The 9-1-1 Checkpoint Clamp Physically Interacts with Polζ and Is Partially Required for Spontaneous Polζ-dependent Mutagenesis in *Saccharomyces cerevisiae*[*]

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The use of translesion synthesis (TLS) polymerases to bypass DNA lesions during replication constitutes an important mechanism to restart blocked/stalled DNA replication forks. Because TLS polymerases generally have low fidelity on undamaged DNA, the cell must regulate the interaction of TLS polymerases with damaged *versus* undamaged DNA to maintain genome integrity. The *Saccharomyces cerevisiae* checkpoint proteins Ddc1, Rad17, and Mec3 form a clamp-like structure (the 9-1-1 clamp) that has physical similarity to the homotrimeric sliding clamp proliferating cell nuclear antigen, which interacts with and promotes the processivity of the replicative DNA polymerases. In this work, we demonstrate both an *in vivo* and *in vitro* physical interaction between the Mec3 and Ddc1 subunits of the 9-1-1 clamp and the Rev7 subunit of the Polζ TLS polymerase. In addition, we demonstrate that loss of Mec3, Ddc1, or Rad17 results in a decrease in Polζ-dependent spontaneous mutagenesis. These results suggest that, in addition to its checkpoint signaling role, the 9-1-1 clamp may physically regulate Polζ-dependent mutagenesis by controlling the access of Polζ to damaged DNA.

The major replicative DNA polymerases are high fidelity enzymes that can be blocked by lesion-containing bases on the template strand (1). Although such blockage can potentially prevent completion of genome duplication, cells can bypass lesions by copying information from the undamaged sister chromatid in a strand switching or recombination type of mechanism. Alternatively, a translesion synthesis (TLS)[5] DNA polymerase can be recruited to insert a nucleotide across from the lesion or to extend a lesion-base mispair (reviewed in Refs. 2 and 3). This unique activity of the TLS polymerases has been attributed to the presence of a large catalytic active site that can accommodate

structurally deformed bases (4). Although it has been suggested that each type of translesion polymerase may be specialized to bypass a certain lesion (or class of lesions) in a relatively error-free manner, many of the TLS polymerases exhibit astoundingly low fidelity on undamaged DNA *in vitro* (reviewed in Refs. 2 and 4). To minimize replication errors on undamaged DNA, the *in vivo* use of translesion polymerases must be restricted so that they are employed only when needed.

Saccharomyces cerevisiae contains three translesion polymerases: Polη, Polζ, and Rev1. Polη has been primarily characterized with respect to its role in the error-free bypass of UV-induced lesions (5) and this bypass appears to require physical interaction with PCNA (6). Yeast strains lacking *REV1* or Polζ demonstrate similar phenotypes in response to DNA damage (4), and it is generally assumed that these two polymerases act in the same pathway of mutagenesis (but see Ref. 7). *In vitro* studies indicate that Rev1 is a G template-specific DNA polymerase (8), but the relevance of this activity to *in vivo* mutagenesis is unclear (9). Polζ, which is comprised of a catalytic subunit encoded by *REV3* and a regulatory subunit encoded by *REV7* (10), is responsible for at least 90% of UV-induced mutagenesis (11) and 50–75% of spontaneous mutagenesis (12). Given the highly mutagenic nature of Polζ, it is vital for the cell to regulate its interaction with DNA.

Recently, it has been appreciated that many TLS polymerases interact with the processivity clamps that tether the replicative DNA polymerases to the template. Pol IV and Pol V of *Escherichia coli*, for example, interact with the β-clamp homodimer, whereas the eukaryotic TLS polymerases Polη, Polι and Polκ physically associate with the PCNA homotrimer (reviewed in Ref. 13). Such interactions have led to the suggestion that a multisubunit processivity clamp might act as a platform during replication to simultaneously tether both replicative and TLS polymerases, thereby allowing ready switching between the two as needed (14). Alternatively, interactions of multiple polymerases with the processivity clamp may be sequential (15), and there is evidence from eukaryotes that post-translational modification of the clamp may be an important regulatory step in polymerase switching (16, 17). Interaction of a TLS polymerase with a processivity clamp would not only keep the TLS polymerase in a location to allow the rapid bypass of DNA lesions, but might also sequester a potentially mutagenic TLS polymerase and restrict its access to undamaged DNA.

The Ddc1, Rad17, and Mec3 proteins of *S. cerevisiae* form a heterotrimeric ring with predicted structural similarity to the PCNA homotrimer (18). This alternative clamp has been termed the 9-1-1 complex based on the names of the homologous *Schizosaccharomyces pombe* and human proteins (Rad9, Rad1, and Hus1, respectively). The 9-1-1 complex is important in the activation of the DNA damage checkpoint and has been recently found to be associated with several DNA repair fac-

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tors, including the Rad14 nucleotide excision repair (NER) protein of *S. cerevisiae* (19), the human FEN1 nuclease (20), the human repair DNA polymerase pol β (21), and the MYH glycosylase of *S. pombe* (22). These diverse interactions may represent a direct function of the 9-1-1 complex in regulating the machineries that process DNA lesions. The 9-1-1 complex can also interact with the DinB TLS polymerase of *S. pombe* and has been shown to be important for DinB activity during replication stress (23). Finally, genetic evidence suggests that the 9-1-1 clamp may be involved in regulating Polζ-dependent damage-induced mutagenesis, as yeast *rad17* or *mec3* mutants have a reduction in UV-induced mutagenesis similar to that of a *rev3* strain (24). The mechanism relating the 9-1-1 complex to Polζ activity in induced mutagenesis is not yet understood, and a possible role of this clamp in Polζ-dependent spontaneous mutagenesis has not been reported.

In the current study, we identify Rev7 as a partner for 9-1-1 complex components in a two-hybrid screen and we provide evidence that the Mec3 and Ddc1 subunits of the *S. cerevisiae* 9-1-1 alternative clamp physically interact with Polζ. We also show that the 9-1-1 complex is required to stably recruit Polζ onto damaged chromosomes, providing an explanation for the role of the complex in UV-induced mutagenesis. Finally, we demonstrate that the 9-1-1 clamp is partially required also for Polζ-dependent spontaneous mutagenesis, supporting a model whereby the checkpoint clamp is an important regulator of TLS both under induced and non-induced mutagenesis conditions.

EXPERIMENTAL PROCEDURES

Media and Growth Conditions—YEP medium (1% yeast extract, 2% Bacto-peptone, 250 μg/ml adenine, 2% agar for plates) was supplemented with either 2% dextrose (YEPD), 2% glycerol, 2% ethanol (YEPGE), or 2% galactose, 2% raffinose (YEPGal). Prototroph selection was on synthetic complete medium containing 2% dextrose (SCD) and lacking the appropriate nutrient (25). Ura$^-$ auxotrophs were selected on SCD-uracil plates containing 1 mg/ml 5-fluoroorotic acid and supplemented with uracil (12 mg/ml final concentration) (26). Geneticin- and hygromycin-resistant transformants were selected on YEPD plates containing 200 μg/ml Geneticin (G418) or 300 μg/ml hygromycin B, respectively. For assessment of β-galactosidase activity, the appropriate medium was supplemented with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal).

Plasmid and Strain Constructions—Bait plasmids for the yeast two-hybrid screening were obtained by amplifying the relevant coding sequences from genomic DNA and ligating the resulting fragments into pEG202 (kind gift from R. Brent). The plasmids used for expressing GST-*MEC3* (pFBL7) and GST-*DDC1* (pFLB11) in *E. coli* were derived by inserting appropriate PCR-amplified genomic DNA fragments into pGEX4-T3 (Amersham Biosciences). Specific information regarding these constructs is available on request.

The yeast two-hybrid screening was performed using the B42/*lexA* system with strain EGY48 (*MATa his3 ura3 trp1 6lexAOP-LEU2*; lexAOP-lacZ reporter on plasmid pBH18-34) as the host strain (27). Strains used for co-immunoprecipitation and immunofluorescence experiments were congenic derivatives of W303*RAD5* (*MATa ade2-1 trp1-1 can1-100 leu2-3,12 his3-11,15 ura3*; kind gift of Marco Foiani). Gene deletions were obtained by PCR-mediated gene replacement (28). C-terminal-tagged versions of chromosomal genes were generated using a one-step PCR method with plasmid pFA6-13Myc-kanMX6 or pFA6-3HA-TRP1 as template (29). Tagged proteins retained wild-type function as assessed by MMS and UV sensitivity. YSS46 (*MATa REV7-13Myc-kanMX6 ddc1Δ*) was a meiotic segregant obtained by crossing strains YSS13 (*MATa REV7-13Myc-kanMX6*) and YDL42 (*MATα*

ddc1Δ::kanMX6). YSS59 (*MATa REV7-13Myc-kanMX6 REV3-3HA-TRP1*) was generated by transformation of YSS13 with the *3HA-TRP1* cassette.

Strains used for the mutation studies were congenic derivatives of SJR922 (*MATα ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔA746*; Ref. 30). Deletion of *RAD1* was as described by Harfe and Jinks-Robertson (31), and a Ura$^-$ derivative was identified on 5-fluoroorotic acid medium to create strain SJR1177. Deletion of *REV3*, *REV7*, *RAD24*, *RAD17*, *DDC1*, *MEC3*, *ELG1*, and *CTF18* was obtained by PCR-mediated gene replacement using pFA6-kanMX2 (28) or pFA6-hphMX4 (32) as appropriate and selecting geneticin- or hygromycin-resistant transformants, respectively. In the *rad1 rad24 ctf18* background, *ELG1* was replaced with the *Kluyveromyces lactis URA3* gene (*URA3-Kl*), which was amplified using the plasmid pCORE (33) as a template. All gene deletions contained a precise deletion of the published open reading frame, with the exception of *REV3*, in which the first and last 60 bp of the coding region remain; *REV7*, in which the first and last 42 bp of the coding region remain; and *CTF18*, in which 301 bp of the 5′ and 161 bp of the 3′ end of the gene remain. Similar disruption strategies of *CTF18* have been shown to completely eliminate Ctf18 function (34, 35).

In Vitro GST Pull-down Experiments—*E. coli* BL21 cells transformed with pGEX4-T3, pFLB7, or pFLB11 were grown at 37 °C to an A_{600} of 0.8. Cells were then shifted to 17 °C for 1 h and induced overnight with 1 mM isopropyl 1-thio-β-D-galactopyranoside at 17 °C. Cells were collected, washed with cold H$_2$O, and resuspended in cold phosphate-buffered saline containing the Roche Complete protease inhibitor mixture and 1 mM phenylmethylsulfonyl fluoride. Cells were disrupted by sonication, and extracts were clarified by centrifugation at 14,000 × g for 15 min. Extracts were incubated for 2 h with glutathione-Sepharose 4B beads and the resin was then washed 5 times with cold phosphate-buffered saline to remove unbound protein. The resin with bound protein was incubated with 50 μl of ^{35}S-labeled Rev7 for 2 h, followed by extensive washing with phosphate-buffered saline. The ^{35}S-labeled Rev7 was produced with TNT T7 Quick for PCR DNA (Promega) following the manufacturer's instructions. Proteins that remained bound to the resin were analyzed by autoradiography after SDS-PAGE.

Chromosome Spreading—Cells were grown in YEPD medium at 28 °C to a concentration of 5 × 10^6 cells/ml and arrested with α-factor (2 μg/ml) for 2 h. α-Factor was then removed by washing, and cells were allowed to re-enter the cell cycle. Cell cycle progression was assessed by performing FACS analysis at defined times after the release. For UV irradiation, cells were spread on YEPD medium at defined times after the α-factor release and irradiated with 100 J/m^2 of UV. Control cells were plated but not treated with UV. Cells were immediately washed off the plates and processed for chromosome spreading as described by Giannattasio *et al.* (19). Immunofluorescence was performed with 9E10 α-Myc, Alexa goat α-mouse, and Alexa donkey α-goat antibodies. 4,6-Diamidino-2-phenylindole staining was used to identify well spread nuclei, and at least 50 such nuclei were blindly counted for coincident rhodamine staining over 5 slides/experiment for each time point.

Co-immunoprecipitation of Proteins from Yeast—G$_1$-arrested cells were released into S phase and UV-irradiated as described above. Cells were immediately washed from plates after UV irradiation and lysed under nondenaturing conditions. Extracts were prepared in phosphate-buffered saline and 10 mg of extract in 1 ml were incubated for 2 h with 7.5 μg of either 9E10 α-Myc or 12CA5 α-HA antibody as appropriate. 30 μl of Protein G-agarose beads were added and incubation was continued for another 2 h. Alternatively, extracts were incubated with a resin previously cross-linked to 4G7/11 α-Ddc1 antibody (36). After
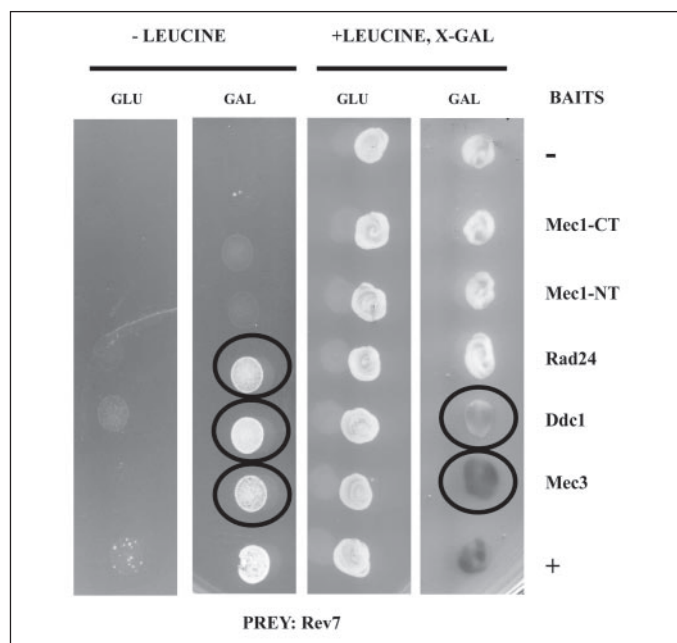
FIGURE 1. **The Rev7 subunit of Polζ interacts with components of the 9-1-1 clamp.** Drops of yeast cultures were spotted on selective plates containing the indicated carbon source (*GLU*, glucose; *GAL*, galactose/raffinose) and incubated for 2 days. Leucine and X-gal were added to the medium as indicated. Mec1-CT and Mec1-NT are bait fusions expressing the C- and N-terminal domains of the Mec1 kinase, respectively. Interaction with a given bait protein results in expression of the *lacZ* reporter gene, which is detected by the chromogenic substrate X-gal (+ *LEUCINE/X-GAL*) and of the *LEU2* reporter gene and growth on plates lacking leucine (− *LEUCINE*). Expression of the more sensitive *LEU2* reporter gene in the absence of *lacZ* expression indicates a weak bait-prey interaction. The negative control (−) corresponds to cells carrying empty bait and prey plasmids. The positive control (+) is a strain carrying p53 and the SV40 T antigen as the bait and prey, respectively.

extensive washing, proteins were separated by SDS-PAGE in 10% polyacrylamide gels. Western blotting was performed using standard techniques and α-Myc (9E10), α-HA (12CA5), or specific antibodies against the protein of interest.

Determination of Spontaneous Reversion Rates and Mutation Spectra—Cultures containing 5 ml of YEPGE were inoculated with single colonies and grown to saturation (2 to 3 days) on a roller drum at 30 °C. Cells were pelleted, washed with 5 ml of H_2O, and resuspended in 1 ml of H_2O. To assess cell viability and Lys^+ reversion, appropriate cell dilutions were plated on YEPD and SCD-lysine plates, respectively. Cells were counted after 2 days of growth for YEPD plates and after 2–3 days for SCD-lysine plates. Reversion rates were determined by the method of the median (37), and 95% confidence intervals of the rates were calculated as described in Spell and Jinks-Robertson (38) using Table B11 of Altman (39).

To generate a mutation spectrum, spontaneous Lys^+ revertants were obtained as described above. To ensure independence of each reversion event, only one colony from each culture was used for analysis. Following purification of revertant colonies, genomic DNA was isolated by glass bead lysis (40) and mutation events were identified by sequencing an appropriate PCR product (see Ref. 30). The reversion rate of a specific category of frameshift event was determined by multiplying the percentage of the event in the reversion spectrum by the corresponding overall reversion rate. To determine the 95% confidence intervals for the event, the high and low confidence intervals for the overall mutation rate were multiplied by the percentage of times the specific event occurred in the spectrum. A table describing the nature of the complex insertions (cins) in each strain is available upon request.
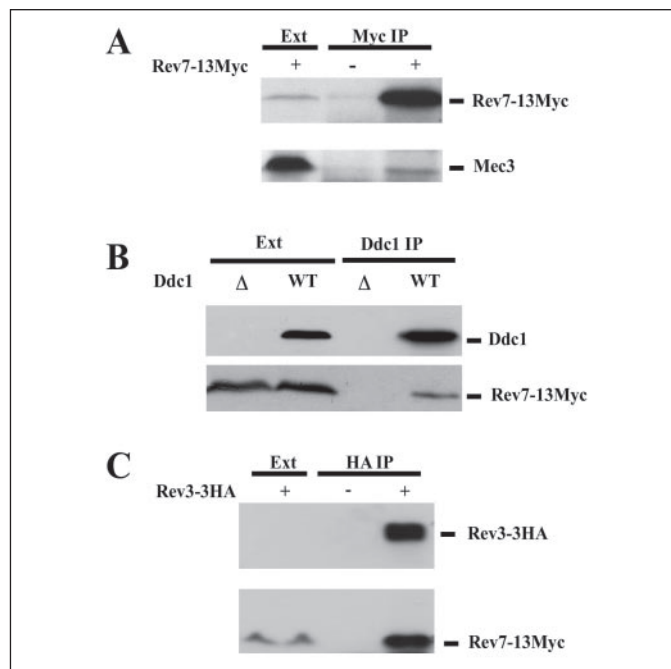

FIGURE 2. **Rev7 interacts *in vivo* with the 9-1-1 clamp and forms a complex with Rev3.** Cells expressing physiological levels of the relevant tagged or untagged protein were UV-irradiated and lysed under nondenaturing conditions. Lanes labeled as *Ext* correspond to the crude extract of the cells expressing the tagged protein. *A*, protein extracts from cells expressing (+) or not expressing (−) Rev7-13Myc incubated with 9E10 anti-Myc antibodies. Proteins recovered using Protein G-agarose beads were analyzed by probing Western blots with Mec3- or Myc-specific antibodies to detect, respectively, Mec3 or Rev7-13Myc. *B*, protein extracts derived from *DDC1* (WT) or *ddc1Δ* (Δ) strains expressing Rev7-13Myc were incubated with anti-Ddc1 antibodies crosslinked to protein G-agarose beads. Western blots were probed with Ddc1- and Myc-specific antibodies to detect, respectively, Ddc1 and Rev7. *C*, protein extracts from Rev7-13Myc cells expressing physiological levels of either HA-tagged Rev3 (+) or untagged Rev3 (−) were incubated with 12CA5 anti-HA antibodies. Proteins recovered using protein G-agarose beads were analyzed by probing Western blots with HA-specific or Myc-specific antibodies to detect Rev3 or Rev7, respectively. Rev3 was detectable only after overnight exposure.

RESULTS

Rev7 Physically Interacts with 9-1-1 Components in Two-hybrid Experiments—Two-hybrid methods have been extensively utilized to screen for protein interactions *in vivo* (41), and we have been systematically applying this technique to identify putative interactors with known checkpoint proteins. In the course of a two-hybrid screening using the Ddc1 subunit of the 9-1-1 complex as bait, we identified several *REV7*-containing plasmids, suggesting that Ddc1 and the Rev7 component of translesion polymerase Polζ may interact *in vivo*. The initial Ddc1-Rev7 interaction identified using a yeast genomic library was confirmed by testing a panel of yeast strains harboring different checkpoint proteins as baits and expressing the Rev7 prey. Cultures of the relevant strains were spotted onto media containing either glucose (prey not expressed) or galactose (prey expressed), and the bait-prey interactions were assessed using *lacZ* and *LEU2* reporters (Fig. 1). Expression of the *lacZ* reporter indicated a strong interaction between Rev7 and the Ddc1 and Mec3 subunits of the 9-1-1 complex, and these interactions were confirmed by plating cells in the absence of leucine. Interaction of Rev7 with Rad17, the third subunit of the 9-1-1 complex, could not be tested because the Rad17 bait construct partially activated the reporter genes in the absence of any prey (data not shown). The two-hybrid analysis also detected a weak interaction between Rev7 and Rad24, the large subunit of the RFC-like clamp loader that loads the 9-1-1 complex onto DNA *in vitro*. No interaction was observed, however, when the Rev7 prey was challenged with control baits expressing

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the N- or C-terminal halves of Mec1. Altogether, the data obtained with the two-hybrid system indicate that the Rev7 component of Polζ interacts with the 9-1-1 clamp, and possibly also with the Rad24-RFC clamp loader.

Rev7 and 9-1-1 Components Can Be Co-immunoprecipitated from Yeast Extracts—The two-hybrid interaction between Rev7 and the 9-1-1 clamp was confirmed by co-immunoprecipitation experiments using UV-irradiated strains expressing physiological levels of Myc-tagged Rev7 (Rev7-13Myc). As shown in Fig. 2*A*, Mec3 was co-immunoprecipitated with Rev7 in extracts derived from the Rev7-13Myc cells, but not from control cells containing untagged Rev7. This interaction was confirmed by the reciprocal experiment (data not shown). Immunoprecipitation with Ddc1-specific antibodies cross-linked to Protein G-agarose beads similarly yielded Rev7-13Myc in the bead-bound fractions (Fig. 2*B*) when wild-type cells were used, but not when the extract was derived from a *ddc1*Δ strain.

The cellular level of soluble Rev3 is very low (compare *Ext* and *IP* lanes in Fig. 2*C*). This could explain why we could not co-immunopre-

cipitate 9-1-1 components with Rev3-HA, whereas we clearly detected an *in vivo* interaction between Rev7-13Myc and Rev3-HA (Fig. 2*C*), in agreement with the finding that Rev7 copurifies with the Rev3 catalytic subunit of Polζ (10).

Rev7 Interacts Directly with 9-1-1 Components in Vitro—To obtain further biochemical evidence to support the *in vivo* interactions of Rev7 with the 9-1-1 components observed by two-hybrid and co-immuno-precipitation analyses, we performed *in vitro* GST pull-down experiments. For this analysis the *REV7* coding sequence was *in vitro* transcribed and translated in the presence of [^{35}S]methionine. Labeled Rev7 was then incubated with GST-Ddc1 or GST-Mec3 fusion protein purified from *E. coli*, and polypeptides associated with glutathione-Sepharose beads were analyzed by autoradiography and Coomassie Blue staining after SDS-PAGE (Fig. 3). This analysis demonstrated that labeled Rev7 can indeed interact with both purified Ddc1 and Mec3, suggesting that the observed *in vivo* interactions are likely to be direct.

The 9-1-1 Complex Influences the Access of Polζ to Damaged Chromosomes—It has been demonstrated that the PCNA-like 9-1-1 complex plays an important role in the cellular response to genotoxic stress (42). In this context, it is possible that the 9-1-1 clamp may modulate the recruitment of specific DNA polymerases required for translesion DNA synthesis in response to DNA damage. We thus analyzed the extent of Rev7 binding to chromosomes under normal *versus* DNA-damaging conditions, and examined whether this loading was influenced by the 9-1-1 complex. Fig. 4 shows the localization of Rev7-13Myc to chromosome spreads. Rev7 (and by inference Polζ) clearly bound to chromosomes with increased efficiency after UV treatment, and this preferential binding to damaged DNA was dependent on the presence of a functional 9-1-1 complex.

To confirm this observation and to obtain more quantitative localization data, the chromosome spreads were repeated using synchronous cultures that had been UV irradiated at different times after release from a G$_1$ arrest. As shown in Fig. 5, the ratio of Rev7-positive nuclei significantly increased in wild-type cells after UV irradiation and this increase was dependent on the presence of Ddc1. The increase in Rev7-positive nuclei was greatest 20 min after release from the α-factor G$_1$ arrest, which corresponded to the time when cells were traversing S phase as assessed by FACS analysis, suggesting that TLS activity may be taking
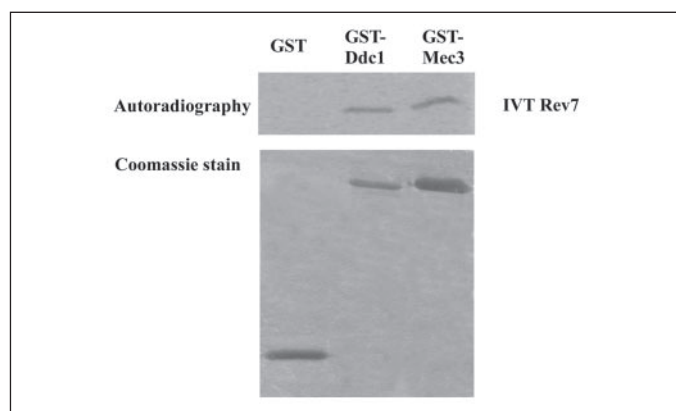

FIGURE 3. **Rev7 interacts *in vitro* with subunits of the 9-1-1 clamp.** Fusion proteins GST-Mec3 and GST-Ddc1 were purified from bacteria and mixed with ^{35}S-labeled Rev7 obtained by *in vitro* transcription/translation. Protein complexes were recovered with glutathione-Sepharose beads and were analyzed by SDS-PAGE and autoradiography. As a negative control to establish the background level of Rev7, GST alone was incubated with labeled Rev7. The Coomassie-stained gel indicates the amounts of purified proteins used in the pull-down experiments.

FIGURE 4. **Rev7 binds to chromosomes after UV irradiation in a Ddc1-dependent manner.** Cells containing Myc-tagged Rev7 were UV irradiated or mock irradiated 20 min after release from an α-factor G$_1$ arrest and chromosome spreads were immediately prepared. The spreads were processed for immunofluorescence with anti-Myc antibodies and DNA was visualized by 4,6-diamidino-2-phenylindole (*DAPI*) staining. Wild-type (*DDC1*) and *ddc1*Δ strains were compared.
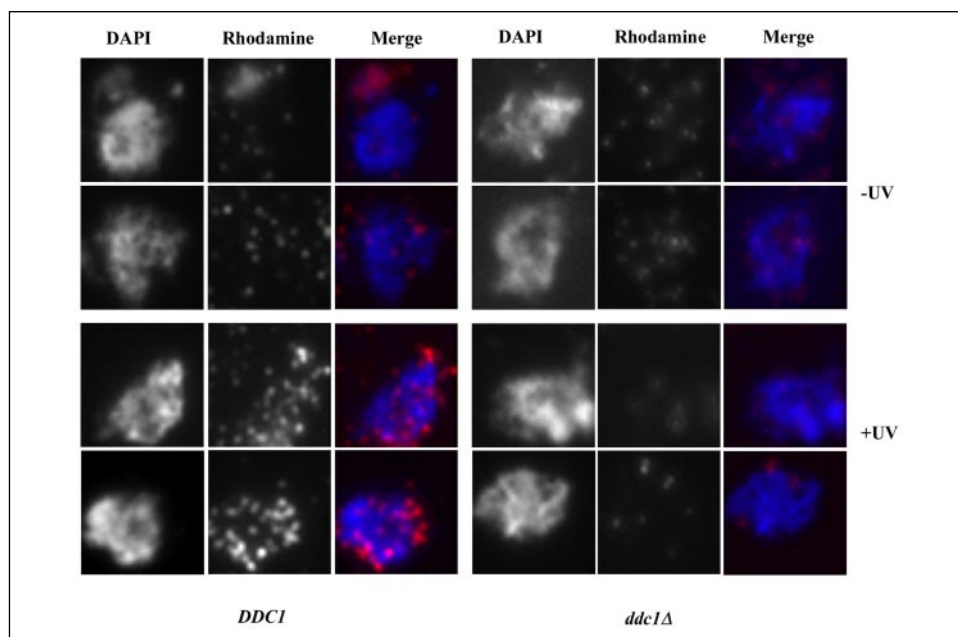
FIGURE 5. **Rev7 binding to damaged chromosomes increases during S phase in a Ddc1-dependent manner.** *DDC1* or *ddc1Δ* strains expressing Myc-tagged Rev7 were arrested in G$_1$ by α-factor treatment. After release from the block, samples were UV irradiated or mock irradiated at the indicated times and processed for chromosome spreads. *A*, FACS analysis of each time sample was done to establish cell cycle position. *B*, the percentage of Myc-positive spreads at each time point was calculated. The *graphs* report the ratio of percentages of positive nuclei in the indicated samples. At 20 min after the release from the G$_1$ arrest, ~60% of the nuclei from the UV-irradiated *DDC1* strain were Rev7-positive.
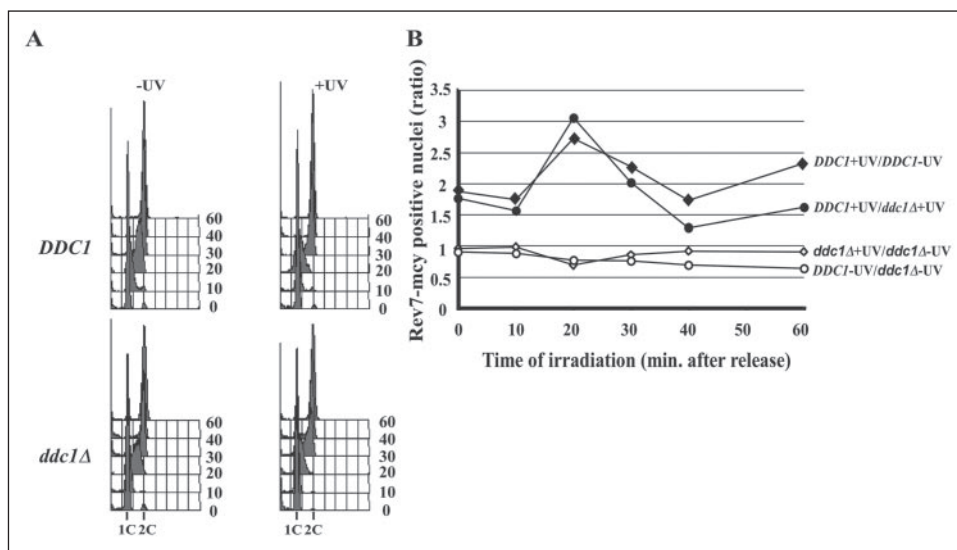
place at the blocked replication fork or at gaps left by the replication machinery.

The Alternative Clamp Is Partially Required for Polζ-dependent Mutagenesis in Vivo—The physical interaction results agree with previous genetic data suggesting a role for 9-1-1 in UV-induced mutagenesis (24). Moreover, the yeast two-hybrid and GST pull-down experiments indicate that the interaction between Rev7 and Ddc1/Mec3 can occur in the absence of exogenous DNA damage. To determine the biological relevance of this interaction in unperturbed cells, we investigated whether elimination of Rad17, Ddc1, or Mec3 affects spontaneous Polζ-dependent mutagenesis. Changes in mutagenesis were assayed using the *lys2ΔA746-1* frameshift allele (30). In an NER-deficient *rad1* background, two major classes of reversion events are detected: simple frameshifts, which correspond to a single nucleotide insertion, and complex frameshifts, in which an insertion is accompanied by a base pair substitution (31) (Fig. 6). Whereas simple frameshifts are likely the result of random polymerase slippage events that occur during processive replication, complex frameshifts occur primarily in two locations in the reversion window, termed hotspots 1 and 2, and are intimately linked with Polζ activity. Complex events are completely absent when Rev3 is eliminated (31) (Fig. 6) or when its catalytic activity is abolished[6] and are believed to reflect lesions that, in the absence of NER, are bypassed in an error-prone manner by Polζ (31). Changes in the rate of complex events at hotspots 1 and 2 provide a specific and sensitive indicator of perturbations in Polζ-dependent mutagenesis (43).

To determine whether loss of the 9-1-1 PCNA-like clamp affects spontaneous Polζ-dependent mutagenesis, we deleted *RAD17*, *MEC3*, or *DDC1* in a *rad1* background. Although the overall *lys2ΔA746* reversion rates of the double mutant strains were not significantly different from that of a *rad1* strain (TABLE ONE), there was a striking decrease in the rate of accumulation of complex frameshift events at hotspots 1 and 2. Deletion of any one of the subunits of the 9-1-1 alternative clamp resulted in about a 3–4-fold drop in the rate of complex frameshifts at these locations compared with that of a *rad1* strain. Examination of the mutation spectrum of a *rad1 rev7* mutant demonstrated that Rev7 is required for most, if not all, complex frameshift events in this assay, as no complex events were detected among the 87 revertants sequenced (Fig. 6). These results demonstrate that the alternative clamp is partially required for Polζ-dependent mutagenesis in this assay, and validate a

biological relevance for the observed physical interaction between Rev7 and Mec3 or Ddc1.

The Rad24 Clamp Loader Protein Does Not Affect Polζ-dependent Mutagenesis in the lys2ΔA746 Frameshift Detection Assay—Given that Polζ-dependent translesion synthesis at hotspots 1 and 2 is partially dependent on the 9-1-1 clamp proteins and that Rad24-RFC can load the alternative clamp onto DNA *in vitro* (18), we predicted that the mutation rate and reversion spectrum of a *rad1 rad24* strain should be similar to that of an NER-defective *rad17*, *mec3* or *ddc1* strain. As expected, the overall reversion rate of a *rad1 rad24* strain did not differ significantly from that of the *rad1*, *rad1 rad17*, *rad1 mec3*, or *rad1 ddc1* strains (TABLE ONE). However, complex events at hotspots 1 and 2 did not decrease significantly in the *rad1 rad24* mutant relative to the *rad1* parent (Fig. 6 and TABLE ONE). The lack of effect of Rad24 loss on Polζ-dependent mutagenesis at hotspots 1 and 2 suggests that the contribution of the 9-1-1 complex to spontaneous mutagenesis is not dependent on the presence of Rad24.

Roles of Ctf18 and Elg1 in Polζ-dependent Mutagenesis—Recently, two additional alternative clamp loader-like complexes have been identified in *S. cerevisiae* and higher eukaryotes: Ctf18-RFC and Elg1-RFC. Like Rad24, Ctf18 and Elg1 form novel complexes with Rfc2-Rfc5 (34, 35, 44–48) and are speculated to function as clamp loaders. Simultaneous elimination of Ctf18, Elg1, and Rad24 dramatically increases sensitivity of yeast to DNA damaging agents and leads to defects in damage-dependent Rad53 phosphorylation above that seen in the corresponding single or double mutant strains (35, 44–46). Given the apparent overlapping roles for these alternative clamp loader-like complexes *in vivo*, we created appropriate triple and quadruple mutant strains to investigate whether loss of two or more of the alternative RFC complexes affects Polζ-dependent mutagenesis.

The overall mutation rate in the *rad1 rad24 ctf18* strain was ~2-fold higher than in a *rad1* or a *rad1 rad24* strain (TABLE ONE). Unexpectedly, the rate of complex events at hotspots 1 and 2 increased 2-fold in the *rad1 rad24 ctf18* triple mutant relative to the *rad1 rad24* double mutant strain (TABLE ONE and data not shown). Elimination of Elg1 in a *rad1 rad24 ctf18* background increased the overall reversion rate of the *lys2ΔA746* frameshift allele nearly 4-fold relative to the triple mutant (TABLE ONE), which is in agreement with other mutation rate studies (44–46, 49). Although there was a clear decrease in the proportion of complex events at hotspots 1 and 2 compared with other frameshifts in the *rad1 rad24 ctf18 elg1* spectrum (Fig. 6), calculation of the

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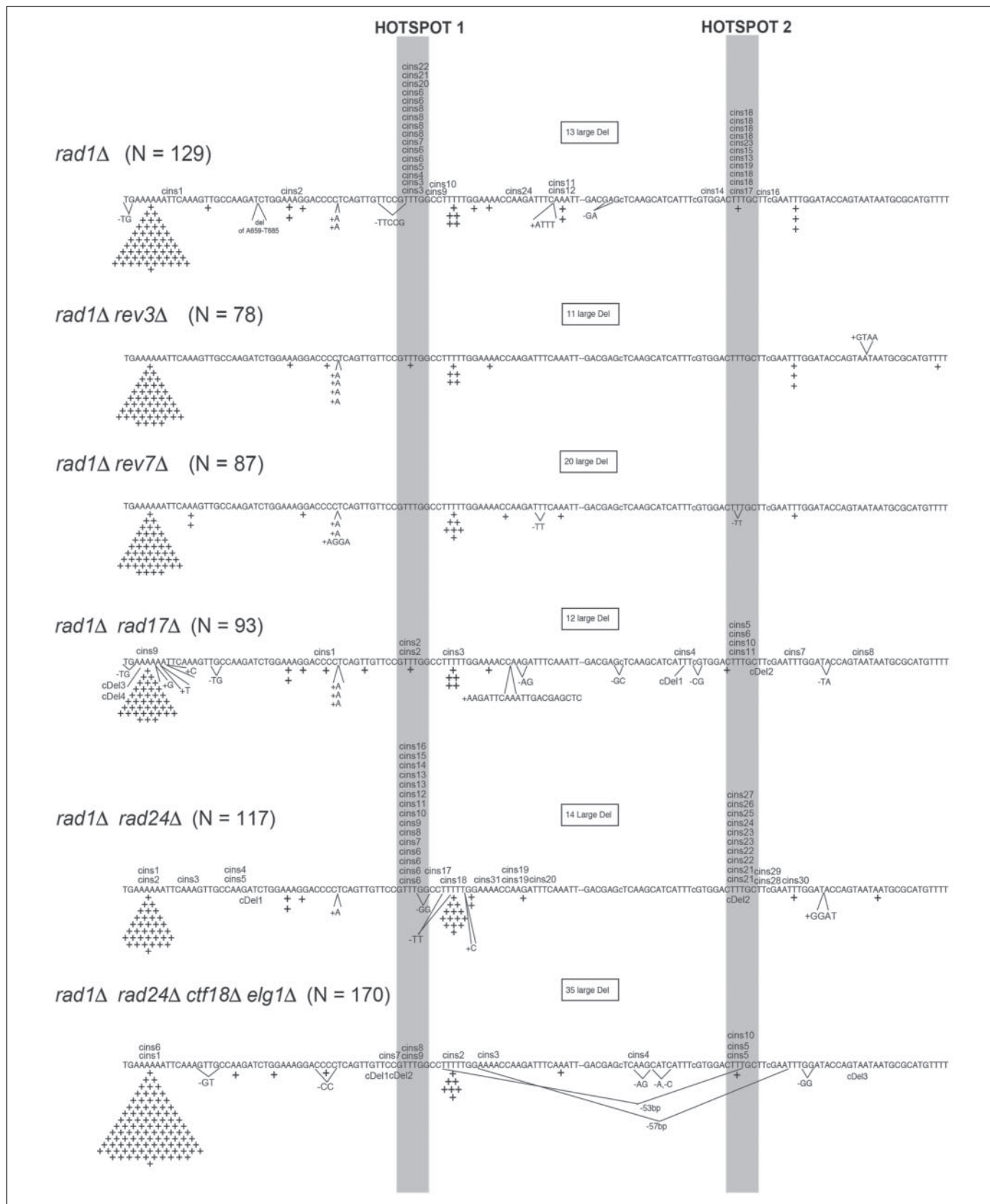[6] S. Yellumahanti and S. Jinks-Robertson, unpublished results.

FIGURE 6. **Reversion spectra of *lys2ΔA746* allele in mutant strains.** The total number of independent revertants sequenced for each strain is indicated *next to* the relevant strain genotype. An insertion of a single nucleotide identical to the surrounding sequence is shown as +. Reversion events in which more than one nucleotide was inserted or events where nucleotides were inserted that were not identical to the surrounding sequence are as indicated *below* the spectrum. Deletions of two or more nucleotides are shown as −, complex deletions are shown as *cDel*, and complex insertions are indicated as *cins*. Individual complex events that had identical sequence changes are given identical numbers within each spectrum. The number of large cleletion events in a given strain is shown *boxed above* each spectrum. The sequences of hotspots 1 and 2 are indicated by the *shaded areas*. The *rad1* and *rad1 rev3* spectra have been published previously (31).

TABLE ONE

Rates of *lys2ΔA746* reversion events in *rad1* derivatives

Mutation rates were calculated using at least 14 independent cultures and 95% confidence intervals are indicated in parentheses. Hotspots 1 and 2 seen in the reversion spectra are abbreviated HS1 and 2. Asterisk indicates significant difference relative to a *rad1* strain.

Strain	All frameshift events	Complex events at HS1 and 2	All other frameshifts		
			Simple	Large Del	Other
	rate $\times 10^{-9}$	rate $\times 10^{-10}$	rate $\times 10^{-10}$		
rad1	4.1 (3.9–4.7)	8.5 (8.2–9.9)	24 (23–28)	4.1 (3.9–4.8)	4.4 (4.2–5.1)
rad1 rev3	1.8* (1.4–2.4)	<0.23*[a] NA[b]	15* (12–20)	2.6* (2.0–3.4)	0.23* (1.8–3.1)
rad1 rev7	2.8 (2.3–5.2)	<0.32*[a] NA	21 (17–38)	6.5* (5.3–12)	1.0* (0.79–1.8)
rad1 rad17	3.5 (2.4–5.4)	2.2* (1.6–3.5)	22 (15–34)	4.5 (3.1–7.0)	6.3 (4.4–9.9)
rad1 mec3	3.9 (3.4–7.7)	3.1* (2.7–6.1)	22 (19–43)	5.8* (5.1–11)	8.2* (7.1–16)
rad1 ddc1	2.8 (2.3–5.8)	3.3* (2.7–6.8)	14 (11–28)	7.2* (5.8–15)	4.1 (3.3–8.5)
rad1 rad24	3.3 (2.6–4.2)	7.0 (5.6–9.0)	17* (13–21)	3.9 (3.1–5.0)	5.4 (4.2–6.8)
rad1 rad24 ctf18	7.9* (6.9–9.8)	14* (12–18)	42* (37–52)	9.2* (8.0–11)	13* (12–17)
rad1 elg1	12* (9.6–14)	12 (9.2–14)	83* (64–97)	9.2* (7.1–11)	20* (15–23)
rad1 rad24 ctf18 elg1	30* (23–48)	8.9 (6.7–14)	200* (150–320)	62* (47–99)	28* (22–45)

[a] Maximum theoretical rate assuming one event.
[b] NA, not applicable.

rate of complex events at hotspots 1 and 2 in the quadruple mutant revealed no significant decrease relative to a *rad1*, *rad1 rad24*, or *rad1 rad24 ctf18* strain (TABLE ONE). These data indicate that neither Rad24-RFC, Ctf18-RFC, nor Elg1-RFC is required for efficient Polζ-dependent translesion synthesis at hotspots 1 and 2.

In contrast to the lack of an effect of loss of all three putative alternative clamp loaders on the rate of complex frameshifts, their simultaneous loss was associated with a 16-fold increase in the rate of large deletion events (TABLE ONE). Because such deletions are often elevated in strains with defects in either PCNA or Polδ (43, 50, 51), we suggest that the dramatic increase in large deletions in the *rad1 rad24 ctf18 elg1* quadruple mutant may reflect overlapping functions of the corresponding clamp loaders during processive DNA replication.

DISCUSSION

The PCNA-like 9-1-1 complex (composed of Ddc1, Mec3, and Rad17 in budding yeast) is required for the initial steps of the DNA damage signaling cascade that leads to the activation of the checkpoint kinases (52). This complex has been shown to interact both physically and functionally with several DNA repair factors, suggesting a direct involvement in the repair of DNA damage as well (19). To identify new pathways functionally connected to the *S. cerevisiae* 9-1-1 complex, we performed an extensive two-hybrid screen using the Ddc1 subunit as bait. In the current study, we report that Rev7, the regulatory subunit of Polζ, interacts with Ddc1 and Mec3 in two-hybrid assays and in GST pulldown experiments. Furthermore, Rev7 can be co-immunoprecipitated with Ddc1 and with Mec3 from crude extracts obtained from UV-damaged yeast cells expressing endogenous levels of the relevant proteins. These data provide biochemical support for the existence of a complex containing 9-1-1 and Polζ. This finding is particularly intriguing because the 9-1-1 complex interacts with the DinB polymerase of *S. pombe* (23), and its human counterpart stimulates the activity of DNA polymerase β (21).

To investigate the significance of the interaction between 9-1-1 and

Polζ, we analyzed Polζ recruitment to damaged chromosomes. Our results demonstrate that Polζ loading increases specifically during S phase following UV irradiation and that this effect requires a fully functional 9-1-1 complex. Deletion of Ddc1 reduced the amount of chromosome-bound Polζ to the basal level, eliminating the UV-induced and S phase-specific quota of bound enzyme. One possible interpretation of these data is that Polζ may normally be loaded onto chromosomes and, when lesions reach a threshold level, is called into action by the 9-1-1 complex. These results are in agreement with published genetic evidence and may provide a molecular mechanism for the requirement for Rad17 and Mec3 in DNA damage-induced mutagenesis (24).

The analysis of 9-1-1 function in Polζ activity was extended by investigating the role of the complex in spontaneous mutagenesis. We previously reported that, in the *lys2ΔA746* reversion assay, loss of the catalytic subunit of Polζ (Rev3) specifically eliminates complex frameshifts that accumulate at two very distinctive hotspots in NER-defective strains (31). Because the Rev3-dependent hotspot events are observed only in the absence of NER, these events are assumed to directly reflect lesion bypass by Polζ. The presence/absence of complex frameshifts at these hotspots can thus be used to assess the activity of Polζ in spontaneous lesion bypass *in vivo* (43). The mutation spectrum obtained in an NER-defective *rev7Δ* strain was indistinguishable from that obtained in a *rev3Δ* background, demonstrating that in this assay both proteins are essential for Polζ activity. Further analysis of Polζ-dependent complex events revealed that deletion of any one of the genes encoding 9-1-1 subunits resulted in a significant decrease in the rate of complex events at the hotspots, indicating that the interactions between 9-1-1 components and Rev7 are indeed of biological relevance to Polζ-dependent activity in spontaneous lesion bypass.

Although the majority of the spontaneous Polζ-dependent complex frameshifts required the 9-1-1 components, it should be noted that ~30% of these events were independent of the 9-1-1 clamp. This could be a reflection of the context of the DNA lesion. For example, an interaction between Polζ and the 9-1-1 clamp may be required if a lesion is

encountered during leading strand synthesis (as a replication-blocking substrate in the presence of PCNA) as opposed to the lagging strand synthesis, where the lesion might instead reside in a gap. Alternatively, perhaps only certain types of lesions require an interaction between the 9-1-1 clamp and Polζ for bypass.

The *S. cerevisiae* 9-1-1 heterotrimer is loaded onto DNA *in vitro* by a modified RFC complex, where Rad24 replaces Rfc1 (18, 53, 54). We were, therefore, surprised to find that deletion of *RAD24* did not affect Polζ-specific spontaneous mutation in an NER-defective background. Two additional RFC-like clamp loaders, Ctf18-RFC and Elg1-RFC, have been recently described in *S. cerevisiae*, and genetic analyses indicate functional overlaps between Rad24, Ctf18, and Elg1 (35, 44–46). We thus assessed Polζ activity in lesion bypass in strains containing deletions of the corresponding genes. Even simultaneous deletion of the *ELG1*, *RAD24*, and *CTF18* genes did not decrease the rate of complex events at the hotspots. It is possible that either an unidentified clamp loader is involved, or that the replicative RFC clamp loader itself might be important for the activity of the 9-1-1 complex under some circumstances. Intriguingly, a physical interaction between PCNA and the 9-1-1 clamp has been detected both *in vivo* and *in vitro* (55, 56).[7] Perhaps when the replicative polymerase and PCNA are stalled at a blocking lesion, the 9-1-1 clamp in conjunction with Polζ is targeted to (possibly a modified form of) PCNA, thereby allowing Polζ to gain access to the DNA lesion for bypass. Finally, an interaction between the Pol32 subunit of the replicative polymerase Polδ and the 9-1-1 complex has been suggested (57), and Pol32 has been shown to be required for both spontaneous and induced Polζ-dependent mutagenesis (43, 50, 57, 58). Regardless of precisely how the 9-1-1 clamp accesses DNA, our data suggest a Rad24-independent process that, at least in the case of spontaneous damage, is relevant to translesion synthesis and may be separable from the more well characterized checkpoint function.

PCNA has been shown to interact directly with some TLS polymerases and to stimulate their activity *in vitro* (16, 59–61). Based on the interaction between Polζ and the 9-1-1 clamp reported here, it would be reasonable to predict that 9-1-1 might stimulate Polζ. However, recent results failed to detect such a stimulation using purified proteins in an *in vitro* assay (62). One possible interpretation may be the existence of a yet undescribed accessory factor that is lacking from the biochemically defined system. Our observation, that the 9-1-1 clamp promotes stable binding of Polζ to damaged DNA *in vivo*, suggests that recruitment, rather then direct stimulation of polymerase activity, may be the relevant *in vivo* function of the 9-1-1 complex. On the other hand, purified PCNA was reported to stimulate Polζ activity (62). Because monoubiquitination of PCNA is completely required for induced and partially required for spontaneous Polζ-dependent mutagenesis (43, 63–65), it is tempting to draw direct parallels between the *in vitro* and *in vivo* PCNA-Polζ results. Caution should be exercised, however, when ascribing *in vivo* relevance to the stimulatory effect of unmodified PCNA on Polζ activity in a purified system that may lack other required accessory factors (*e.g.* Rev1). As noted by others (13), an alternative role of PCNA monoubiquitination may be to displace a replicative polymerase so that the blocked 3′ end becomes accessible to the appropriate bypass machinery. The experiments reported here not only add another layer of complexity to lesion bypass by Polζ, but also enlarge the role of the 9-1-1 checkpoint clamp during the cellular responses to DNA damage.

How might checkpoints and TLS polymerases cooperate in maintaining genome stability in the absence of exogenous damage? One possibility is that checkpoint factors, including the 9-1-1 clamp, might be associated with the replication fork (66). When a replicative polymerase is blocked by a lesion, checkpoint factors would stabilize the stalled fork; the 9-1-1 clamp might also be involved in preventing further attempts by Polδ or Polε to insert new nucleotides. At the same time, the 9-1-1 clamp, in cooperation with PCNA or Pol32, might drive a polymerase switch to replace replicative enzymes with TLS polymerases. If the lesion is bypassed, replicative polymerases would replace the TLS polymerase to continue DNA synthesis; if not, sufficient single-stranded DNA might be generated by the replicative helicase to trigger a checkpoint cascade. Alternatively the replicative polymerase may restart replication downstream of the lesion, leaving a gap containing the damage, the 9-1-1 clamp, and possibly PCNA behind. This complex would then help recruit a TLS polymerase to the gapped region to complete DNA synthesis. Altogether, the available data strongly suggest that Polζ may be considered as a repair component that associates with both 9-1-1 and PCNA clamps. Clearly, a focus of future experiments will be to determine the precise role of the 9-1-1 checkpoint clamp in Polζ-dependent mutagenesis, and how the 9-1-1 complex interfaces with PCNA in this process.

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The 9-1-1 Checkpoint Clamp Physically Interacts with Polζ **and Is Partially Required for Spontaneous Pol** ζ**-dependent Mutagenesis in** *Saccharomyces cerevisiae*

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