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Role of budding yeast Rad18 in repair of HO-induced double-strand breaks

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Abstract

The Rad6-Rad18 complex mono-ubiquitinates proliferating cell nuclear antigen (PCNA) at the lysine 164 residue after DNA damage and promotes DNA polymerase η (Pol η)- and Pol ζ /Rev1-dependent DNA synthesis. Double-strand breaks (DSBs) of DNA can be repaired by homologous recombination (HR) or non-homologous end-joining (NHEJ), both of which require new DNA synthesis. HO endonuclease introduces DSBs into specific DNA sequences. We have shown that Pol ζ and Rev1 localize to HO-induced DSBs in a Mec1-dependent manner and promote Ku-dependent DSB repair. However, Pol ζ and Rev1 localize to DSBs independently of PCNA ubiquitination. Here we provide evidence indicating that Rad18-mediated PCNA ubiquitination stimulates DNA synthesis by Pol ζ and Rev1 in repair of HO-induced DSBs. Ubiquitination defective PCNA mutation or *rad18 Δ* mutation confers the same DSB repair defect as *rev1 Δ* mutation. Consistent with a role in DSB repair, Rad18 localizes to HO-induced DSBs in a Rad6-dependent manner. Unlike Pol ζ or Rev1, Pol η is dispensable for repair of HO-induced DSBs. Ku and DNA ligase IV constitute a central NHEJ pathway. We also show that Pol ζ and Rev1 act in the same pathway as DNA ligase IV, suggesting that Pol ζ and Rev1 are involved in DNA synthesis during NHEJ. Our results suggest that Pol ζ -Rev1 accumulates at regions near DSBs independently of PCNA ubiquitination and then interacts with ubiquitinated PCNA to facilitate DNA synthesis.

Keywords

translesion DNA synthesis; DNA repair; DNA damage checkpoint; Mec1; Tel1

Introduction

DNA damage that stalls DNA replication poses a major threat to genomic stability and cellular viability. To tolerate such replication blocks, cells are equipped with specialized DNA polymerases that can bypass DNA damage by a process known as translesion synthesis (TLS) [1,2]. The TLS DNA polymerases include DNA polymerase η (Pol η) and Pol ζ . Pol η , encoded by *RAD30* in budding yeast and XPV in human, performs relatively error-free bypassing of pyrimidine dimers [3,4]. Pol ζ consists of the catalytic subunit Rev3 and the accessory subunit Rev7 [5-7]. In budding yeast, Pol ζ interacts physically with Rev1 [8], a deoxycytidyl transferase that inserts a C opposite an abasic site [9]. It has been proposed that Rev1 carries out the insertion step across from the lesion and that Pol ζ subsequently extends from the lesion

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[9]. Recent evidence indicates that Pol η and Pol ζ -Rev1 are also involved in other repair pathways than TLS [8,10,11].

In budding yeast, Rad6 and Rad18 are required for replication of damaged DNA templates [2]. Rad6, a ubiquitin-conjugating enzyme, exists *in vivo* in a tight complex with Rad18 [12, 13]. Proliferating cell nuclear antigen (PCNA) is the eukaryotic sliding clamp required for processive DNA synthesis [14]. The Rad6-Rad18 complex mediates mono-ubiquitination at the lysine 164 residue of PCNA [15] and promotes DNA synthesis by Pol η and Pol ζ after DNA damage [16,17]. However, some instances of DNA synthesis may not require modification of lysine 164 [18]. PCNA is loaded onto DNA by replication factor C, which couples ATP hydrolysis with the opening and closing of the PCNA ring around the DNA [14]. PCNA loaded on DNA can be efficiently ubiquitinated by the Rad6-Rad18 complex [19,20]. Ubiquitination of PCNA might create a platform to recruit the TLS polymerases [21,22]. Alternatively, ubiquitinated PCNA could function as a processivity factor for the TLS polymerases [19]. We have shown that Pol ζ and Rev1 localize to DSBs and contribute to Ku-dependent repair of DSBs [8]. Pol ζ and Rev1 are found to associate with DSBs in a Mec1-dependent manner but independently of PCNA ubiquitination [8]. However, it remains to be determined whether PCNA ubiquitination activates Pol ζ /Rev1-dependent DNA synthesis in DSB repair.

In this study, we have investigated whether Rad18 or PCNA ubiquitination is required for Pol ζ /Rev1-dependent DSB repair. Although accumulation of Pol ζ and Rev1 at DSBs does not require PCNA ubiquitination, DSB repair activities of Pol ζ and Rev1 are fully dependent on Rad18 function. Consistent with the role in DSB repair, Rad18 localizes to sites near DSB lesions. These results suggest that Rad18 activates the Pol ζ /Rev1 pathway after DSB induction.

Materials and Methods

Plasmids and Strains

To generate pGAL-ACT1, the *ACT1* coding sequence was amplified by polymerase chain reaction (PCR) and cloned into YCpG22 [23]. YCpG22, containing a *GALI-10* promoter region, is a derivative of YCplac22 [24]. YCpA-GAL-HO was described previously [8]. YCpT-GAL-HO is obtained from J.A. Nickoloff. Epitope tagging of *RAD18* was performed by a PCR-based strategy [25]. Cells expressing myc-tagged Rad18 protein are as resistant to UV irradiation as wild-type cells, indicating that myc-tagged Rad18 protein is fully functional (data not shown). Cells carrying *dnl4 Δ* , *pol4 Δ* , *rad1 Δ* , *rad6 Δ* , *rad30 Δ* or *siz1 Δ* mutation were obtained by transformation with PCR fragments marked with *HphMX4* [26], *KanMX4* [27], *TRP1* [8] or *URA3* [28]. Disruption of each gene was confirmed by PCR. Constructions of other mutations are described elsewhere [8,29]. The strains used here are listed in Table 1.

Sensitivity to HO-induced DSBs

Sensitivity to HO-induced DSBs was examined as previously described [8,30]. Cells were transformed with the YCpA-GAL-HO plasmid, and grown overnight in sucrose-medium selectable for the plasmid. Cultures were serially diluted in water and aliquots of diluted cultures were spread out on plates containing glucose (2%) or sucrose-galactose (2%) medium selectable for the plasmid. *HO* expression from YCpA-GAL-HO is inducible in sucrose-galactose medium, but is repressed by the addition of glucose. Independent transformants were examined at least three times, and viability was calculated in a condition where 100-200 colonies are formed on a plate. Dilution experiments showed that survivors form colonies proportionally if less than 2×10^6 cells were spread out on a plate. Sensitivity to HO-induced DSBs results in a reduced plating efficiency on inducible galactose plates relative to repressible glucose plates. In this assay, cells suffer continuous *HO* expression. However, cells can survive after *HO* expression if they carry mutations in the HO recognition sequence or lose the HO

cleavage site at the *ADH4* locus. Rad52-dependent recombination could delete the HO recognition site, because cells contain no donor sequence containing the HO cleavage site. We note that all the genes distal to the *ADH4* locus on chromosome VII are not essential for cell proliferation. During NHEJ, annealing of ssDNA regions can delete non-homologous sequences [31], resulting in loss of the HO cleavage site. In addition, annealing of ssDNA tracts could generate gaps, which are filled in by DNA synthesis. Subsequently, error-prone DNA synthesis might introduce mutations at the HO cleavage site. Therefore, cell survival after *HO* expression depends on DSB repair processes. However, this assay system fails to monitor simple joining of DSB ends, which regenerates the HO cleavage site.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation was performed as previously described [8] using anti-myc (9E10) antibodies. The PCR reaction was performed under non-saturating conditions, in which the rate of PCR amplification was proportional to the substrate concentration and cycling. The sequences of primers for the HO set at the *ADH4* locus were 5'-TCTATTAATGAGCCGAGACCGGTA-3' and 5'-CGCATGTGAATGACACACGAAAGT-3', those for the *SMC2* locus were 5'-AAGAGAACTTTAGTCAAAACATGGG-3' and 5'-CCATCACATTATACTAACTACGG-3'.

Results

Overlapping role of Rev1 and Rev3 with DNA ligase IV in DSB repair

We have shown that Pol ζ (the Rev3-Rev7 complex) and Rev1 are required for Ku-dependent repair of DSBs [8], using an experimental system in which HO endonuclease induces a single DSB at the *ADH4* locus (Fig. 1A). Ku and DNA ligase IV constitute a central NHEJ pathway [32,33]. In budding yeast, DNA ligase IV is composed of the catalytic subunit Dnl4 (Lig4) and the co-factor Lif1 [33]. To further support that Pol ζ and Rev1 act in DSB repair, we examined whether *REV1* and *REV3* act in the same pathway as *DNL4* (Fig. 1B). In budding yeast, DSBs are repaired primarily by the Rad52-dependent HR pathway; therefore, the efficiency of NHEJ can be assessed in a *rad52 Δ* background [30,34,35]. In the assay system (Fig. 1A), wild-type and *rad52 Δ* mutant cells retain 20% and 0.2% viability after *HO* expression, respectively [8]. Cells carrying the *rad52 Δ* mutation or those containing the *rev1 Δ* , *rev3 Δ* , *dnl4 Δ* or *hdf1 Δ* mutation were transformed with the GAL-HO plasmid. Transformed cells were grown in sucrose to maintain the *GAL* promoter inactive. Aliquots of the culture were then plated on medium containing galactose to induce *HO* expression or medium containing glucose to repress *HO* expression. Consistent with the idea that Ku and DNA ligase IV constitute a central NHEJ pathway, introduction of the *hdf1 Δ* single, *dnl4 Δ* single or *hdf1 Δ dnl4 Δ* double mutation into *rad52 Δ* mutants caused a further 20-fold reduction in survival after DSB induction. The *rev1 Δ* or *rev3 Δ* mutation decreased viability of *rad52 Δ* mutants by 5-fold [8], but neither mutation decreased viability of *rad52 Δ dnl4 Δ* double mutants. In budding yeast, Rad1 contributes to DSB repair independently of Ku or Rad52 [36]. If *rad52 Δ hdf1 Δ* cells were survived by DSB repair, introduction of the *rad1 Δ* mutation should further reduce the cell viability. Consistently, *rad52 Δ hdf1 Δ rad1 Δ* triple mutants were more sensitive (3-fold) than *rad52 Δ hdf1 Δ* double mutants (Fig. 1B). Taken together, these observations support the possibility that Pol ζ and Rev1 contribute to DNA synthesis in NHEJ of HO-induced DSBs.

The viability assay here depends on the hypothesis that cells survived from continuous *HO* expression after aberrant repair of the HO cleavage site. However, some *rad52 Δ hdf1 Δ rad1 Δ* triple mutants carrying the GAL-HO plasmid proliferated on galactose medium. We addressed how the *rad52 Δ hdf1 Δ rad1 Δ* triple mutants survived on galactose medium. There are two explanations for cell survival without DSB repair. One possibility could be that cells

lost the intact GAL-HO plasmid; for example, they might have received mutations in the GAL-HO construct or reversion mutations in the *ade1* mutation on the chromosome. The GAL-HO plasmid used for the viability assay carries *ADE1* as a selection marker. We thus re-tested whether *rad52Δ hdf1Δ rad1Δ* survivors retain viability on galactose medium by introducing a *TRP1*-marked GAL-HO plasmid (Table 2). Another possibility could be that cells obtained mutations in genes involved in galactose metabolism, thereby causing a defect in *HO* expression. We addressed this possibility by introducing a *TRP1*-marked GAL-ACT1 plasmid (Table 2). *ACT1* encodes actin, which is one of major components of the cytoskeleton. Overexpression of *ACT1* from the GAL promoter blocks cell proliferation [37]. Most of the *rad52Δ hdf1Δ rad1Δ* survivors carrying the GAL-ACT1 plasmid failed to proliferate on galactose medium. One out of 39 tested survivors proliferated well on galactose, suggesting that this survivor acquired mutation(s) affecting galactose metabolism. Correspondingly, this survivor retained viability on galactose even after transformation with the *TRP1*-marked GAL-HO plasmid. Besides, only one survivor retained viability on galactose medium after introduction of the *TRP1*-marked GAL-HO plasmid. Thus, *rad52Δ hdf1Δ rad1Δ* survivors principally lost the intact GAL-HO plasmid for cell proliferation, suggesting that *rad52Δ hdf1Δ rad1Δ* mutants are defective in repair of HO-induced DSBs.

We also examined survivors from each of *rad52Δ* single, *rad52Δ rev1Δ* double or *rad52Δ hdf1Δ* double mutants (Table 2). All the *rad52Δ* survivors carrying the GAL-ACT1 plasmid did not grow well on galactose. However, most survivors carrying the *TRP1*-marked GAL-HO plasmid retained viability on galactose medium. These results suggest that *rad52Δ* survivors largely received mutations in the HO recognition site. Similar results were obtained with the *rad52Δ rev1Δ* survivors. Notably, half of *rad52Δ hdf1Δ* survivors appeared to acquire mutations at the HO restriction site. These results are consistent with the current view that the Ku-dependent NHEJ pathway plays an important role in DSB repair in the absence of the HR pathway and Rad1 contributes to DSB repair as a third pathway in budding yeast.

Involvement of Rad18 and PCNA ubiquitination in DSB repair

The Rad6-Rad18 complex ubiquitinates PCNA at Lys-164, and thereby activates Polη and Polζ functions in budding yeast. Indeed, neither *rad30Δ* nor *rev3Δ* mutations increase sensitivity to MMS or UV light of cells carrying the ubiquitination-defective PCNA (*pol30-K164R*) mutation [16,17]. We therefore examined the effect of *pol30-K164R* mutation on the DSB repair as above (Fig. 2A). Although the *pol30-K164R* mutation alone did not significantly affect cellular death after DSB induction (data not shown), it decreased viability of *rad52Δ* cells similarly to the *rev1Δ* mutation. The *pol30-K164R* mutation did not further enhance the viability loss in *rad52Δ hdf1Δ* double mutants. Introduction of the *rev1Δ* mutation did not affect viability in *rad52Δ pol30-K164R* double mutants. Thus, the *pol30-K164R* mutation behaves like the *rev1Δ* mutation in DSB repair. We next examined the effect of *rad18Δ* mutation on the DSB repair (Fig. 2B). The *rad18Δ* mutation conferred the same phenotype as the *pol30-K164R* mutation; both mutations increased cell death in *rad52Δ* mutants but did not affect viability in *rad52Δ hdf1Δ* double mutants after DSB induction. Moreover, introduction of the *pol30-K164R* mutation did not increase viability loss in *rad52Δ rad18Δ* double mutants. Again, *rev1Δ* mutation did not affect the *rad52Δ rad18Δ* double mutants. Cells carrying the *rad52Δ rad6Δ* double or *rad52Δ rad6Δ rad18Δ* triple mutation showed the same viability loss as *rad52Δ rad18Δ* double mutants, consistent with the view that Rad6 and Rad18 act as a complex in the same pathway (Fig. 2C). PCNA is also SUMOylated by the Siz1-Ubc9 complex at Lys-164 [15]. Both PCNA ubiquitination and SUMOylation have been shown to contribute to Polζ/Rev1-dependent DNA synthesis [16]. We monitored effects of *siz1Δ* mutation on cellular viability in *rad52Δ* and *rad52Δ rad18Δ* backgrounds (Fig. 2D). No apparent defect was associated with the *siz1Δ* mutation, suggesting that Siz1-dependent SUMOylation is not

essential for repair of HO-induced DSBs. Together, these results support a model in which Rad6-Rad18 complex promotes the Pol ζ /Rev1-dependent DSB repair by ubiquitinating PCNA.

Localization of Rad18 to the HO-induced DSB in a Rad6-dependent manner

The Rad6-Rad18 complex binds to DNA [12,13], and ubiquitinates PCNA loaded on DNA [19,20]. To confirm the function of the Rad6-Rad18 complex in DSB repair, we investigated whether the Rad6-Rad18 complex localizes to the HO-induced DSB by chromatin immunoprecipitation (ChIP) assay. Cells expressing Rad18-myc were transformed with the GAL-HO plasmid [8]. Transformants were grown initially in sucrose to repress *HO* expression and then incubated with galactose to induce *HO* expression. Cells were collected after a 3 hr incubation with galactose, and extracts prepared after formaldehyde cross-linking were subjected to immunoprecipitation with anti-myc antibodies. Co-precipitated DNA was extracted and amplified by polymerase chain reaction (PCR) using the HO1 primer set to amplify a region near the HO restriction site in the *ADH4* locus on chromosome VII. As a control, PCR was performed to amplify the *SMC2* locus on chromosome VI, which contains no cleavage site (Fig. 1A). PCR amplification with the HO1 primer set was detected in cells carrying the GAL-HO plasmid after incubation with galactose (Fig. 3A). In contrast, there was no PCR amplification from the *SMC2* locus after incubation with galactose (Fig. 3A). PCR amplification was not observed in untagged cells or cells lacking an HO cleavage site at the *ADH4* locus (data not shown). These results indicate that Rad18 associates with sites near the HO-induced DSB. To address the role of Rad6-Rad18 complex formation, we next examined the effect of *rad6 Δ* mutation on Rad18 association with the HO-induced DSB. Rad18 association with the DSB was significantly decreased in *rad6 Δ* mutants (Fig. 3A). This association defect was not due to decreased expression of Rad18; the *rad6 Δ* mutation did not affect the expression level of Rad18 (data not shown). These results suggest that Rad18 associates with DSBs in the form of the Rad6-Rad18 complex, and support the role of the Rad6-Rad18 complex in DSB repair.

Localization of Rad18 to DSBs through a Mec1- and Tel1-independent mechanism

The ATR homolog Mec1 plays a central role in DNA damage checkpoint responses in budding yeast [38]. We have shown that Pol ζ and Rev1 associate with regions near DSBs in a Mec1-dependent manner [8]. We therefore asked whether Mec1 also controls the Rad18 association with DSBs (Fig. 3B). However, Rad18 association with DSBs was not impaired in *mec1 Δ* mutants. The ATM homolog Tel1 is also involved in the cellular response to DSBs, although Tel1 plays a minor role [38]. Checkpoint response to DSBs is essentially abolished in *mec1-81 tel1 Δ* double mutants [29]. We then examined the effect of *mec1-81 tel1 Δ* mutations on Rad18 association with DSBs (Fig. 3B). Rad18 associated efficiently with DSBs even in *mec1-81 tel1 Δ* mutants. Thus, activation of the checkpoint responses is not required for Rad18 localization to the DSB.

Effect of *rad30 Δ* or *pol4 Δ* mutation on repair of the HO-induced DSB

Ubiquitination of PCNA activates the Pol η (Rad30) pathway as well as the Pol ζ -Rev1 pathway in budding yeast [16,17]. Similar to *rev1 Δ* or *rev3 Δ* mutation [8], *rad30 Δ* mutation by itself did not affect cellular viability in the presence of Rad52 after DSB induction (data not shown). We then examined the effect of *rad30 Δ* mutation on the DSB repair in *rad52 Δ* mutants (Fig. 4A). Unlike *rev1 Δ* or *rev3 Δ* mutation, introduction of the *rad30 Δ* mutation did not enhance viability loss in *rad52 Δ* mutants. Moreover, the *rad30 Δ* mutation did not increase cell death in *rev1 Δ rad52 Δ* double mutants. These results are consistent with the above finding that the viability loss in *rev1 Δ rad52 Δ* or *rev3 Δ rad52 Δ* double mutants is not distinguishable from that of *pol30-K164R rad52 Δ* or *rad18 Δ rad52 Δ* double mutants (see Fig. 1 and Fig. 2). We note that *rad52 Δ rev1 Δ rad30 Δ* triple mutants are more sensitive to UV light than either of

rad52Δ rev1Δ or *rad52Δ rad30Δ* double mutants (data not shown). Thus, Rad30 does not play an apparent role in repair of the HO-induced DSB.

Pol4, a family X DNA polymerase, plays a critical role in DNA synthesis during NHEJ [39, 40]. To address the relationship between the Polζ-Rev1 and Pol4 pathways, we investigated the effect of *rev1Δ* and *pol4Δ* mutations on the DSB repair in *rad52Δ* mutants (Fig. 4B). Consistent with the previous finding [39], introduction of the *pol4Δ* mutation enhanced viability loss in *rad52Δ* mutants. Interestingly, the *rev1Δ pol4Δ* double mutation caused more significant viability loss in *rad52Δ* mutants than either of the *rev1Δ* or *pol4Δ* single mutation. These results suggest that Polζ-Rev1 and Pol4 act redundantly during DNA synthesis in repair of HO-induced DSBs.

Discussion

Rad18-mediated PCNA ubiquitination has been proposed to promote Polζ- and Rev1-dependent DNA synthesis. PCNA ubiquitination has been shown to mediate Rev1-PCNA interaction [41]. However, localization of Polζ and Rev1 to DSB lesions does not require Rad18 function or PCNA ubiquitination [8]. It has thus remained possible that Polζ and Rev1 contribute to DSB repair independently of PCNA ubiquitination. In this study, we have used a system in which cells receive pure DSBs after *HO* expression and found that the Rad18-mediated PCNA ubiquitination activates the Polζ/Rev1 pathway in DSB repair as well. These observations support the model in which Polζ-Rev1 first accumulates at regions near DSBs and then interacts with ubiquitinated PCNA to facilitate DNA synthesis. Exposure to DSB inducing agents, for example ionization radiation (IR), also induces single-strand breaks, base modifications and DNA adducts, which can be repaired by the TLS pathway. For example, the introduction of a *rev1Δ* mutation significantly reduces viability of *rad52Δ hdf1Δ* double mutant cells after IR (data not shown). Therefore, it is difficult to characterize functions of Polζ-Rev1 and Rad18 in DSB repair using IR. HO endonuclease, which generates pure DSBs, enabled us to investigate a role of Polζ-Rev1 and Rad18 in DSB repair.

If simple re-joining of DNA ends does not occur, DSBs are processed by exonuclease activities, generating ssDNA tract at the DSB ends. The Rad6-Rad18 complex has been shown to bind to single-stranded DNA *in vitro* [12,13]. This biochemical property of the Rad6-Rad18 complex is consistent with our observation that Rad18 localizes to DSBs. During the revision of this paper, Davies et al. [42] also reported that Rad18 localizes to DSBs. Requirement of Rad18 for DSB repair has been shown in chicken DT 40 cells [43], suggesting that Rad18 associates with DSB lesions in higher eukaryotes as well. The Rad6-Rad18 complex has been proposed to ubiquitinate PCNA loaded on DNA [19,20], because only a small fraction of PCNA is modified after DNA damage. Even though Rad18 localizes to DSBs, PCNA modification has not been detected in cells suffering HO-induced or *EcoRI*-induced DSBs by immunoblotting analysis (data not shown)[44]. It is not known what fraction of PCNA is loaded on DNA at a DSB lesion or how many DSBs are generated after *EcoRI* expression. If only a few PCNA molecules are loaded on DNA, it might be difficult to detect PCNA ubiquitination.

Annealing of ssDNA tracts at DNA ends may generate gaps with 3'-termini. Replication factor C can load PCNA on gapped DNA substrates [45]. The Rad6-Rad18 and Polζ-Rev1 complexes accumulate at sites near DSB ends [8]. Once loaded, PCNA could be ubiquitinated by the Rad6-Rad18 complex. In turn, ubiquitinated PCNA might stimulate the activity of nearby Polζ or Rev1 [19]. Previous studies have demonstrated that Pol4 plays an important role in gap-filling, by extending the DNA strand from mismatched 3'-termini [39,40]. Similarly, Polζ efficiently extends from mismatched termini [46]. Polζ is a processive enzyme [46], whereas Pol4 is not [40]. Thus, Polζ-Rev1 and Pol4 could play distinct roles in gap-filling during NHEJ. Recent studies have established the model in which the cyclin-dependent kinase, Cdc28, stimulates

resection of DNA ends to generate ssDNA tracts at DSBs [47,48]. This model explains why NHEJ operates preferentially in G1. In the absence of DNA degradation, however, NHEJ could rejoin the DNA ends without gap-filling. Thus, DNA polymerase activities might be more dispensable for NHEJ in G1 phase than in other cell-cycle stages. Consistently, Rev1 and Rad18 do not play an apparent role in repair of HO-induced DSBs in G1 (data not shown), although Ku plays a critical role [49]. Since Mec1 localizes to sites of DNA damage by interacting with RPA-coated ssDNA [50,51], Mec1 associate with DSBs in S and G2/M phase more efficiently than in G1 phase [52]. We have shown that Mec1-dependent phosphorylation promotes Pol ζ -Rev1 association with DSBs [8]. Mec1-mediated Pol ζ -Rev1 accumulation might assure efficient gap-filling during NHEJ in S or G2/M phase.

PCNA ubiquitination activates the Pol η pathway as well as the Pol ζ /Rev1 pathway [16,17]. However, Pol η (Rad30) is dispensable for our DSB repair assay. These findings suggest that PCNA ubiquitination does not always activate the Pol η pathway. Association of Pol η with the HO-induced DSB has not been detected by ChIP assay (data not shown), whereas Pol ζ and Rev1 association is observed [8]. Association of human Pol η with damage sites is dependent on PCNA ubiquitination [21,22]. As discussed above, ubiquitination of PCNA has not been detected after DSB induction. It is therefore possible that each DSB lesion contains only a few ubiquitinated PCNA molecules. In this case, Pol η might fail to accumulate at DSBs in budding yeast. By contrast, Pol ζ -Rev1 accumulates at DSBs independently of PCNA ubiquitination. Therefore, Pol ζ -Rev1 could interact with ubiquitinated PCNA at DSBs.

Although there are only two TLS polymerases (Pol ζ and Pol η) in budding yeast, human cells express more varied TLS polymerases including Polk and Polt [1]. These TLS polymerases may play specific roles at sites of DNA damage, because they possess different biochemical properties. However, it still remains unclear how these TLS polymerases act coordinately in DNA repair. Our results suggest that ubiquitination-independent accumulation helps Pol ζ -Rev1 to act differently from Pol η in budding yeast.

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References

1. Friedberg EC, Wagner R, Radman M. Specialized DNA polymerases, cellular survival, and the genesis of mutations. *Science* 2002;296:1627–1630. [PubMed: 12040171]
2. Prakash S, Prakash L. Translesion DNA synthesis in eukaryotes: a one- or two-polymerase affair. *Genes Dev* 2002;16:1872–1883. [PubMed: 12154119]
3. Johnson RE, Prakash S, Prakash L. Efficient bypass of a thymine-thymine dimer by yeast DNA polymerase, Poleta. *Science* 1999;283:1001–1004. [PubMed: 9974380]
4. Masutani C, Kusumoto R, Yamada A, Dohmae N, Yokoi M, Yuasa M, Araki M, Iwai S, Takio K, Hanaoka F. The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta. *Nature* 1999;399:700–704. [PubMed: 10385124]
5. Nelson JR, Lawrence CW, Hinkle DC. Thymine-thymine dimer bypass by yeast DNA polymerase zeta. *Science* 1996;272:1646–1649. [PubMed: 8658138]
6. Gibbs PE, McGregor WG, Maher VM, Nisson P, Lawrence CW. A human homolog of the *Saccharomyces cerevisiae* REV3 gene, which encodes the catalytic subunit of DNA polymerase zeta. *Proc Natl Acad Sci U S A* 1998;95:6876–6880. [PubMed: 9618506]
7. Murakumo Y, Roth T, Ishii H, Rasio D, Numata S, Croce CM, Fishel R. A human REV7 homolog that interacts with the polymerase zeta catalytic subunit hREV3 and the spindle assembly checkpoint protein hMAD2. *J Biol Chem* 2000;275:4391–4397. [PubMed: 10660610]

8. Hirano Y, Sugimoto K. ATR homolog Mec1 controls association of DNA polymerase zeta-Rev1 complex with regions near a double-strand break. *Curr Biol* 2006;16:586–590. [PubMed: 16546083]
9. Nelson JR, Lawrence CW, Hinkle DC. Deoxycytidyl transferase activity of yeast REV1 protein. *Nature* 1996;382:729–731. [PubMed: 8751446]
10. Kawamoto T, Araki K, Sonoda E, Yamashita YM, Harada K, Kikuchi K, Masutani C, Hanaoka F, Nozaki K, Hashimoto N, et al. Dual roles for DNA polymerase eta in homologous DNA recombination and translesion DNA synthesis. *Mol Cell* 2005;20:793–799. [PubMed: 16337602]
11. McIlwraith MJ, Vaisman A, Liu Y, Fanning E, Woodgate R, West SC. Human DNA polymerase eta promotes DNA synthesis from strand invasion intermediates of homologous recombination. *Mol Cell* 2005;20:783–792. [PubMed: 16337601]
12. Bailly V, Lamb J, Sung P, Prakash S, Prakash L. Specific complex formation between yeast RAD6 and RAD18 proteins: a potential mechanism for targeting RAD6 ubiquitin-conjugating activity to DNA damage sites. *Genes Dev* 1994;8:811–820. [PubMed: 7926769]
13. Bailly V, Lauder S, Prakash S, Prakash L. Yeast DNA repair proteins Rad6 and Rad18 form a heterodimer that has ubiquitin conjugating, DNA binding, and ATP hydrolytic activities. *J Biol Chem* 1997;272:23360–23365. [PubMed: 9287349]
14. Waga S, Stillman B. The DNA replication fork in eukaryotic cells. *Annu Rev Biochem* 1998;67:721–751. [PubMed: 9759502]
15. Hoege C, Pfander B, Moldovan GL, Pyrowolakis G, Jentsch S. RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* 2002;419:135–141. [PubMed: 12226657]
16. Stelter P, Ulrich HD. Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. *Nature* 2003;425:188–191. [PubMed: 12968183]
17. Haracska L, Torres-Ramos CA, Johnson RE, Prakash S, Prakash L. Opposing effects of ubiquitin conjugation and SUMO modification of PCNA on replicational bypass of DNA lesions in *Saccharomyces cerevisiae*. *Mol Cell Biol* 2004;24:4267–4274. [PubMed: 15121847]
18. Chen CC, Moteji A, Hasegawa Y, Myung K, Kolodner R, D'Andrea A. Genetic analysis of ionizing radiation-induced mutagenesis in *Saccharomyces cerevisiae* reveals TransLesion Synthesis (TLS) independent of PCNA K164 SUMOylation and ubiquitination. *DNA Repair (Amst)* 2006;5:1475–1488. [PubMed: 16990054]
19. Garg P, Burgers PM. Ubiquitinated proliferating cell nuclear antigen activates translesion DNA polymerases eta and REV1. *Proc Natl Acad Sci U S A* 2005;102:18361–18366. [PubMed: 16344468]
20. Haracska L, Unk I, Prakash L, Prakash S. Ubiquitylation of yeast proliferating cell nuclear antigen and its implications for translesion DNA synthesis. *Proc Natl Acad Sci U S A* 2006;103:6477–6482. [PubMed: 16611731]
21. Kannouche PL, Wing J, Lehmann AR. Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. *Mol Cell* 2004;14:491–500. [PubMed: 15149598]
22. Watanabe K, Tateishi S, Kawasuji M, Tsurimoto T, Inoue H, Yamaizumi M. Rad18 guides pol eta to replication stalling sites through physical interaction and PCNA monoubiquitination. *EMBO J* 2004;23:3886–3896. [PubMed: 15359278]
23. Kondo T, Matsumoto K, Sugimoto K. Role of a complex containing Rad17, Mec3, and Ddc1 in the yeast DNA damage checkpoint pathway. *Mol Cell Biol* 1999;19:1136–1143. [PubMed: 9891048]
24. Gietz RD, Sugino A. New yeast-*Escherichia coli* shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. *Gene* 1988;74:527–534. [PubMed: 3073106]
25. Knop M, Siegers K, Pereira G, Zachariae W, W B, Nasmyth K, Schiebel E. Epitope tagging of yeast genes using a PCR-based strategy: more tags and improved practical routines. *Yeast* 1999;15:963–972. [PubMed: 10407276]
26. Goldstein AL, McCusker JH. Three new dominant drug resistance cassettes for gene disruption in *Saccharomyces cerevisiae*. *Yeast* 1999;15:1541–1553. [PubMed: 10514571]
27. Wach A, Brachat A, Pohlmann R, Philippsen P. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* 1994;13:1793–1808. [PubMed: 7747518]
28. Reid RJ, Lisby M, Rothstein R. Cloning-free genome alterations in *Saccharomyces cerevisiae* using aptamer-mediated PCR. *Methods Enzymol* 2002;350:258–277. [PubMed: 12073317]

29. Nakada D, Shimomura T, Matsumoto K, Sugimoto K. The ATM-related Tel1 protein of *Saccharomyces cerevisiae* controls a checkpoint response following phleomycin treatment. *Nucleic Acids Res* 2003;31:1715–1724. [PubMed: 12626713]
30. Milne GT, Jin S, Shannon KB, Weaver DT. Mutations in two Ku homologs define a DNA end-joining repair pathway in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1996;16:4189–4198. [PubMed: 8754818]
31. Kramer KM, Brock JA, Bloom K, Moore JK, Haber JE. Two different types of double-strand breaks in *Saccharomyces cerevisiae* are repaired by similar RAD52-independent, nonhomologous recombination events. *Mol Cell Biol* 1994;14:1293–1301. [PubMed: 8289808]
32. Lieber MR, Ma Y, Pannicke U, Schwarz K. The mechanism of vertebrate nonhomologous DNA end joining and its role in V(D)J recombination. *DNA Repair (Amst)* 2004;3:817–826. [PubMed: 15279766]
33. Daley JM, Palmbo PL, Wu D, Wilson TE. Nonhomologous End Joining in Yeast. *Annu Rev Genet* 2005;39:431–451. [PubMed: 16285867]
34. Boulton SJ, Jackson SP. *Saccharomyces cerevisiae* Ku70 potentiates illegitimate DNA double-strand break repair and serves as a barrier to error-prone DNA repair pathways. *Embo J* 1996;15:5093–5103. [PubMed: 8890183]
35. Wilson TE, Grawunder U, Lieber MR. Yeast DNA ligase IV mediates non-homologous DNA end joining. *Nature* 1997;388:495–498. [PubMed: 9242411]
36. Ma JL, Kim EM, Haber JE, Lee SE. Yeast Mre11 and Rad1 proteins define a Ku-independent mechanism to repair double-strand breaks lacking overlapping end sequences. *Mol Cell Biol* 2003;23:8820–8828. [PubMed: 14612421]
37. Liu H, Krizek J, Bretscher A. Construction of a GAL1-regulated yeast cDNA expression library and its application to the identification of genes whose overexpression causes lethality in yeast. *Genetics* 1992;132:665–673. [PubMed: 1468625]
38. Zhou BB, Elledge SJ. The DNA damage response: putting checkpoints in perspective. *Nature* 2000;408:433–439. [PubMed: 11100718]
39. Wilson TE, Lieber MR. Efficient processing of DNA ends during yeast nonhomologous end joining. Evidence for a DNA polymerase beta (Pol4)-dependent pathway. *J Biol Chem* 1999;274:23599–23609. [PubMed: 10438542]
40. Bebenek K, Garcia-Diaz M, Patishall SR, Kunkel TA. Biochemical properties of *Saccharomyces cerevisiae* DNA polymerase IV. *J Biol Chem* 2005;280:20051–20058. [PubMed: 15778218]
41. Wood A, Garg P, Burgers PM. A ubiquitin-binding motif in the translesion DNA polymerase Rev1 mediates its essential functional interaction with ubiquitinated proliferating cell nuclear antigen in response to DNA damage. *J Biol Chem* 2007;282:20256–20263. [PubMed: 17517887]
42. Davies AA, Huttner D, Daigaku Y, Chen S, Ulrich HD. Activation of ubiquitin-dependent DNA damage bypass is mediated by replication protein a. *Mol Cell* 2008;29:625–636. [PubMed: 18342608]
43. Szuts D, Simpson LJ, Kabani S, Yamazoe M, Sale JE. Role for RAD18 in homologous recombination in DT40 cells. *Mol Cell Biol* 2006;26:8032–8041. [PubMed: 16923963]
44. Chen S, Davies AA, Sagan D, Ulrich HD. The RING finger ATPase Rad5p of *Saccharomyces cerevisiae* contributes to DNA double-strand break repair in a ubiquitin-independent manner. *Nucleic Acids Res* 2005;33:5878–5886. [PubMed: 16224103]
45. Podust VN, Hubscher U. Lagging strand DNA synthesis by calf thymus DNA polymerases alpha, beta, delta and epsilon in the presence of auxiliary proteins. *Nucleic Acids Res* 1993;21:841–846. [PubMed: 8451186]
46. Johnson RE, Washington MT, Haracska L, Prakash S, Prakash L. Eukaryotic polymerases iota and zeta act sequentially to bypass DNA lesions. *Nature* 2000;406:1015–1019. [PubMed: 10984059]
47. Ira G, Pelliccioli A, Balijja A, Wang X, Fiorani S, Carotenuto W, Liberi G, Bressan D, Wan L, Hollingsworth NM, et al. DNA end resection, homologous recombination and DNA damage checkpoint activation require CDK1. *Nature* 2004;431:1011–1017. [PubMed: 15496928]
48. Aylon Y, Liefshitz B, Kupiec M. The CDK regulates repair of double-strand breaks by homologous recombination during the cell cycle. *EMBO J* 2004;23:4868–4875. [PubMed: 15549137]

49. Wu D, Topper LM, Wilson TE. Recruitment and dissociation of nonhomologous end joining proteins at a DNA double-strand break in *Saccharomyces cerevisiae*. *Genetics* 2008;178:1237–1249. [PubMed: 18245831]
50. Zou L, Elledge SJ. Sensing DNA damage through ATRIP recognition of RPA-ssDNA complexes. *Science* 2003;300:1542–1548. [PubMed: 12791985]
51. Nakada D, Hirano Y, Tanaka Y, Sugimoto K. Role of the C terminus of Mec1 checkpoint kinase in its localization to sites of DNA damage. *Mol Biol Cell* 2005;16:5227–5235. [PubMed: 16148046]
52. Barlow JH, Lisby M, Rothstein R. Differential regulation of the cellular response to DNA double-strand breaks in G1. *Mol Cell* 2008;30:73–85. [PubMed: 18406328]

Fig. 1. Effect of *dnl4Δ*, *rev1Δ* and *rev3Δ* mutation on cellular viability after DSB induction
 (A) Schematic of the HO cleavage site at *ADH4* locus (*ADH4cs*). An HO cleavage site, marked with *HIS2*, was introduced at the *ADH4* locus on chromosome VII. The HO1 primer pair amplifies a region 1 kb away from the HO cleavage site. An arrow represents the telomere. (B) Viability after DSB induction. Wild-type (WT) and mutant cells were transformed with YCpA-GAL-HO. Transformed cells were grown in sucrose, and then plated out on either glucose or galactose medium selectable for the plasmid. Viability was estimated from colony formation ability on galactose medium. The bars represent standard deviations.

Fig. 2. Role of PCNA ubiquitination in cell survival after DSB induction

Effects of *pol30-K164R* (A), *rad18Δ* (B), *rad6Δ* (C) or *siz1Δ* (D) mutations on cellular viability were analyzed as in Fig. 1B. The *pol30-K164R* mutation is abbreviated as *pol30-K/R*.

Fig. 3. Association of Rad18 with the HO-induced DSBs

(A) Association of Rad18 with HO-induced DSBs in wild-type and *rad6Δ* mutant cells. Cells expressing Rad18-myc were transformed with the GAL-HO plasmid. Cells were grown to a log-phase in sucrose. The culture was split into two portions. One half was incubated with galactose to induce *HO* expression, while another half was maintained in sucrose to keep the promoter inactive. Aliquots of cells were collected at 3 hr after *HO* induction and subjected to ChIP analysis. PCR was done with the HO1 primer set for the *HO* cleavage site at the *ADH4* locus and the SMC2 primer set for the control locus. PCR products from the respective input extracts are shown below.

(B) Effect of Rad18 association with DSBs in *mec1Δ* and the *mec1-81 tel1Δ* mutants. Cells were transformed with the GAL-HO plasmid and analyzed as in A. The strains carry an *sml1Δ* mutation to rescue the viability loss associated with *mec1* mutations.

Fig. 4. Relationship of Polζ-Rev1 with Rad30 and Pol4 in the DSB repair
Effects of *rad30Δ* (A) or *pol4Δ* mutations (B) on cellular viability were analyzed as in Fig. 1B.

Table 1**Strains used in this study**

KSC2043	<i>rad52Δ::URA3</i>
KSC2047	<i>rad52Δ::URA3 hdf1Δ::LEU2</i>
KSC2274	<i>rad52Δ::URA3 dnl4Δ::HphMX</i>
KSC2275	<i>rad52Δ::URA3 hdf1Δ::LEU2 dnl4Δ::HphMX</i>
KSC2276	<i>rad52Δ::URA3 hdf1Δ::LEU2 rad1Δ::URA3</i>
KSC2044	<i>rad52Δ::URA3 rev1Δ::KanMX</i>
KSC2045	<i>rad52Δ::URA3 rev3Δ::LEU2</i>
KSC2277	<i>rad52Δ::URA3 dnl4Δ::HphMX rev1Δ::KanMX</i>
KSC2278	<i>rad52Δ::URA3 dnl4Δ::HphMX rev3Δ::LEU2</i>
KSC2279	<i>pol30-K164R::URA3</i>
KSC2280	<i>rad52Δ::URA3 pol30-K164R::URA3</i>
KSC2281	<i>rad52Δ::URA3 hdf1Δ::LEU2 pol30-K164R::URA3</i>
KSC2282	<i>rad52Δ::URA3 pol30-K164R::URA3 rev1Δ::KanMX</i>
KSC1859	<i>rad18Δ::LEU2</i>
KSC2283	<i>rad52Δ::URA3 rad18Δ::LEU2</i>
KSC2284	<i>rad52Δ::URA3 hdf1Δ::LEU2 rad18Δ::LEU2</i>
KSC2285	<i>rad52Δ::URA3 rad18Δ::LEU2 rev1Δ::KanMX</i>
KSC2286	<i>rad52Δ::URA3 pol30-K164R::URA3 rad18Δ::LEU2</i>
KSC2287	<i>siz1Δ::HphMX</i>
KSC2288	<i>rad52Δ::URA3 siz1Δ::HphMX</i>
KSC2289	<i>rad52Δ::URA3 rad18Δ::LEU2 siz1Δ::HphMX</i>
KSC2290	<i>rad52Δ::URA3 rad6Δ::KanMX</i>
KSC2291	<i>rad52Δ::URA3 rad6Δ::KanMX rad18Δ::LEU2</i>
KSC2293	<i>RAD18-myc::TRP1</i>
KSC2294	<i>RAD18-myc::TRP1 rad6Δ::KanMX</i>
KSC2296	<i>RAD18-myc::TRP1 sml1Δ::LEU2</i>
KSC2297	<i>RAD18-myc::TRP1 sml1Δ::LEU2 mec1Δ::LEU2</i>
KSC2298	<i>RAD18-myc::TRP1 sml1Δ::LEU2 mec1-81 tel1Δ::KanMX</i>
KSC2299	<i>rad30Δ::TRP1</i>
KSC2300	<i>rad52Δ::URA3 rad30Δ::TRP1</i>
KSC2301	<i>rad52Δ::URA3 rev1Δ::KanMX rad30Δ::TRP1</i>
KSC2302	<i>pol4Δ::TRP1</i>
KSC2303	<i>rad52Δ::URA3 pol4Δ::TRP1</i>
KSC2304	<i>rad52Δ::URA rev1Δ::KanMX pol4Δ::TRP1</i>

All the strains are isogenic to KSC1516 (MATa-inc *ADH4cs::HIS2 ade1 his2 leu2 trp1 ura3*).

Table 2
Type of survivors carrying the YCpA-GAL-HO plasmid

Genotype	n	Plasmid	
		pGAL-ACT1 ^a	YCpT-GAL-HO ^b
<i>rad52Δ</i>	40	0	39
<i>rad52Δ rev1Δ</i>	40	0	40
<i>rad52Δ hdf1Δ</i>	40	0	24
<i>rad52Δ hdf1Δ rad1Δ</i>	39	1	2 ^c

Survivors from each of *rad52Δ*, *rad52Δ rev1Δ*, *rad52Δ hdf1Δ* or *rad52Δ hdf1Δ rad1Δ* mutants in Fig. 1B were transformed with *TRP1*-marked pGAL-ACT1 or YCpT-GAL-HO. After introduction of pGAL-ACT1, survivors were transferred to galactose medium selectable for the plasmid to monitor cell proliferation. Survivors carrying YCpT-GAL-HO were subjected to the viability assay using galactose medium selectable for the plasmid as in Fig. 1B. Cells that retained more than 90% viability are assessed as viable. *n*, number of survivors tested.

^aNumber of proliferated survivors is shown.

^bNumber of viable survivors is shown.

^cOne of viable survivors was found to proliferate on galactose after transformation with pGAL-ACT1.