesion bypass comes from the study of Hartwell and colleagues (Paulovich et al. 1998), in which they showed that the frequency of UV-induced mutations shows a considerable reduction in a nucleotide excision repair (NER)-defective yeast strain in the absence of checkpoint clamp and clamp loader proteins. Since UV-induced mutations result from TLS mediated by Pol ζ opposite cyclobutane pyrimidine dimers (CPDs) and (6-4) photoproducts, one possible interpretation of these observations is that these checkpoint proteins help maintain a stably paused replication fork at UV lesions for TLS to occur.

In this study, we investigate the role of the Mec1-dependent replication checkpoint in TLS in UV-damaged yeast cells. We find that in wild-type yeast cells, proficient TLS occurs in the absence of Mec1 kinase or the components of checkpoint clamp and clamp loader. In NER-defective cells, however, the proficiency of Pol ζ -mediated TLS is reduced in the absence of these checkpoint proteins. To decipher how checkpoint proteins modulate TLS proficiency in NER-deficient cells, we analyzed the impact that Rev1 phosphorylation, which we show to be dependent on these checkpoint proteins, has on the proficiency of Pol ζ -mediated TLS. We conclude that although the replication checkpoint is not essential for TLS to occur, checkpoint protein-mediated Rev1 phosphorylation contributes to increasing the proficiency of Pol ζ -mediated TLS, the need for which becomes evident in the absence of NER.

Results

Mec1 kinase is dispensable for UV mutagenesis in wild-type yeast cells

Since (6-4) photoproducts are removed much more rapidly by NER than are CPDs, UV mutations in wild-type yeast result predominantly from TLS opposite CPDs (Sancar 1996; You et al. 2001). In yeast, Pol η and Pol ζ promote TLS opposite CPDs, wherein Pol η acts primarily in an error-free manner and Pol ζ functions in a more

mutagenic manner. Whereas Pol η can carry out proficient and relatively error-free TLS through CPDs (Johnson et al. 1999, 2000b; Washington et al. 2000, 2003), Pol ζ contributes to their bypass by extending from the correct or incorrect nucleotide inserted opposite the 3' pyrimidine of the CPD by another Pol (Johnson et al. 2000a).

To determine whether activation of the replication checkpoint is required for TLS, we analyzed the frequency of UV-induced *CAN1*^S to *can1*^r forward mutations in yeast cells carrying deletion mutations of the various checkpoint genes. Since Mec1 kinase is the initiator of replication checkpoint and is indispensable for maintaining replication fork stability in DNA-damaged yeast cells, we first examined whether Mec1 was required for UV mutagenesis in wild-type cells. However, because *MEC1* is essential for cell viability, but viability can be restored to *mec1* Δ cells by the *sml1* Δ mutation, we examined UV mutagenesis in the *sml1* Δ and *sml1* Δ *mec1* Δ strains. UV sensitivity or UV mutagenesis is not affected by the *sml1* Δ mutation (cf. Figs. 1 and 2). The *mec1* Δ mutation confers an increase in UV sensitivity, but the frequency of UV-induced mutations remains about the same in the *sml1* Δ *mec1* Δ strain as in the *sml1* Δ strain (Fig. 1). Since UV-induced mutations in NER-proficient cells result predominantly from Pol ζ action opposite CPDs and, to a much lesser extent, opposite (6-4) photoproducts, this result indicates that proficient Pol ζ -mediated TLS opposite both these UV lesions can operate in the absence of Mec1. To verify that UV mutations in the *sml1* Δ *mec1* Δ strain arise from Pol ζ action, we examined UV-induced mutation frequencies in the *sml1* Δ *mec1* Δ *rev3* Δ strain and, as expected, the incidence of UV mutagenesis was reduced in this strain to the same extent as in the *rev3* Δ strain (Fig. 1). Because of the role of Pol η in error-free TLS opposite CPDs, the frequency of UV-induced mutations rises in the *rad30* Δ strain, which lacks Pol η (McDonald et al. 1997; Yu et al. 2001). The incorporation of the *rad30* Δ mutation into the *sml1* Δ *mec1* Δ strain led to an increase in the UV sensitivity of the latter strain, and the frequency of UV-induced mutations was enhanced in the *sml1* Δ *mec1* Δ *rad30* Δ strain compared with that in the *rad30* Δ strain (Fig. 1). The increase in UV mutagenesis is particularly evident at the higher UV dose examined (10 J/m²), where we observe an approximately fourfold increase in mutation frequencies in the *sml1* Δ *mec1* Δ *rad30* Δ strain over that in the *rad30* Δ strain. Since the absence of Pol η from the *sml1* Δ *mec1* Δ strain leads to an enhancement of UV sensitivity and confers an increase in the frequency of UV-induced mutations, these observations imply that Pol η is able to function in TLS in the absence of Mec1.

In yeast cells treated with DNA-damaging agents, PCNA is monoubiquitinated at its Lys-164 residue by the Rad6–Rad18 enzyme complex, and genetic studies in yeast have shown that PCNA monoubiquitination is essential for TLS (Hoegge et al. 2002; Stelter and Ulrich 2003; Haracska et al. 2004). The Lys-164-linked ubiquitin moiety is subsequently modified by Lys-63-linked polyubiquitination, which additionally requires the Mms2–Ubc13 ubiquitin-conjugating enzyme complex together

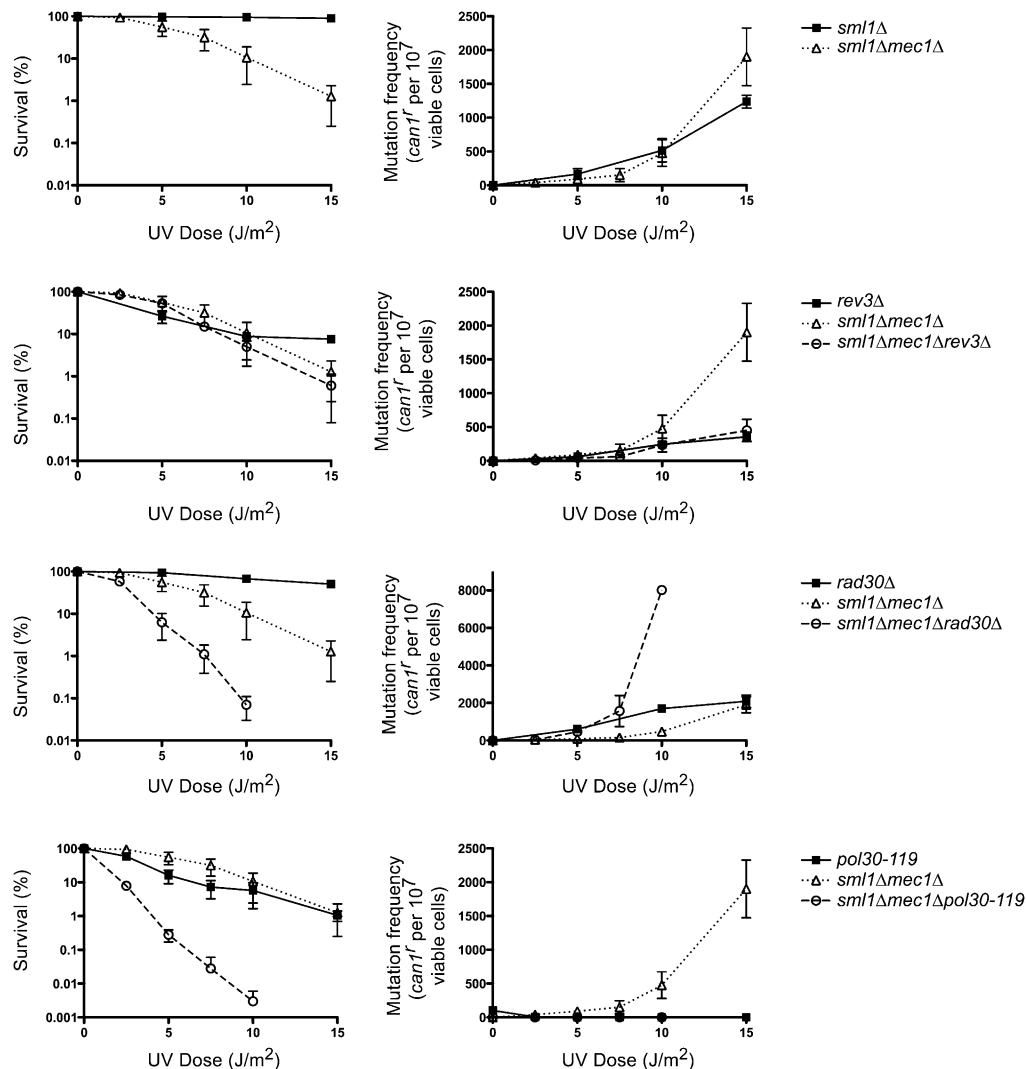


Figure 1. Mec1 is not required for UV mutagenesis. UV survival and the frequencies of UV-induced *can1^r* mutations were determined in the *sml1Δ* and *sml1Δ mec1Δ* strains. The *sml1Δ* strain exhibits the same UV survival and UV mutagenesis as the wild-type strain. Because the *sml1Δ* mutation restores viability to *mec1Δ* cells, all the *mec1Δ* strains harbor the *sml1Δ* mutation. The data represent averages of three independent experiments and error bars represent standard deviations of determinations. In this figure and Figures 2, 5, and 6, the apparent absence of error bars in some cases is because the error bars are too small.

with Rad5, which acts as the ubiquitin ligase in the Mms2–Ubc13–Rad5 complex (Hoege et al. 2002). Since PCNA monoubiquitination is required for TLS and PCNA polyubiquitination is required for template switching mediated by the helicase function of Rad5, both of these Rad6–Rad18 lesion bypass pathways are inhibited by the *pol30-119* mutation wherein the Lys-164 residue of PCNA has been changed to arginine, and therefore cannot be ubiquitinated (Haracska et al. 2004). To establish that UV mutations in the *mec1Δ* strain arise from TLS and not from a role of Polζ in some other as-yet-undefined pathway, we determined whether UV-induced mutations that arise in the *mec1Δ* strain require PCNA ubiquitination. As shown in Figure 1, a drastic reduction in UV mutagenesis occurs in the *sml1Δ mec1Δ pol30-119* strain similar to that in the *pol30-119* strain, indicating

that UV-induced mutations in the *mec1Δ* strain arise from PCNA ubiquitination-dependent Polζ-mediated TLS.

Although Rad6–Rad18-dependent lesion bypass by TLS or by template switching does not occur in the *pol30-119* mutant, this mutation does not impart the same high degree of UV sensitivity as the *rad6Δ* or *rad18Δ* mutations. This is because the Lys-164 residue of PCNA also undergoes the SUMO modification in DNA-damaged yeast cells (Hoege et al. 2002) and, in the absence of this modification, the Rad52-dependent lesion bypass pathway becomes activated (Haracska et al. 2004). Our observation that the *sml1Δ mec1Δ pol30-119* mutant exhibits a large synergistic enhancement of UV sensitivity compared with the UV sensitivity for the *sml1Δ mec1Δ* and *pol30-119* mutants raises the possibility that

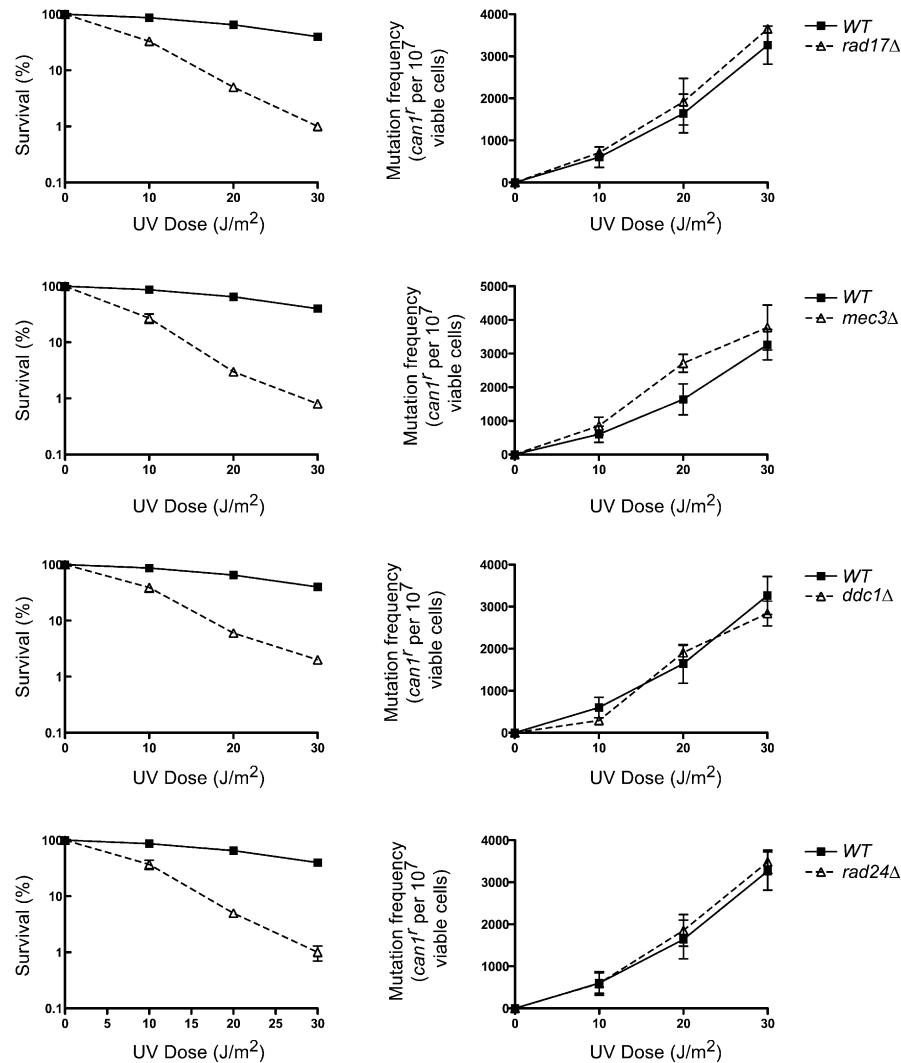


Figure 2. The Rad17, Mec3, and Ddc1 subunits of checkpoint clamp and Rad24 subunit of checkpoint clamp loader are not required for UV mutagenesis. UV survival and the frequencies of UV-induced *can1⁺* mutations were determined in the wild-type and isogenic *rad17Δ*, *mec3Δ*, *ddc1Δ*, and *rad24Δ* strains. The data represent averages of three independent experiments and error bars represent standard deviations.

the Mec1-dependent checkpoint pathway plays an important role in promoting lesion bypass by the Rad52-dependent recombination pathway.

Checkpoint clamp and clamp loader are not required for UV mutagenesis in wild-type yeast cells

To determine whether the checkpoint clamp and clamp loader proteins were also dispensable for TLS, we examined the frequency of UV-induced *can1⁺* mutations in *rad17Δ*, *mec3Δ*, *ddc1Δ*, and *rad24Δ* strains. As shown in Figure 2, although all of these checkpoint mutations confer an increase in UV sensitivity, they have no effect on UV mutagenesis. Thus, our data show that neither the Mec1 kinase nor the various components of checkpoint clamp and clamp loader are required for proficient TLS in yeast cells.

Rev1 phosphorylation requires the Mec1–Ddc2 kinase and the components of check point clamp and clamp loader

Rev1, a Y family DNA Pol, physically associates with Polζ and plays an indispensable structural role in Polζ-mediated TLS (Acharya et al. 2006). Since Rev1 has been shown recently to be phosphorylated in response to DNA damage, and since this phosphorylation requires Mec1 (Sabbioneda et al. 2007), we considered the possibility that Rev1 phosphorylation might contribute to increasing the proficiency of Polζ-mediated TLS, the need for which may become more acute when the lesion load rises, as in the absence of NER. Furthermore, we speculated that if Rev1 phosphorylation were found to also require the checkpoint clamp and clamp loader, then that might provide a rationale for the reduction in UV

mutagenesis that has been reported to occur in the absence of these checkpoint proteins in an NER-defective yeast strain (Paulovich et al. 1998).

To determine if the checkpoint clamp and clamp loader affect an increase in the proficiency of Pol ζ -mediated TLS via their role in Rev1 phosphorylation, we first examined the status of Rev1 phosphorylation in the absence of these checkpoint proteins following treatment with 4NQO. Rev1 phosphorylation is induced in wild-type yeast cells following treatment with 4NQO (Fig. 3A). As has been reported previously, damage-inducible Rev1 phosphorylation is absent in the *mec1* Δ strain (Sabbioneda et al. 2007). Since Ddc2 is the nonkinase subunit of Mec1 and is essential for Mec1 function in checkpoint activation, we examined if Ddc2 was also required for Rev1 phosphorylation. As expected, we find that Rev1 phosphorylation does not occur in the 4NQO-treated *ddc2* Δ strain (Fig. 3A). In contrast to the requirement of Mec1–Ddc2 for Rev1 phosphorylation, however, efficient Rev1 phosphorylation was found to occur in *rad53* Δ cells following 4NQO treatment (Fig. 3A).

Next, we examined if the components of checkpoint clamp, Rad17, Mec3, and Ddc1, were required for damage-induced Rev1 phosphorylation. As shown in Figure 3B, we found no evidence of Rev1 phosphorylation in the absence of any of these proteins. Also, our results show that Rad24, which represents the largest subunit specific for checkpoint clamp loader, is required for Rev1 phosphorylation. In summary, damage-inducible Rev1 phosphorylation requires the Mec1–Ddc2 kinase complex and the components of checkpoint clamp and clamp loader, but not the Rad53 kinase.

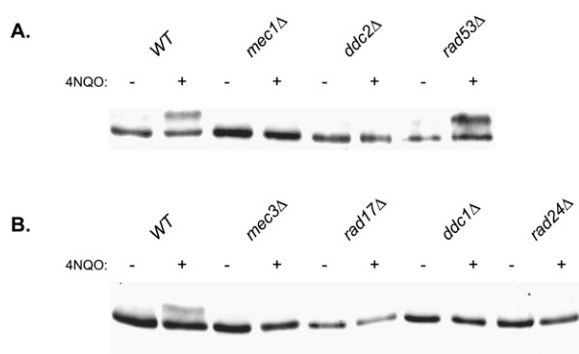


Figure 3. Requirement of checkpoint proteins for DNA damage-induced Rev1 phosphorylation. The Rev1 protein tagged at the C terminus with 4HA was expressed from plasmid pVP64 in the wild-type or the checkpoint mutant strain. Exponentially growing cells were treated (+) or not (–) with 4NQO (2 μ g/mL) for 15 min. Cells were then washed with water and total protein extract was obtained in the presence of protease inhibitors and phosphatase inhibitors. Protein extracts were analyzed on 6% acrylamide gels (77:1 acrylamide:bis-acrylamide), and Rev1 was detected by Western blotting with anti-HA antibody using standard techniques. (A) Rev1 phosphorylation is inhibited in the absence of Mec1–Ddc2 kinase but not in the absence of Rad53 kinase. (B) Rev1 phosphorylation does not occur in *mec3* Δ , *rad17* Δ , *ddc1* Δ , and *rad24* Δ strains.

Rev1 phosphorylation modulates the efficiency of Pol ζ -mediated TLS in NER-defective cells

To determine the contribution that Rev1 phosphorylation makes to Pol ζ -mediated TLS, we sought to identify the phosphorylation site in Rev1 so that the effects of loss of phosphorylation could then be assessed by mutational studies. Rev1 has seven consensus (S/T)Q sites that are the potential targets of Mec1 kinase (Fig. 4A). We individually changed each of these serine or threonine residues to an alanine and examined the phosphorylation status of Rev1 protein encoded by each of these mutant alleles. As shown in Figure 4B, 4NQO-inducible Rev1 phosphorylation does not occur when the Ser-31 residue present in the SQ1 site is changed to alanine, whereas mutational inactivation of the remaining six SQ/TQ sites has no effect on Rev1 phosphorylation.

Next, we examined the effects of mutational inactivation of Rev1 phosphorylation on UV sensitivity and on UV-induced *can1*^r forward mutations in wild-type and NER-defective *rad14* Δ cells. As shown in Figure 5A, neither the UV sensitivity nor the induction of UV mutagenesis is affected by the *rev1S31A* mutation in the SQ1 site when carried in the wild-type strain. However, the introduction of the *rev1S31A* mutant allele into the *rad14* Δ strain conferred a reduction in the frequency of UV-induced *can1*^r mutations compared with that in the *rad14* Δ strain carrying the wild-type *REV1* gene (Fig. 5B). Even though the *rev1S31A* mutation lowers the incidence of UV mutagenesis in the *rad14* Δ strain, it has no perceptible effect on the UV sensitivity of the *rad14* Δ strain (Fig. 5B). We infer from these observations that Rev1 phosphorylation contributes to an increase in the proficiency of Pol ζ -mediated TLS, the need for which becomes more evident upon the inactivation of NER. Presumably, however, the increase in lesion bypass proficiency afforded by Rev1 phosphorylation is not substantial enough to confer a significant impact on UV resistance.

Defects in replication checkpoint proteins affect Pol ζ 's proficiency in TLS via Rev1 phosphorylation

Since the checkpoint proteins; the Mec1 kinase; Rad17, Mec3, and Ddc1, which form the checkpoint clamp; and Rad24, the clamp loader subunit, are all required for damage-induced Rev1 phosphorylation, and since the lack of Rev1 phosphorylation confers a reduction in UV mutagenesis in NER-defective cells, we surmised that the stimulatory effects of checkpoint proteins on UV mutagenesis that have been reported to occur in the absence of NER derive from their effects on Rev1 phosphorylation. If that were the case, then we would expect that the phosphorylation-defective *rev1S31A* mutation and the absence of checkpoint proteins that are required for Rev1 phosphorylation would affect UV mutagenesis in the *rad14* Δ strain in a similar fashion and that they would exhibit an epistatic relationship in their effects on UV mutagenesis. To check the validity of this idea, we first compared the frequencies of UV-induced *can1*^r mutations in the *rad14* Δ *sml1* Δ and *rad14* Δ *sml1* Δ *mec1* Δ strains.

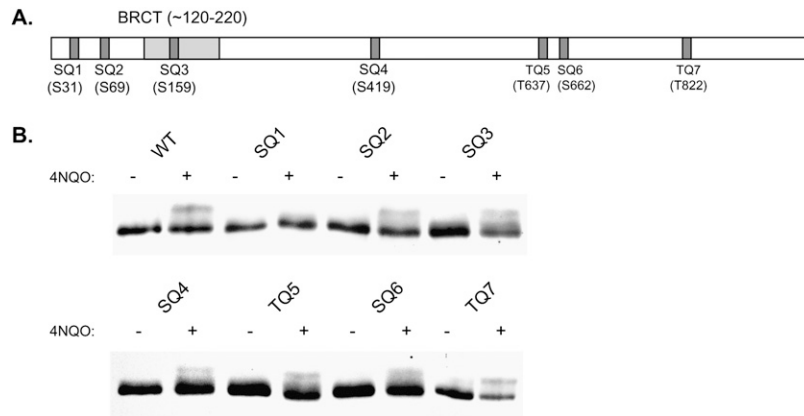


Figure 4. Identification of the Rev1 phosphorylation site. (A) Schematic map of seven potential (S/T)Q phosphorylation sites in Rev1. The position of the BRCT domain is also indicated. (B) The effect of mutations in different potential phosphorylation sites (SQ or TQ) on Rev1 phosphorylation. Only the mutation in the SQ1 site (S31 → A) affects 4NQO-induced Rev1 phosphorylation. 4NQO-induced Rev1 phosphorylation was examined in yeast cells expressing the 4HA-tagged wild-type (WT) or mutant Rev1 protein. The S or T residue of (S/T)Q was changed to an alanine at each of the sites.

As shown in Figure 6A, a reduction in UV mutagenesis occurs in the *rad14Δ sml1Δ mec1Δ* strain compared with that in *rad14Δ sml1Δ* cells; however, the absence of Mec1 confers a greater reduction in UV mutagenesis in *rad14Δ* cells than does the *rev1S31A* mutation. Also, we find that the introduction of the *rev1S31A* mutation in the *rad14Δ sml1Δ mec1Δ* strain has no perceptible effect on UV mutagenesis or on UV sensitivity (Fig. 6A). These observations raise the possibility that in addition to affecting the proficiency of Polζ-mediated TLS via Rev1 phosphorylation, the Mec1 kinase modulates phosphorylation of some other proteins that function with Polζ.

Next, we examined whether the defects in checkpoint clamp and clamp loader subunits also affect UV mutagenesis similarly to the defects in Mec1 kinase. A comparison of UV mutagenesis in *rad14Δ rad17Δ*, *rad14Δ mec3Δ*, *rad14Δ ddc1Δ*, and *rad14Δ rad24Δ* strains with that in *rad14Δ* cells shows that a reduction in UV mutagenesis is incurred in *rad14Δ* cells in the absence

of checkpoint clamp and clamp loader proteins (Fig. 6B). And, importantly, we find that the introduction of the *rev1S31A* mutation in *rad14Δ* cells lacking these checkpoint proteins confers no further change in UV mutation frequencies. Also, the UV sensitivity of *rad14Δ* cells lacking the checkpoint clamp or clamp loader proteins is not affected upon the introduction of *rev1S31A* mutation (Fig. 6B). However, similar to the effects of the *mec1Δ* mutation, the absence of checkpoint clamp and clamp loader proteins produces a greater defect in UV mutagenesis than that conferred by the *rev1S31A* mutation. We conclude from these observations that checkpoint proteins affect the proficiency of Polζ-mediated TLS at least in part via their role in Rev1 phosphorylation, and surmise that they affect the phosphorylation of some other protein(s) involved in Polζ-mediated TLS as well.

To identify the other protein factor(s) that, in addition to Rev1, could be the substrate for Mec1-mediated phosphorylation, we determined whether any of the proteins

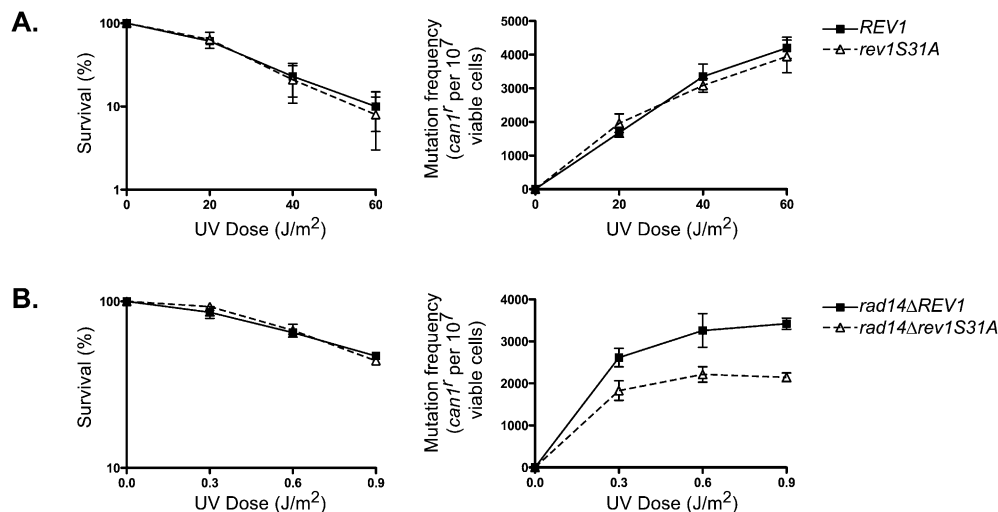


Figure 5. The phosphorylation-defective *rev1S31A* mutation affects the proficiency of UV mutagenesis in NER-defective yeast cells. UV survival and UV-induced *can1⁺* mutation frequencies were examined in *rev1Δ* cells carrying the wild-type (WT) or *rev1S31A* mutant gene on plasmid pVP21 in NER-proficient cells (A) or NER-defective *rad14Δ* cells (B). The data represent averages of three independent experiments and error bars represent standard deviations.

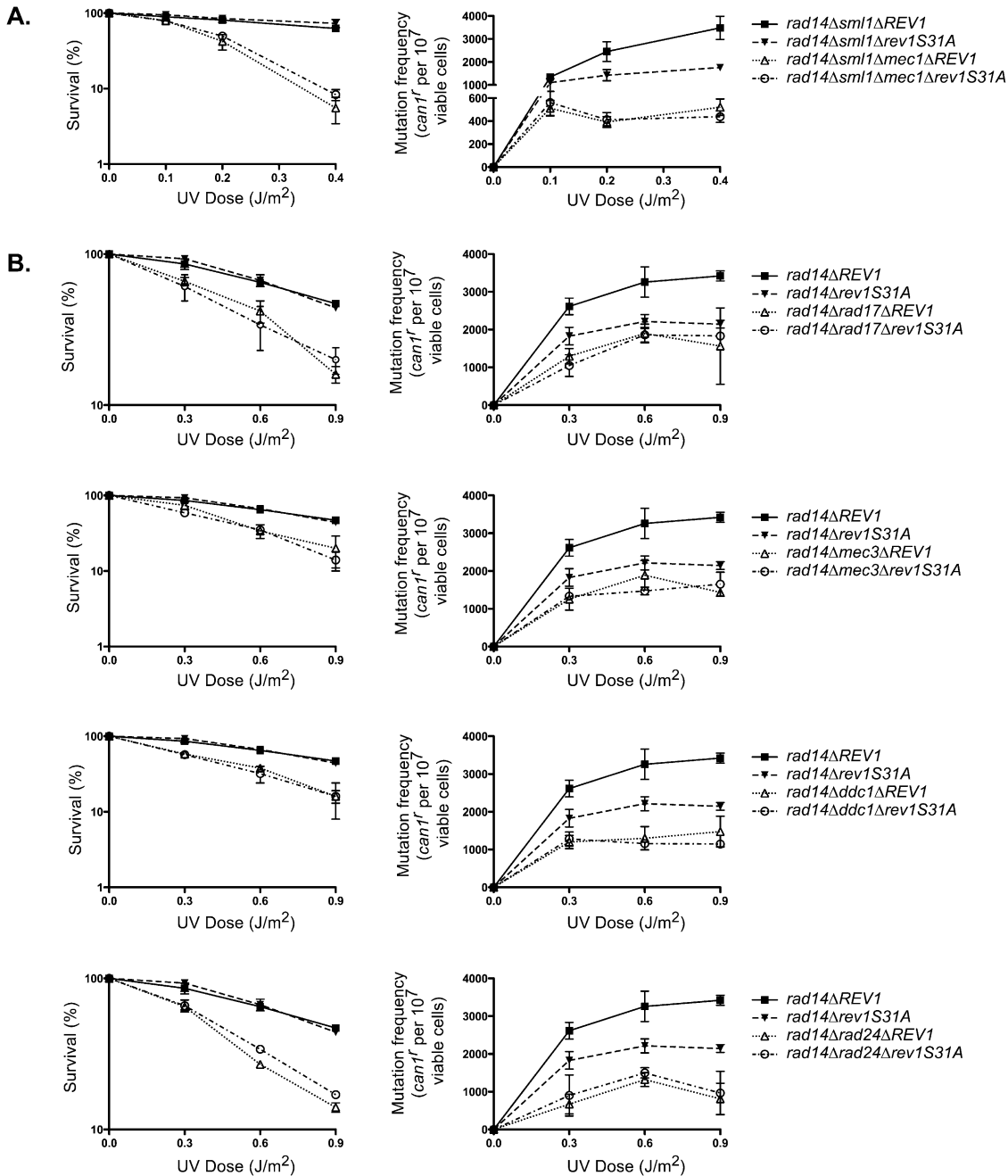


Figure 6. The effects of the *rev1S31A* mutation on UV mutagenesis in *rad14Δ* cells in the absence of checkpoint proteins. (A) The *mec1Δ* mutation adversely affects UV mutagenesis in *rad14Δ* cells and it exhibits epistasis over *rev1S31A* mutation for UV mutagenesis in *rad14Δ* cells. (B) The effects of deletions of Rad17, Mec3, and Ddc1 subunits of checkpoint clamp and of the Rad24 subunit of checkpoint clamp loader on UV mutagenesis in *rad14Δ* cells are epistatic over effects of the *rev1S31A* mutation. UV-induced *can1^r* mutation frequencies were examined in *rad14Δ* cells carrying genomic deletions of checkpoint genes and also harboring the genomic *rev1Δ* mutation, and carrying the wild-type *REV1* or *rev1S31A* mutant gene on plasmid pVP21. The data represent averages of three independent experiments and error bars are standard deviations of these determinations.

that are known to function in Polζ-dependent TLS are phosphorylated in response to DNA damage. Of the proteins required for Polζ-dependent TLS, Rev3 contains seven potential (four SQ and three TQ) Mec1 phosphorylation sites spread throughout the protein, and Rev7 contains one SQ site toward its C terminus. In addition to

the direct involvement of Polζ and Rev1 in Polζ-dependent TLS, the Rad6–Rad18 enzyme complex plays a regulatory role in TLS mediated by Polζ or Polη. In this enzyme complex, Rad6 contains one SQ site toward its C terminus and Rad18 contains two adjacent TQ, SQ sites in the middle of the protein. In addition, Pol32 is also

required for Pol ζ function in TLS; however, it lacks any potential Mec1 phosphorylation site. Pol η functions in a pathway alternate to Pol ζ for mediating TLS through UV-induced lesions, and it has two potential Mec1 phosphorylation sites: one SQ site in the middle and another SQ site toward the C terminus of the protein.

The various C-terminally tagged proteins were expressed from their chromosomal loci in yeast cells and, using methods similar to those we used for Rev1 studies, the phosphorylation status of these TLS proteins was examined in yeast cells treated with 4-NQO (see the Supplemental Material; Supplemental Tables 4, 5). However, as shown in Supplemental Figure 2, we could detect no evidence for phosphorylation of Rev3, Rev7, Rad30, or Rad6 in undamaged or 4NQO-damaged yeast cells. Since the expression level of genomic HA-tagged Rad18 was quite low and we were unable to establish its phosphorylation status, we examined whether mutational inactivation of the two potential Mec1 phosphorylation sites present in Rad18 was detrimental to Pol ζ -mediated TLS or to Pol η function. However, in an NER-proficient yeast strain, UV survival or the frequency of UV-induced *can1^f* mutations was not affected by the T282A–S284A double mutation made to inactivate the two potential TQ and SQ phosphorylation sites in the *RAD18* gene (Supplemental Fig. 3A). Since the effect of the phosphorylation-defective mutation in the *REV1* gene (*rev1 S31A*) became apparent only in the NER-defective *rad14 Δ* cells, we also determined the effects of the *rad18* T282A–S284A mutation in *rad14 Δ* cells. Here, also, we found that the UV survival and the frequency of UV-induced *can1^f* mutations was almost identical in the *rad14 Δ rad18 Δ* strain regardless of whether it carried the wild-type *RAD18* gene on plasmid pVP121 or the mutant *rad18* gene on plasmid pVP124 (Supplemental Fig. 3B).

These observations lead us to conclude that Rev1 is unique among the known TLS factors in being regulated by Mec1-mediated phosphorylation, and that there still remains an as-yet-unidentified protein that functions in TLS and whose phosphorylation by Mec1 modulates TLS proficiency.

Discussion

The DNA damage-inducible replication checkpoint is dispensable for TLS in wild-type yeast cells

Based on genetic studies in yeast, the idea that checkpoint proteins, in particular the checkpoint clamp and clamp loader, are required for TLS has received considerable attention. In addition to the previous study (Paulovich et al. 1998) that suggested that, in *S. cerevisiae*, the components of checkpoint clamp and clamp loader were required for UV-induced mutagenesis and hence for Pol ζ -mediated TLS, two other studies have implicated the involvement of these checkpoint proteins in TLS. In one of the studies, the Mec3 and Ddc1 checkpoint clamp subunits of *S. cerevisiae* were shown to interact physically with the Rev7 accessory subunit of Pol ζ , and the loss of checkpoint clamp and clamp loader proteins was found

to have an effect on the rate of Pol ζ -dependent spontaneous mutagenesis of complex frameshifts (Sabbioneda et al. 2005). In another study, the *Schizosaccharomyces pombe* DinB TLS Pol was found to coprecipitate with the checkpoint clamp components, and the enhanced spontaneous mutagenesis that occurs in a Pol α mutant was shown to be reduced in the absence of checkpoint clamp loader subunit (Kai and Wang 2003).

In this study, we carry out a comprehensive analysis of the role of Mec1 kinase and the subunits of checkpoint clamp and clamp loader on UV-induced mutagenesis. Since the genetic bases of UV mutagenesis is well established, and since it requires PCNA ubiquitination, the analysis of UV mutagenesis provides a useful measure of TLS. For spontaneous mutagenesis, such as the study of complex frameshifts in *S. cerevisiae* or that of the Pol α mutant in *S. pombe* (Kai and Wang 2003; Sabbioneda et al. 2005), it remains unclear whether these mutations result from TLS or whether they arise by some other means; as, for example, the elevated spontaneous mutability of the Pol α mutant could result from the role of *S. pombe* DinB in mismatch extension, as the related human Pol κ has been shown to carry out proficient mismatch extension (Washington et al. 2002). In that case, the primer terminus with a mispaired nucleotide resulting from the incorporation of a wrong nucleotide by the Pol α mutant could be extended by DinB. That could then suggest a role for checkpoint proteins in modulating DinB function in mismatch extension and not in TLS.

Our studies indicate that Mec1 kinase is not required for TLS carried out opposite UV lesions in wild-type yeast cells, and neither are the Rad17, Mec3, and Ddc1 subunits of the checkpoint clamp or the Rad24 subunit of checkpoint clamp loader. Although the suggestion has been made for the direct involvement of checkpoint clamp in the binding of TLS Pols for mediating lesion bypass (for example, see Kai and Wang 2003; Sabbioneda et al. 2005), such a role is difficult to reconcile with the fact that Rad6–Rad18-mediated PCNA ubiquitination is essential for TLS and that the likely role of this PCNA modification is to facilitate the exchange of the replicative Pol with the TLS Pol (Zhuang et al. 2008). And, importantly, since the ability of a TLS Pol to bind PCNA is obligatory for its function in lesion bypass, that renders the possibility of a direct involvement of the checkpoint clamp in TLS implausible (Haracska et al. 2001; Acharya et al. 2008).

Although there is strong evidence that replication forks collapse in MMS-treated *mec1 Δ* or *rad53 Δ* yeast cells (Tercero and Diffley 2001; Tercero et al. 2003), and that Mec1 and Rad53 are required to delay replication in UV-irradiated *rad14 Δ* cells (Neecke et al. 1999), it has remained unclear which of the various lesion bypass processes—TLS and/or template switching—depend on the activation of replication checkpoint for fork stabilization to occur. Our results indicating the lack of requirement of Mec1-mediated replication checkpoint for TLS could be explained if the functions of TLS Pols were closely coordinated with the replication ensemble, since that would then provide for relatively rapid and efficient

means of lesion bypass. In that case, the stalling of the replication fork may not persist for a long enough period for checkpoint activation to occur. Alternatively, if the replicative Pols were to leave a gap opposite the DNA lesion resuming synthesis downstream, we would expect the persistence of gapped DNA to be sufficient to generate the signal necessary for checkpoint activation to occur; the ensuing delay in replication and in cell cycle progression would then provide the time interval needed for gap filling by TLS.

Replication checkpoint proteins affect TLS proficiency in NER-defective yeast cells

Our studies indicating a reduction in UV mutagenesis in *rad14Δ* cells in the absence of Mec1 kinase or the components of checkpoint clamp and clamp loader are in concert with previous studies in which similar results with the deletions of checkpoint clamp and clamp loader proteins were observed (Paulovich et al. 1998). Since UV lesions are not repaired in NER-defective cells, all of the unrepaired lesions have to be bypassed by TLS or by template switching; consequently, the frequency of UV-induced mutations is greatly enhanced in *rad14Δ* cells resulting from Polζ-mediated TLS opposite DNA lesions.

The persistence of unrepaired UV lesions in *rad14Δ* cells causes the inhibition of S-phase progression dependent on Mec1 and Rad53 but not on the checkpoint clamp and clamp loader (Neecke et al. 1999). In the *mec1* and *rad53* mutants, progression through S phase in UV-irradiated *rad14Δ* cells occurs from replication initiation from late origins. Presumably, in the absence of the Mec1-mediated replication checkpoint in UV-irradiated *rad14Δ* cells, many of the replication forks collapse and unregulated DNA synthesis resumes from late firing origins (Neecke et al. 1999). Since the activation of the replication checkpoint in UV-irradiated *rad14Δ* cells requires the Mec1 kinase but UV mutagenesis can still occur in *rad14Δ* cells in the absence of Mec1 kinase, TLS in *rad14Δ* cells can transpire without the intervention of a replication checkpoint. The checkpoint proteins, Mec1, and the components of checkpoint clamp and clamp loader, however, modulate the proficiency of Polζ-mediated TLS, at least in part through Rev1 phosphorylation, the need for which becomes evident in the absence of NER (see below).

Checkpoint protein-mediated Rev1 phosphorylation modulates the efficiency of Polζ function in UV mutagenesis

Although Rev1 is a Y family Pol, its DNA polymerase activity is very specific for the insertion of a C opposite template G (Nelson et al. 1996a; Haracska et al. 2002), and Rev1 can promote replication through N²-dG minor groove DNA adducts by its proficient ability for inserting a C opposite them, from which another DNA Pol can then carry out the extension reaction (Nair et al. 2008). Even though opposite UV-induced DNA lesions Rev1's DNA polymerase activity plays no role in TLS, Rev1 functions as an indispensable structural element enabling

Polζ to carry out its role in TLS opposite them. Rev1 physically interacts with the Rev3 catalytic subunit of Polζ, and Rev1 binding enhances the proficiency of Polζ for extension from nucleotides inserted opposite a DNA lesion by another DNA Pol (Acharya et al. 2006).

The observations that damage-inducible Rev1 phosphorylation requires—in addition to Mec1/Ddc2 kinase—the components of checkpoint clamp and clamp loader, and that Rev1 phosphorylation occurs at the SQ1 N-terminal site, have allowed us to draw inferences as to how the effects of these checkpoint proteins on UV mutagenesis are related to their role in Rev1 phosphorylation. The results indicating that Rev1 phosphorylation positively modulates the proficiency of Polζ-mediated TLS in NER-defective cells but not in NER-proficient cells, and that the checkpoint proteins involved in mediating Rev1 phosphorylation also affect TLS proficiency only in NER-defective cells, strongly suggest that checkpoint-mediated Rev1 phosphorylation contributes to the proficiency of Polζ-mediated TLS. Furthermore, the epistasis of deletions of checkpoint proteins over the *rev1S31A* mutation for effects on UV mutagenesis implies that the checkpoint proteins contribute to increasing the proficiency of Polζ-mediated TLS at least in part through Rev1 phosphorylation. Since deletions of checkpoint proteins generate a greater reduction in UV mutagenesis in *rad14Δ* cells than the *rev1S31A* mutation, that raises the possibility of checkpoint proteins additionally activating some other protein involved in Polζ-mediated TLS by phosphorylating it, the identity of which remains to be determined.

Since both CPDs and (6-4) photoproducts would persist in DNA in NER-defective yeast cells and be subject to replicative bypass, it remains to be determined whether phosphorylated Rev1 is much more stimulatory to Polζ's role in extending opposite from both types of DNA lesions than the unphosphorylated form, or whether this effect is more pronounced for a (6-4) photoproduct than for a CPD. Alternatively, or in addition, phosphorylated Rev1 may be more effective in complex formation with Polζ and its associated protein factors.

Replication checkpoint, lesion bypass, and Rev1 phosphorylation

Our observations that the checkpoint proteins, Mec1, and the components of checkpoint clamp and clamp loader are not essential for UV mutagenesis, but that checkpoint-induced Rev1 phosphorylation affects the proficiency of UV mutagenesis—which becomes evident in the absence of NER—pose the question as to where at the stalled fork might Rev1 phosphorylation occur in cells during replication. Since the inhibition of S-phase progression in UV-irradiated *rad14Δ* cells requires the Mec1 and Rad53 kinases and not the proteins of the checkpoint clamp and clamp loader (Neecke et al. 1999), whereas Rev1 phosphorylation requires the Mec1 kinase and the checkpoint clamp and clamp loader but not the Rad53 kinase, we infer that Rev1 phosphorylation can occur in the absence of replication checkpoint. The

transient stalling of the replication fork at the lesion site could generate a signal for the loading of checkpoint clamp by the clamp loader on the gapped DNA on the lagging strand, following which the Mec1/Ddc2 kinase would bind the clamp, phosphorylating Rev1 and other proteins present in the replication ensemble. According to this scenario, Rev1 phosphorylation might occur after Rev1 and the other proteins involved in Pol ζ -mediated TLS have been assembled at the site of stalled replication fork. Further, we surmise that, although the transient stalling of the replication fork that ensues during TLS may be adequate for activating Rev1 phosphorylation, it does not produce the threshold level of signal needed for checkpoint activation to occur.

Another question to consider is which of the lesion bypass processes become inoperative in the absence of damage-induced replication checkpoint. Since lesion bypass in the absence of TLS occurs by Rad5-, Mms2-, and Ubc13-dependent (Torres-Ramos et al. 2002; Gangavarapu et al. 2006; Blastyak et al. 2007) or by Rad51-, Rad52-, and Rad54-dependent (Prakash 1981; Gangavarapu et al. 2007) template-switching pathways, the possibility remains that these processes require the establishment of a replication checkpoint to ensure that the integrity of the replication fork stalled at the lesion site is maintained. Such a possibility is suggested also from the greatly elevated UV sensitivity and enhanced UV mutagenesis of *mec1 Δ rad30 Δ* cells, as that could result from the inhibition of Rad5-, Mms2-, Ubc13-, Rad51-, Rad52-, and Rad54-dependent template-switching pathways in the absence of Mec1. Moreover, the greatly enhanced UV sensitivity of the *mec1 Δ pol30-119* double mutant over that of *mec1 Δ* or *pol30-119* mutants is in line with a role for the Mec1-mediated replication checkpoint in Rad51-, Rad52-, and Rad54-mediated lesion bypass by recombinational means.

Conclusions

This study shows that the Mec1 kinase and the components of checkpoint clamp and clamp loader are not required for TLS in yeast cells, and we infer from our results that the intervention of the replication checkpoint is not needed for TLS to occur. The replication checkpoint proteins, however, modulate the proficiency of Pol ζ -mediated TLS at least in part by effecting Rev1 phosphorylation. The dependence of Pol ζ 's ability to carry out efficient TLS on checkpoint proteins as well as on Rev1 phosphorylation can be discerned in *rad14 Δ* cells, where the lesion load is greatly accentuated because of the absence of NER, and therefore the need for more proficient TLS becomes more acute.

Materials and methods

Yeast strains

All yeast strains are derived from DBY747 (*MATa his3- Δ 1 leu2-3 leu2-112 ura3-52*) or from the isogenic EMY74.7 (*MATa his3- Δ 1 leu2-3 leu2-112 trp1 Δ ura3-52*) strain. Genomic deletions of checkpoint genes were made in the wild-type yeast strain

EMY74.7 (Supplemental Table 1). For Rev1 phosphorylation assays, the 4-HA-tagged wild-type Rev1 or mutant Rev1 was expressed from a YCplac111-based plasmid (Supplemental Fig. 1) in different yeast strains (Supplemental Table 2). UV-induced mutagenesis studies for examining the effects of the phosphorylation-defective *rev1S31A* mutation were carried out in yeast strains in which the chromosomal copy of *REV1* has been deleted, and the wild-type Rev1 or the Rev1 S31A mutant protein was expressed from the YCplac111-based plasmid (Supplemental Table 3).

Plasmids for Rev1 expression

The *REV1* ORF plus 1050 nucleotides (nt) of its upstream region that include the *REV1* promoter and 500 nt of the downstream region that include the *REV1* transcription terminator was cloned into the vector YCplac111 (pTB364) (Gietz and Sugino 1988), which carries the *LEU2* gene, the autonomous replicating sequence *ARS1*, and the centromeric *CEN4* region (Supplemental Fig. 1A), generating plasmid pVP21.

A BglII restriction site was added by site-directed mutagenesis right before the stop codon of *REV1*. A 33-mer oligonucleotide duplex containing the HA-coding sequence and a BamHI and a BglII restriction site (5'-GATCATTACCCGTATGATGTTCCG GATTACGCA-3'/5'-GATCTGCGTAATCCGGAACATCATAC GGGTAAT-3') was inserted into the BglII restriction site, adding an HA tag in fusion with *REV1*, and regenerating a unique BglII restriction site (Supplemental Fig. 1B). This step was repeated three times in order to get four HA tags in fusion with *REV1*, generating plasmid pVP64 (Supplemental Fig. 1A).

Rev1 has seven (S/T)Q sites that are known to be the target of PI-3 protein kinases such as Mec1 (Fig. 4A). We individually inactivated these seven potential phosphorylation sites by site-directed mutagenesis by changing the serine or threonine residue to an alanine: S31 \rightarrow A (SQ1), S69 \rightarrow A (SQ2), S159 \rightarrow A (SQ3), S419 \rightarrow A (SQ4), T637 \rightarrow A (TQ5), S662 \rightarrow A (SQ6), and T822 \rightarrow A (TQ7). Each of these mutations was introduced into plasmid pVP64, leading to an HA-tagged version of these mutant proteins.

Analysis of damage-induced Rev1 phosphorylation

An overnight culture of cells expressing 4HA-tagged wild-type or mutationally altered Rev1 at (S/T)Q sites (Supplemental Table 2) from plasmid pVP64 (Supplemental Fig. 1) was grown in synthetic complete (SC) medium lacking leucine (SC – leu) and used to inoculate 50 mL of yeast extract–peptone–dextrose (YPD) media. Once the cells had reached exponential phase ($\sim 10^7$ cells per milliliter), 25 mL of the culture were treated with 4NQO (2 μ g/mL) for 15 min, and 25 mL remained untreated. Cells were then washed once with water and once with 1 \times PBS, 10% glycerol, and 1 mM EDTA before protein extraction. Cells were broken in a bead beater with 3 vol of 1 \times PBS + 10% glycerol + protease inhibitors + phosphatase inhibitor and 2 vol of zirconia/silica beads. Cell debris was removed by centrifugation, and total protein concentration was determined using the Bio-Rad protein assay.

Seventy-five micrograms of the crude protein extract were loaded on a 6% acrylamide/bisacrylamide (77:1) gel. The combination of this low cross-linking percentage with a long migration of the gel (15 cm for the 100-kDa marker) improved the resolution of the phospho-forms of Rev1. Rev1 was detected by Western blotting with anti-HA antibody (Abcam, ab91110).

UV survival and mutagenesis

To examine the effects of checkpoint mutations, the wild-type and isogenic derivatives of genomic deletions of checkpoint

genes (Supplemental Table 1) were grown to logarithmic phase in SC medium supplemented with 10 amino acids plus adenine and uracil (SC + 12). Cultures were washed by centrifugation, followed by sonication to disperse cell clumps. After sonication, the cell suspension was centrifuged again and resuspended to a density of $\sim 2 \times 10^8$ cells per milliliter. Cells were diluted, and serial dilutions were spread on SC + 12 plates for viability determinations and on SC + 12 plates lacking arginine but containing canavanine (SC – arg + can) for determination of *CAN1^S* to *can1^T* mutation frequencies. The plates were UV-irradiated and incubated in the dark for 5 d prior to counting of colonies. The effects of the *rev1S31A* mutation on UV mutagenesis were examined in *rev1Δ* cells carrying the wild-type *REV1* gene or the *rev1S31A* mutant gene on plasmid pVP21 (Supplemental Table 3). To maintain selection for the plasmid, yeast strains were grown in SC – leu and UV survival and the frequency of UV-induced *can1^T* mutations were assessed by plating cells on SC – leu and SC – leu – arg + can media, respectively.

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