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Histone H4 LRS mutations can attenuate UV mutagenesis without affecting PCNA ubiquitination or sumoylation

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ABSTRACT

UV is a significant environmental agent that damages DNA. Translesion synthesis (TLS) is a DNA damage tolerance pathway that utilizes specialized DNA polymerases to replicate through the damaged DNA, often leading to mutagenesis. In eukaryotic cells, genomic DNA is organized into chromatin that is composed of nucleosomes. To date, if and/or how TLS is regulated by a specific nucleosome feature has been undocumented. We found that mutations of multiple histone H4 residues mostly or entirely embedded in the nucleosomal LRS (loss of ribosomal DNA-silencing) domain attenuate UV mutagenesis in *Saccharomyces cerevisiae*. The attenuation is not caused by an alteration of ubiquitination or sumoylation of PCNA (proliferating cell nuclear antigen), the modifications well-known to regulate TLS. Also, the attenuation is not caused by decreased chromatin accessibility, or by alterations of methylation of histone H3 K79, which is at the center of the LRS surface. The attenuation may result from compromised TLS by both DNA polymerases ζ and η, in which Rad6 and Rad5 are but Rad18 is not implicated. We propose that a feature of the LRS is recognized or accessed by the TLS machineries either during/after a nucleosome is disassembled in front of a lesion-stalled replication fork, or during/before a nucleosome is reassembled behind a lesion-stalled replication fork.

1. INTRODUCTION

Cells are equipped with multiple pathways, including cell cycle checkpoints, DNA repair, and damage tolerance, to reduce the deleterious consequences of DNA damage caused by endogenous and exogenous agents [1,2]. Ultraviolet (UV) is a significant DNA-damaging agent that primarily produces cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs). In eukaryotic cells, postreplication repair (PRR) is a Rad6-dependent DNA damage tolerance pathway that is activated when single-stranded DNA accumulates at lesion-stalled DNA replication forks or at gaps created by repriming downstream of the initial stalling lesion [3,4]. Error-prone translesion synthesis (TLS), which may result in mutagenesis, and error-free template switching (TS) are two PRR pathways. In *Saccharomyces cerevisiae*, TLS is primarily accomplished by polymerase ζ (Polζ) and polymerase η (Polη) [5–7]. Polζ is responsible for most DNA damage-induced mutagenesis and a substantial portion (half or more) of spontaneous mutations by extending termini across DNA lesions or at mismatches, hairpins or other structural features of template DNA that are difficult to overcome by normal replicative DNA polymerases [5]. Polη catalyzes largely (>90%) error-free TLS of CPDs [8]. Polη can also insert a G opposite the 3' nucleotide of a 6-4PP, which can then be extended by Polζ to finish

Abbreviations: 6-4PP, 6-4 photoproduct; Can^r, canavanine resistant; CPD, cyclobutane pyrimidine dimer; LRS, loss of ribosomal DNA-silencing; MNase, micrococcal nuclease; NER, nucleotide excision repair; Paf1C, RNA polymerase II-associated factor 1 complex; PCNA, proliferating cell nuclear antigen; Polη, DNA polymerase η; Polζ, DNA polymerase ζ; PRR, postreplication repair; TLS, translesion synthesis; TS, template switching; UV, ultraviolet; WT, wild type.

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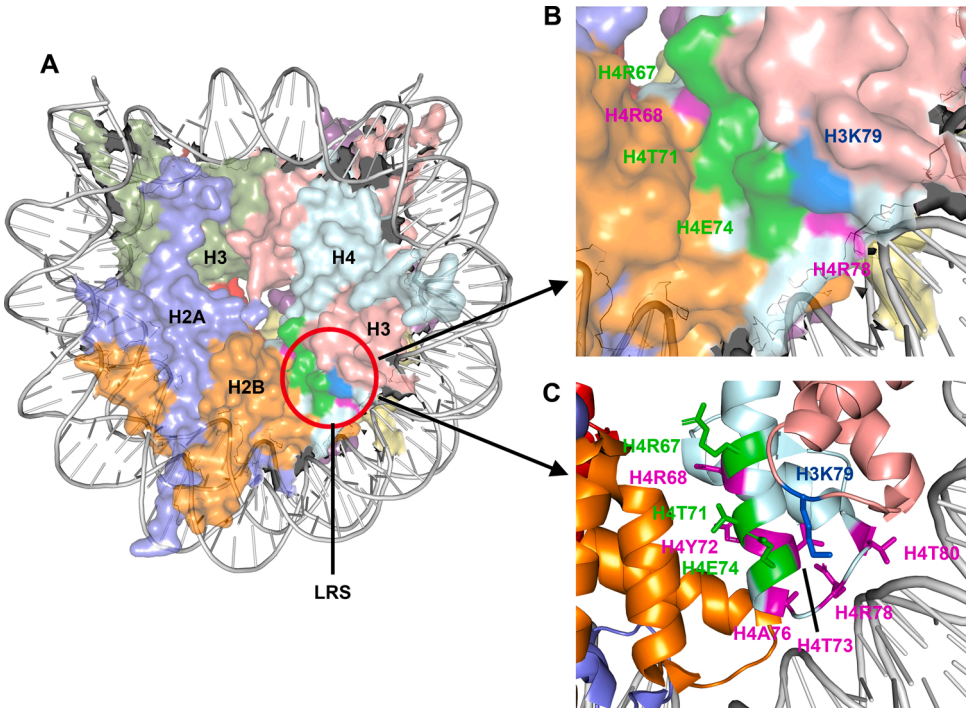


Fig. 1. The LRS domain of a nucleosome. (A) Structure of a yeast nucleosome (PDB,1ID3). The approximate LRS domain is indicated by the red circle. (B and C) The LRS domain and surrounding nucleosomal areas shown as surface and cartoon structures, respectively. Histone H4 residues of interest on the nucleosome surface are shown in green, and those mostly or entirely embedded in the nucleosome are shown in magenta.

bypassing the photoproduct [9]. TS uses a newly synthesized sister chromatid as a template for synthesis and requires the participations of homologous recombination proteins Rad51 and Rad52 [10–12].

In *S. cerevisiae*, all branches of PRR have been known to be regulated by ubiquitination and sumoylation of the proliferating cell nuclear antigen (PCNA), an essential processivity factor for DNA replication and repair [13,14]. In response to DNA damage, PCNA is mono-ubiquitinated at K164 by the E3 ubiquitin ligase Rad18 in complex with the E2 ubiquitin conjugase Rad6 [13]. The mono-ubiquitin can be extended into a regulatory, K63-linked poly-ubiquitin chain by the E3 ubiquitin ligase Rad5 in complex with the E2 ubiquitin conjugase complex Ubc13-Mms2 [13]. Mono-ubiquitination of PCNA promotes both Polζ- and Polη-dependent TLS, while poly-ubiquitination promotes TS [13,14]. PCNA can also be sumoylated at K164 and K127, which are completely and partially dependent on the E3 SUMO ligase Siz1, respectively [13]. The sumoylation of PCNA promotes the recruitment of Rad18, thereby facilitating damage-induced ubiquitination [10,15]. Sumoylation of PCNA can also lead to the recruitment of Srs2, a DNA helicase that can disrupt Rad51 presynaptic filaments and thus prevents TS [16,17].

Eukaryotic genomes are organized into chromatin [18]. The basic building block of chromatin is the nucleosome, which consists of DNA wrapped around a histone octamer comprised of one $(H3\text{-}H4)_2$ tetramer and two H2A-H2B dimers. All cellular events that involve DNA transactions, including DNA replication, repair and transcription, have to respond to and overcome the constraints of chromatin structures. The inhibitory effects of chromatin structures on nucleotide excision repair (NER) [19–21], DNA double strand break repair and damage checkpoint signaling [22] have been well-documented. Limited studies suggested that chromatin structures may also inhibit PRR. For example, chromatin relaxation, either by KAP-1, a transcriptional repressor whose phosphorylation relaxes chromatin, or by treatment with trichostatin A, a histone deacetylase inhibitor, have been shown to promotes PCNA ubiquitination and TLS [23]. Treatment of cells with DRAQ5, a DNA intercalating dye that disrupts chromatin structure, causes a dramatic immobilization of Polη [24]. Also, the chromatin remodeling complexes INO80 and RSC have been shown to promote PCNA ubiquitination [25,26] and Rad51-mediated processing of recombination intermediates [25].

LRS (loss of ribosomal DNA-silencing) is a nucleosome domain composed of certain residues of histones H3 and H4 (Fig. 1). The LRS has been known to be required for heterochromatin formation and transcriptional repression at specific yeast loci [27,28]. Here we report that mutations of histone H4 residues in the LRS domain can attenuate TLS and UV mutagenesis, without significantly affecting PCNA ubiquitination or sumoylation. Our finding challenges the traditional view that chromatin structures just passively inhibit TLS and the TLS machinery battles to overcome the inhibition. Instead, the chromatin feature conferred by the LRS may actively promote TLS.

2. MATERIAL AND METHODS

2.1. Plasmids and yeast strains

Plasmids and yeast strains expressing wild type histones used in this study are shown in Supplementary Tables S1 and S2, respectively. Histone H4 LRS mutants and their isogenic wild strains were created by transforming the pDM9-bearing strains (Supplementary Table S2) with pHTF2-derivatived plasmids, which express wild type histone H3 and histone H4 LRS mutants (Supplementary Table S1) and pHTF2, which expresses wild type histones H3 and H4. Plasmid pDM9 was then removed from the transformed cells by selection with 5-fluoroorotic acid.

2.2. UV sensitivity assay

Yeast cells were grown in synthetic dextrose (SD) medium at 30°C to saturation, sequentially 10-fold diluted and spotted onto YPD (1% yeast extract, 2% peptone and 2% dextrose) plates. After different doses of UV irradiation, the plates were incubated in the dark at 30°C for 3-6 days before being photographed.

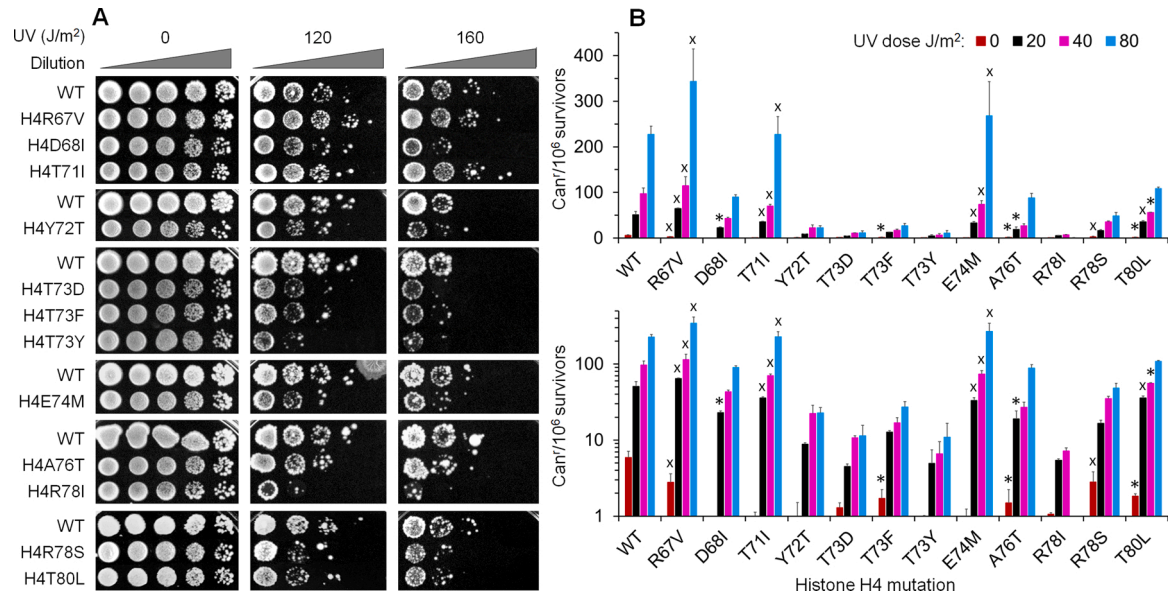


Fig. 2. All the UV sensitive histone H4 LRS mutations, except for the E74M mutation, significantly attenuate UV mutagenesis. (A) UV sensitivities of WT and histone H4 LRS mutant cells. (B) UV induced Can^r mutation frequencies. Top and bottom panels are linear and logarithmic scale plots, respectively. Error bars indicate standard errors. The mutation frequencies of all the LRS mutant cells are very significantly different ($p < 0.01$) from those of the WT cells at the corresponding UV doses, except for those marked with × ($p > 0.05$) or * ($0.01 < p < 0.05$).

2.3. UV-induced mutagenesis assay

Cells were cultured in YPD medium to late log phase ($OD_{600} = 1.0$), pelleted, washed with and resuspended in H_2O. After irradiation with different doses of 254 nm UV, the cells were serially 10-fold diluted. To determine the numbers of viable cells, the diluted cells were plated onto SD plates without arginine and canavanine. To determine the numbers of canavanine resistant (Can^r) mutants, the diluted cells were spread on SD plates without arginine but with 60 mg/L of canavanine. Plates were counted after 3-6 days of incubation in the dark at 30°C. At least three independent mutagenesis experiments were carried out for each of the yeast strains of interest. The mutation frequencies in the histone H4 mutants were compared with those in the wild type (WT) cells at the corresponding UV doses by using the two-tailed Student's t-test.

2.4. Western blot

Cells were cultured to late log phase. If UV irradiation was required, half of the cultures were irradiated with 120 J/m^2 of 254 nm UV. The UV irradiated and unirradiated cells were incubated in YPD medium at 30°C. Aliquots were taken at different times of the incubation. Whole protein extracts were prepared from the aliquots and proteins of interest were detected by Western blots using procedures described previously [29]. Anti-FLAG antibody (M2) was from Sigma. Antibodies against mono-, di- and tri-methylated H3K79 and total histone H3 were from Abcam.

2.5. Chromatin accessibility assay

Micrococcal nuclease (MNase) cleavage of chromatin DNA was done essentially as described previously [30]. Briefly, yeast cells were grown in SD medium at 30°C to late log phase. Half of the culture was irradiated with 120 J/m^2 of 254 nm UV followed by incubation at 30°C for 1 hour. Cells from 45 ml of the irradiated and unirradiated samples were treated with 50 units of Zymolyase (Zymo Research) in 5 ml zymolyase buffer (50 mM Tris, pH 7.8, 1 M sorbitol, 5 mM β-ME, 0.5 mM PMSF) at 30°C for 40 min. The resulting spheroplasts from each sample were then suspended in 2 ml of MNase buffer (50 mM Tris, pH 7.8, 1 M sorbitol, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM β-ME, 0.5 mM PMSF and 0.075% v/v NP-40), divided into 300 µl aliquots and digested with varying (0, 49, 148, 444, 1333 and 4000) units of MNase for 10 min at 37°C. The reactions were terminated by mixing with 60 µl of stop solution (6% SDS, 250 mM EDTA) and immediately incubated at 65°C for 3 hours. The genomic DNA was isolated from the aliquots and fractionated on 1.2% agarose gels.

3. RESULTS

3.1. Histone H4 LRS mutations can attenuate UV mutagenesis

Through random mutagenesis, we identified multiple UV sensitive or resistant histone H4 mutations in the nucleosomal LRS domain (Fig. 1) [31]. We found that the histone H4 H75E mutation significantly attenuates global genomic NER and Rad26-independent transcription-coupled NER. However, all the other mutations do not significantly affect NER or a NER subpathway [31], indicating that most of the LRS mutations may be implicated in other DNA repair and/or damage tolerance pathways.

To determine if the LRS mutations are implicated in PRR, we first measured UV mutagenesis by analyzing Can^r mutation frequencies in WT and the LRS mutant cells. The R67V and T71I mutations, which mildly increase UV resistance (2-5 fold) (Fig. 2A) [31], did not significantly affect the UV mutagenesis (Fig. 2B). In contrast, all the UV sensitive LRS mutations tested, except for the E74M mutation, significantly attenuated the UV mutagenesis (Fig. 2B). The T73D, T73F, T73Y and R78I mutations, which increase cell UV sensitivity 10-50 fold, attenuated the UV mutagenesis more dramatically than the D68I, A76T, R78S and T80L mutations, which are mildly (~ 5 fold) UV sensitive (Fig. 2A and B). The Y72T mutation, which is mildly UV sensitive (~ 5 fold), also dramatically attenuated the UV mutagenesis (Fig. 2A and B). Of note, all the LRS mutations that significantly attenuated the UV mutagenesis are mostly (D68I, R78I and R78S) or entirely (all the other mutations) embedded in the nucleosome (Figs. 1 and 2). In contrast, the R67V, T71I and E74M mutations, which did not significantly affect the UV mutagenesis, are on the nucleosome surface (Figs. 1 and 2).

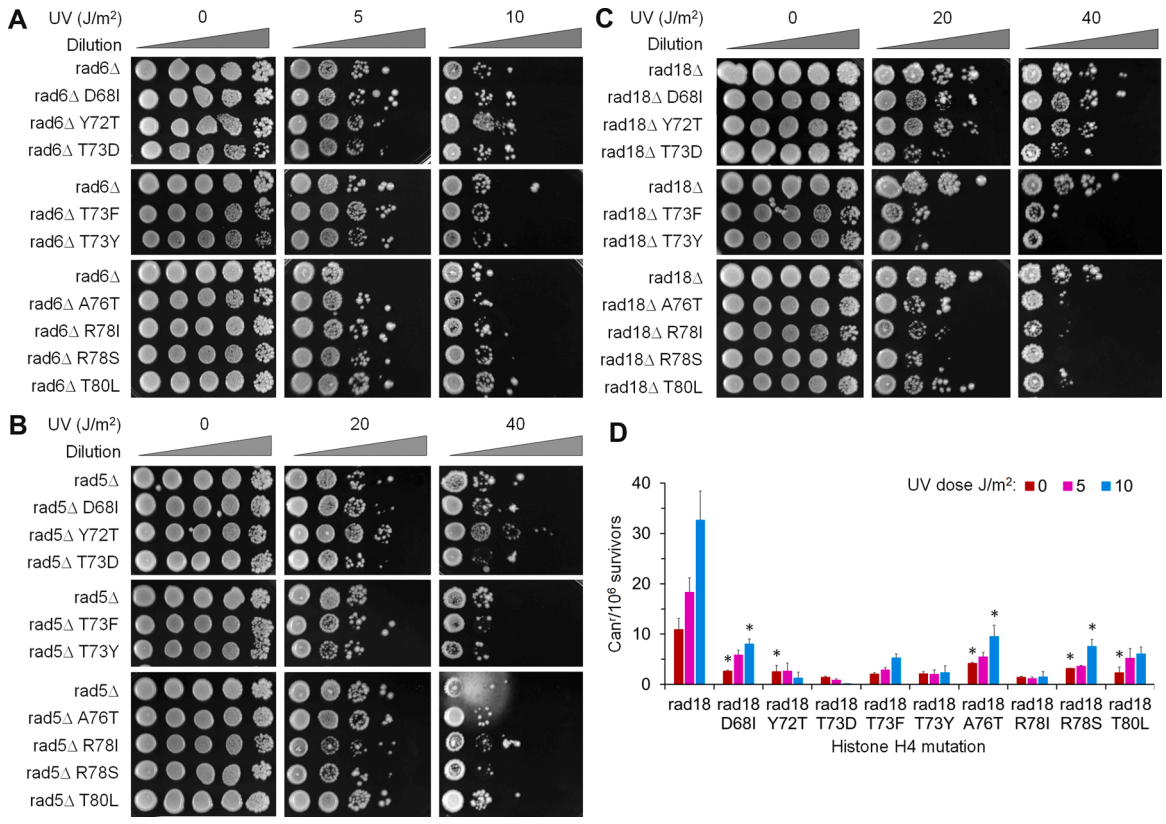


Fig. 3. All the UV sensitive histone H4 LRS mutations that attenuate UV mutagenesis are largely epistatic to *rad6Δ* and *rad5Δ*, but not *rad18Δ*. (A-C) UV sensitivities of *rad6Δ* (A), *rad5Δ* (B) and *rad18Δ* (C) cells with or without the indicated histone H4 LRS mutations. (D) UV induced Can^r mutation frequencies. Error bars indicate standard errors. The mutation frequencies of all the *rad18Δ* cells with the LRS mutations are very significantly different ($p < 0.01$) from those without the LRS mutation, except for those marked with * ($0.01 < p < 0.05$).

3.2. All the histone H4 LRS mutations that attenuate UV mutagenesis are largely epistatic to rad6Δ and rad5Δ, but not rad18Δ

We next determined epistatic interactions of the LRS mutations with those implicated in PRR. Rad6 is an E2 ubiquitin conjugase that is critical for both TLS and TS [32]. Indeed, *rad6* mutants are highly deficient in PRR of UV-damaged DNA [12] and exhibit no UV mutagenesis [33,34]. Rad5 is a SWI/SNF family ATPase and an E3 ubiquitin ligase that is required for TS [35,36]. Rad5 also plays a structural role in TLS, where neither its ubiquitin ligase activity nor its ATPase activity is required [37–39]. None of the LRS mutations that attenuate the UV mutagenesis (Fig. 2) dramatically enhanced UV sensitivities of *rad6Δ* cells (Fig. 3A). The enhancements of the UV sensitivity of *rad5Δ* cells by all the LRS mutations were very mild if any (< 3 fold) (Fig. 3B). The Y72T mutation enhanced the UV resistance of both *rad6Δ* and *rad5Δ* cells to certain extents (Fig. 3A and B), presumably due to derepression of a non-PRR mechanism that remains to be characterized.

Rad18 is an E3 ubiquitin ligase that forms a complex with Rad6, and plays an important role in both TLS and TS of various DNA lesions [40]. However, *rad18* mutations do not significantly affect UV mutagenesis [33,41] and increase spontaneous mutagenesis [42–44]. All the LRS mutations that attenuated UV mutagenesis in otherwise WT cells (Fig. 2), except for the D68I and Y72T mutations, synergistically increased the UV sensitivity of *rad18Δ* cells (Fig. 3C). All the LRS mutations, including the D68I and Y72T mutations, attenuated UV mutagenesis in *rad18Δ* cells (Fig. 3D). Therefore, the D68I and Y72T mutations are not truly epistatic to *rad18Δ*. Besides attenuating UV mutagenesis, the D68I and Y72T mutations may derepress a DNA repair or damage tolerance mechanism that remains to be elucidated.

Taken together, our results so far indicate that the histone H4 LRS mutations that attenuate UV mutagenesis are largely implicated in a Rad6- and Rad5-dependent but not Rad18-dependent PRR mechanism.

3.3. The attenuation of UV mutagenesis by the histone H4 LRS mutations may be caused by compromised Polζ- and Polη-dependent TLS, but not by enhanced error-free TS

In *S. cerevisiae*, all UV mutagenesis is dependent on Polζ, which is most efficient in extending primer termini opposite a variety of lesions or mismatches [5,6,9,45]. Polζ is composed of the catalytic subunit Rev3 and the accessory subunit Rev7. Rev1 itself is a deoxycytidyl transferase that incorporates dCTPs opposite abasic or damaged G sites [46–49]. Rev1 also interacts with Rev3, Rev7 and Polη, and has an essential structural role in Polζ-dependent error-prone TLS [50–52]. Rev3 was barely detectable on Western blots (Fig. 4A), in line with its very low abundance [53]. The LRS mutations, including the Y72T and T73D mutations that most severely attenuate UV mutagenesis (Figs. 2 and 3), did not significantly affect the expression levels of Rev3, Rev1 or Rev7 (Fig. 4A-C; data not shown). All the LRS mutations that attenuate UV mutagenesis (Figs. 2 and 3) synergistically increased the UV sensitivity of *rev3Δ* cells (Fig. 4F), indicating that the LRS mutations also attenuate a Polζ-independent mechanism.

Polη encoded by *RAD30* catalyzes error-free TLS opposite CPDs [54], and *rad30Δ* cells have elevated UV mutagenesis [55–57]. Polη also cooperates with Polζ for TLS of 6-4PPs, which is mutagenic if the 3' nucleotide of a 6-4PP is not C [9]. Polη primarily inserts a G opposite the 3' nucleotide of a 6-4PP and Polζ can extend the G by incorporating a correct nucleotide opposite the 5' nucleotide of the 6-4PP. In agreement with previous reports [7,58], the Polη protein level did not significantly change following UV irradiation (Fig. 4D; data not shown). However, all the LRS mutant cells had a lower level of Polη (Fig. 4D and E). *rad30Δ* cells were more UV sensitive than *rev3Δ* cells (Fig. 4F and G), indicating

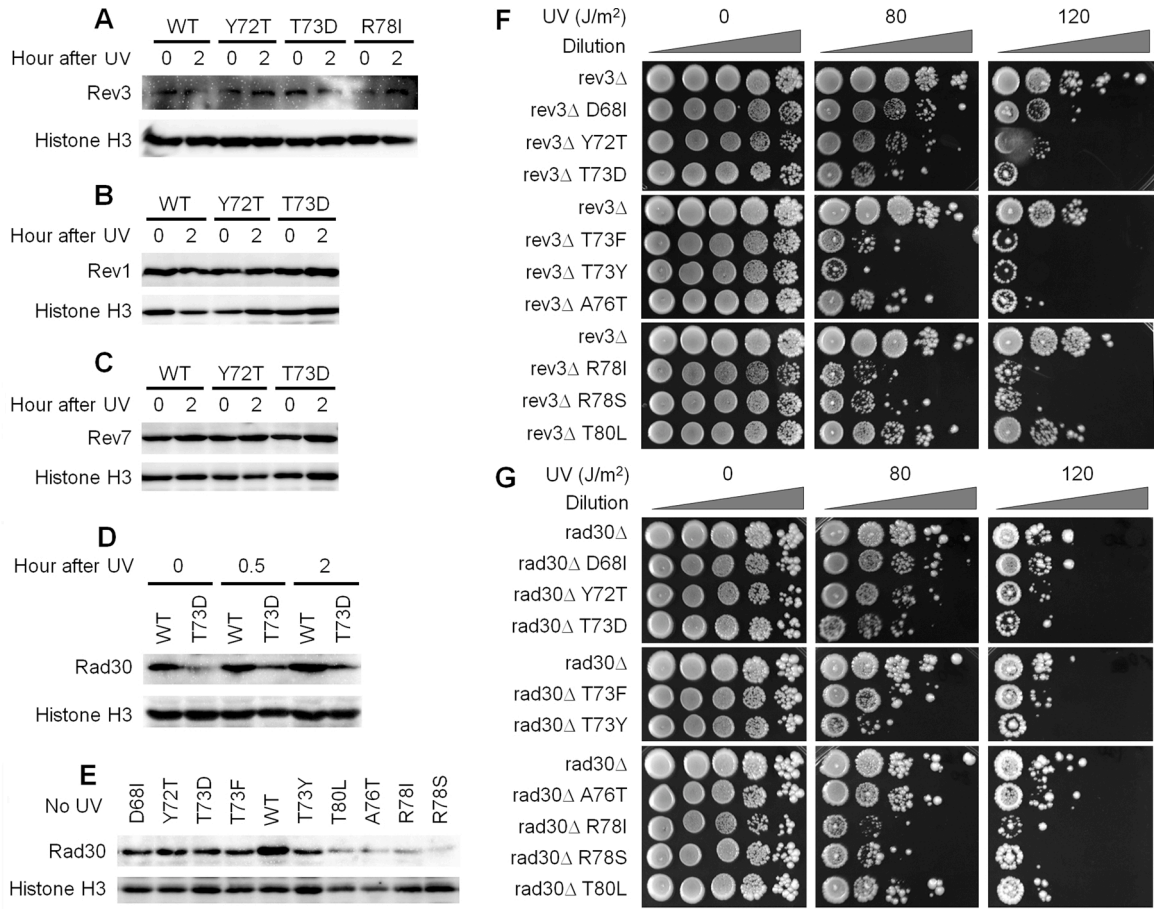


Fig. 4. The histone H4 LRS mutations that attenuate UV mutagenesis may affect the Polη-dependent TLS as well. (A-E) Levels of 6×FLAG-tagged Rev3 (A), and 3×FLAG-tagged Rev1 (B), Rev7 (C) and Rad30 (D and E) in the indicated cells. Histone H3 serves as the loading control. (F and G) UV sensitivities of *rev3Δ* (F) and *rad30Δ* (G) cells with or without the indicated histone H4 LRS mutations.

that the Polη-dependent TLS contributes more to cell UV resistance than does the Polζ-dependent TLS. Most of the LRS mutations mildly (less than additively) or had not at all (the D68I and A76T) increased the UV sensitivity of *rad30Δ* cells (Fig. 4G). This indicates that most of the LRS mutations are strongly implicated in the Polη-dependent TLS, which may be at least in part due to decreased cellular levels of the Polη protein. This may also explain why the LRS mutations synergistically increase the UV sensitivity of *rev3Δ* cells.

Activation of DNA damage checkpoint has been shown to promote Polζ-dependent error-prone TLS [59,60], as well as the Rad51-dependent error-free TS [61]. Rad24 is a DNA damage sensor that is required for the activation of the DNA damage checkpoint. All the LRS mutations additively or synergistically increased the UV sensitivity of *rad24Δ* cells (Supplementary Fig. 1A), indicating that the LRS mutations may not significantly affect the DNA damage checkpoint activation.

Decreased UV mutagenesis in the LRS mutant cells might also be caused by enhanced error-free TS. Therefore, we tested epistatic interactions of the LRS mutations with *rad51Δ* and *rad52Δ*. Rad51 and Rad52 are required for homologous recombinational repair of DNA double-strand breaks and the formation of recombination intermediates during TS [10,62,63]. All the LRS mutations that attenuate UV mutagenesis additively increased the UV sensitivities of *rad51Δ* and *rad52Δ* cells (Supplementary Fig. 1B and C). This indicates that the attenuation

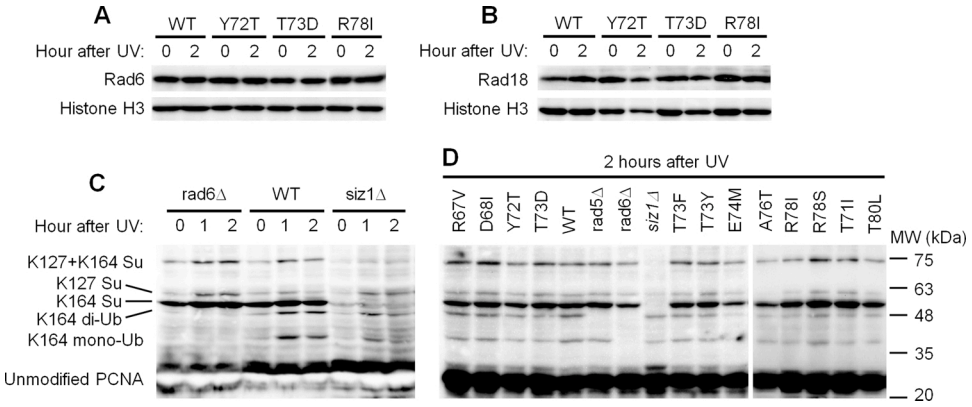
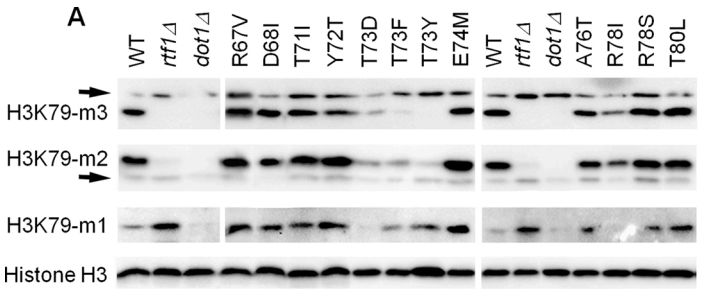


Fig. 5. The histone H4 LRS mutations do not significantly affect cellular levels of Rad6 or Rad18, or the ubiquitination or sumoylation of PCNA. (A and B) Levels of 3×FLAG-tagged Rad6 and Rad18, respectively, in the indicated cells. Histone H3 serves as the loading control. (C) Levels of unmodified and ubiquitinated (Ub) and sumoylated (Su) 3×FLAG-tagged PCNA in the indicated cells at different times after UV irradiation. (D) Levels of the different forms of 3×FLAG-tagged PCNA in the indicated cells 2 hours after UV irradiation. Note that the sumoylated PCNA bands migrated much slower than would be expected for their molecular weights. Also note that the images are assembled from two Western blots.



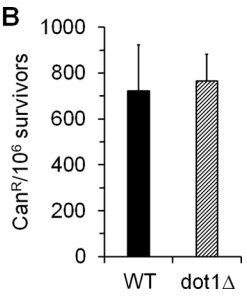


Fig. 6. The attenuation of UV mutagenesis by the histone H4 LRS mutations is unrelated to the alteration of histone H3 K79 methylation. (A) Levels of mono-, di- and tri-methylations (m1, m2 and m3) of histone H3 K79 in the indicated cells. Arrows on the left indicate nonspecific bands. Histone H3 serves as the loading control. Note that the images are assembled from multiple Western blots. (B) Can^r mutation frequencies in the WT (Y452) and isogenic *dot1Δ* cells following 80 J/m^2 of UV irradiation.

of UV mutagenesis by the LRS mutations are not caused by enhanced Rad51- and Rad52-dependent error-free TS. Our observation that most of the LRS mutations synergistically interact with *rad18Δ* (Fig. 3C), which abolishes PCNA mono- and poly-ubiquitinations that is required for TS, supports the idea that the LRS mutations do not significantly affect the TS branch of PRR.

3.4. The attenuations of UV mutagenesis by the histone H4 LRS mutations are not due to significant alteration of PCNA ubiquitination or sumoylation

Both TLS and TS have been well known to be tightly regulated by ubiquitination and sumoylation of PCNA [13,14]. Mono- and poly-ubiquitinations of PCNA are catalyzed by Rad6-Rad18 and Ubc13-Mms2-Rad5, respectively. Sumoylation of PCNA is primarily catalyzed by Siz1 [13,14]. The expression levels of Rad6 and Rad18 were not significantly affected even in the Y72T, T73D and R78I mutant cells (Fig. 5A and B; data not shown), which are severely defective in UV mutagenesis (Figs. 2 and 3). As expected, following UV irradiation, mono- and di-ubiquitination of PCNA were induced in WT but not *rad6Δ* cells, and the di-ubiquitination did not occur in *rad5Δ* cells (Fig. 5C and D). Also as expected, PCNA sumoylation at K164 was abolished, while those at K127 and at both K164 and K127 diminished in *siz1Δ* cells (Fig. 5C and D). The LRS mutations caused little, if any, changes of PCNA ubiquitination or sumoylation (Fig. 5D). This indicates that the attenuated UV mutagenesis in the LRS mutant cells is not due to alteration of the PCNA modifications.

3.5. The attenuations of UV mutagenesis by the histone H4 LRS mutations are unrelated to alteration of histone H3 K79 methylation

At the center of the nucleosomal LRS domain is histone H3 K79 (Fig. 1), which can be methylated by the methyltransferase Dot1 [64].

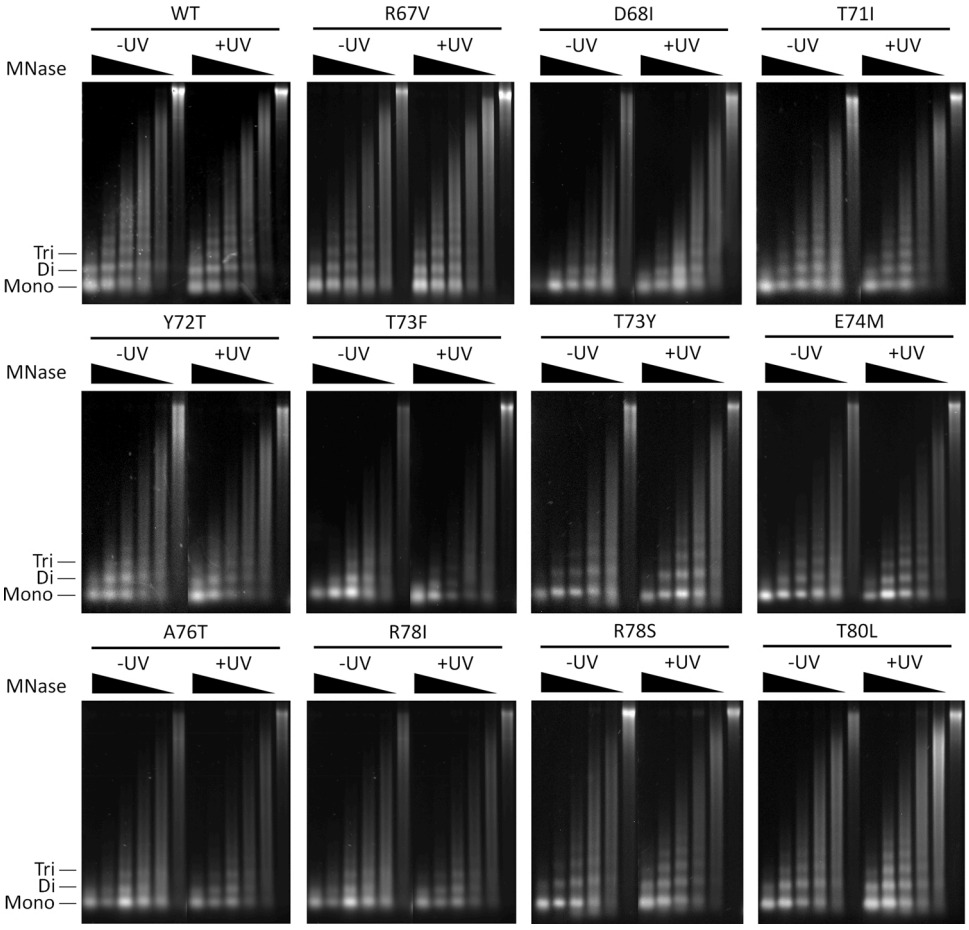


Fig. 7. All UV sensitive histone H4 LRS mutations increase chromatin accessibility. Permeabilized spheroplasts of the indicated cells were treated with 0, 49, 148, 444, 1333 and 4000 units of MNase in 300 μl reactions for 10 min at 37 °C. Mono-, di and tri-nucleosomal DNA fragments are indicated on the left.

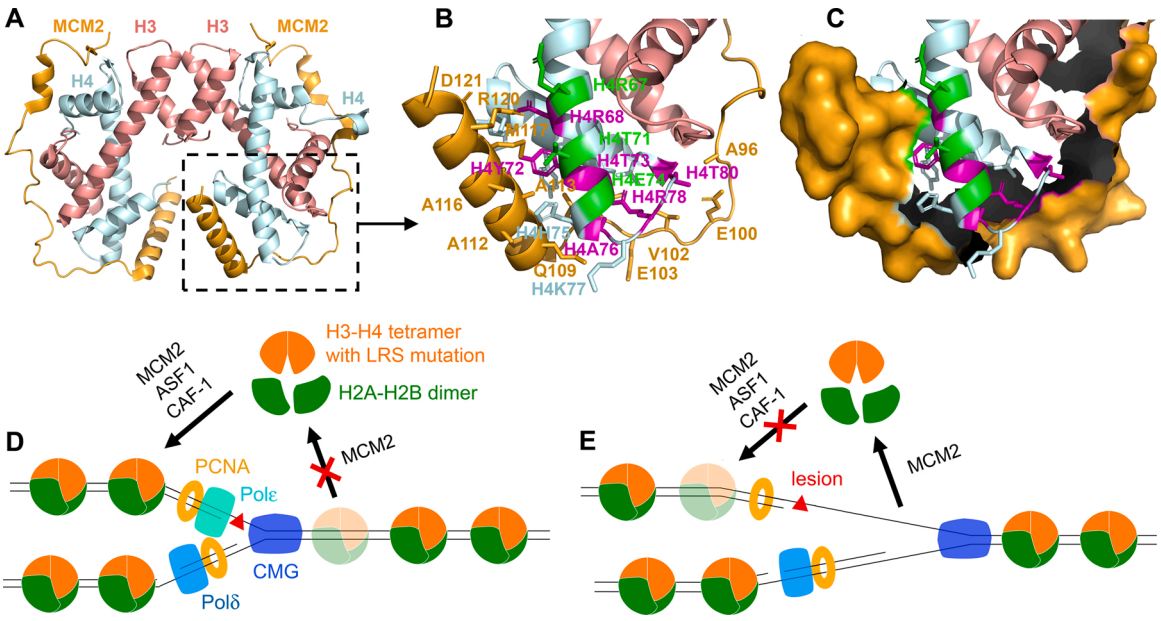


Fig. 8. Histone H4 LRS mutations might attenuate TLS by compromising nucleosome disassembly and/or reassembly at a lesion-stalled replication fork. (A) Structure of a histone H3-H4 tetramer complexed with two MCM2 molecules (5BNV) [67]. (B and C) Cartoon and surface structures illustrating interactions between the histone H4 LRS domain and MCM2. Side chains of histone H4 and MCM2 residues on the interaction surfaces are shown. Histone H4 residues that attenuate UV mutagenesis are shown in magenta, and those that do not significantly affect UV mutagenesis are shown in green. (D) Histone H4 LRS mutations may compromise nucleosome disassembly by MCM2 in front of a lesion-stalled replication fork of normal replicative polymerases ε and δ, thereby attenuating the loading/functionalities of TLS polymerases η and ζ (undepicted). (E) Histone H4 LRS mutations may compromise nucleosome reassembly by MCM2, ASF1 and CAF-1 behind a lesion-stalled replication fork, thereby attenuating the loading/functionalities of TLS polymerases η and ζ.

As expected, no mono-, di- or tri-methylation of histone H3 K79 can be detected in *dot1Δ* cells (Fig. 6A). In agreement with previous reports (e.g. [65]), cells lacking Rtf1, one of the 5 subunits of the RNA polymerase II-associated factor 1 complex (Paf1C), showed virtually no di- or tri-methylation but increased mono-methylation of histone H3 K79 (Fig. 6A). By modulating mono-ubiquitination of histone H2B K123, Paf1C is known to promote tri- and di-methylations but is dispensable for mono-methylation of histone H3 K79 [66]. The T73D, T73F, T73Y and R78I mutations severely decreased tri-, di- and/or mono-methylations of histone H3 K79 (Fig. 6A). This may be due to the fact that the histone H4 T73 and R78 residues are located underneath the histone H3 K79 residue in the nucleosome (Fig. 1). However, the R78S and all the other LRS mutations including the Y72T mutation that drastically attenuated UV mutagenesis do not dramatically affect histone H3 K79 methylations (Fig. 6A). To determine if the histone H3 K79 methylation affects UV mutagenesis, we measured UV-induced Can^r mutation frequencies in *dot1Δ* cells. As can be seen in Fig. 6B, the UV mutagenesis in *dot1Δ* cells was similar to that in the WT cells. These results indicate that the attenuation of UV mutagenesis by the histone H4 LRS mutations is unrelated to the alteration of histone H3 K79 methylation.

3.6. All the UV sensitive histone H4 LRS mutations increase chromatin accessibility

Chromatin structures pose constraints on all aspects of DNA transactions [18]. Chromatin structures have been shown to inhibit PRR [23,24]. We wondered if the LRS mutations decrease chromatin accessibility thereby attenuating TLS and UV mutagenesis. Partial digestion of chromatin with MNase, which preferentially cleaves the linker DNA between nucleosomes, generates DNA ladders reflecting different numbers of associated nucleosomes. Having a decreased accessibility of chromatin will generate longer and/or sharper nucleosomal DNA ladders. The histone H4 R67V mutation, which is located on the nucleosome surface, mildly increased UV resistance and had no deficiency in UV mutagenesis (Fig. 2), did not significantly affect chromatin accessibility (Fig. 7, compare the nucleosomal DNA ladders between the R67V and WT cells). However, MNase treatments generated shorter and/or blurrier nucleosomal DNA ladders in all the other LRS mutant cells (including the E74M) than in the WT cells (Fig. 7). This may reflect the fact that the LRS is required for heterochromatin formation and transcriptional repression at specific yeast loci (which entail chromatin compaction) (27,28). These results indicate that the attenuations of UV mutagenesis by the LRS mutations are not due to decreased chromatin accessibility. Instead, certain intact structures posed by the LRS domain may actually play a positive role in TLS and mutagenesis.

4. DISCUSSION

We showed that histone H4 LRS mutations can attenuate UV mutagenesis without affecting ubiquitination or sumoylation of PCNA. Instead of being located on the nucleosome surface, all of the UV mutagenesis-deficient LRS mutations are mostly or entirely embedded in the nucleosome (Figs. 1 and 2). Therefore, the TLS machinery may not recognize or access a feature of the LRS when the nucleosome is intact. Instead, the LRS feature may be recognized or accessed during/after a nucleosome is disassembled or during/before a nucleosome is assembled. During DNA replication, nucleosomes must be disassembled ahead of the replication fork and immediately reassembled behind the replication fork [67–69]. MCM2, a component of the CMG (Cdc45-MCM-GINS) complex, and ASF1 and CAF-1 are histone H3-H4 chaperones involved in the disassembly and reassembly of nucleosomes. MCM2 captures parental H3-H4 tetramers and passes them to ASF1 and CAF-1 to reassemble nucleosomes behind the replication fork [67–69]. Histone chaperones are crucial for mitigating DNA replication stresses, including those caused by DNA damage [70]. Indeed, CAF-1 is rapidly recruited to chromatin following UV irradiation [71]. Structural studies showed that a region (residues 96-121) of MCM2 extensively interacts with the histone H4 LRS residues that would otherwise be mostly embedded in the intact nucleosome (Figure 8A-C) [67].

Therefore, it is likely that the histone H4 LRS mutations will affect interactions with MCM2, compromising the disassembly (Figure 8D) and/or reassembly of nucleosomes at lesion-stalled replication forks (Figure 8E). The compromised disassembly and/or reassembly of nucleosomes may attenuate the recruitment and/or functionality of Polη, Polζ and other accessory factors for TLS. Also, the decreased level of Polη in the LRS mutant cells (Fig. 4D and E) may at least in part exacerbate the TLS deficiency. Future studies are needed to test this model.

We found that the LRS mutations that attenuate UV mutagenesis are largely epistatic to *rad6Δ* and *rad5Δ*, but not *rad18Δ* (Fig. 3). This indicates that these mutations affect the TLS mechanism(s) that is/are largely dependent on Rad6 and Rad5, but not Rad18. It has been known a long time ago that *rad6* mutations can abolish UV mutagenesis, whereas *rad18* mutations do not significantly affect the mutagenesis [33,41]. How Rad6 functions independently of Rad18 in TLS of UV lesions has been unknown. Rad6 also interacts with the ubiquitin ligase Bre1 to mono-ubiquitinate histone H2B K123 [72], and with the ubiquitin ligase Ubr1 to poly-ubiquitinate proteins containing unacetylated N-terminal residues causing their subsequent degradation [73]. Mono-ubiquitination of histone H2B K123 has recently been shown to contribute to recombination-mediated DNA damage tolerance but not TLS [74]. The interaction of Rad6 with Ubr1 does not contribute to PRR [75,76]. Rad6 may have an as-yet-unidentified substrate implicated in TLS of UV lesions. This substrate may directly or indirectly affect nucleosome disassembly and/or reassembly at a lesion-stalled replication fork (Figure 8), in which the LRS mutations are also implicated.

Rad5 is an E3 ubiquitin ligase and a DNA-dependent ATPase [37,38]. In addition to promoting overall error-free TS through poly-ubiquitinating PCNA, Rad5 directly interacts with Rev1 and promotes Polζ-dependent TLS [39,77,78]. Although it is dispensable for Polη-dependent error-free TLS of CPDs, Rad5 is required for TLS of 6-4PPs [38], which can be error-prone and requires the sequential actions of Polη and Polζ [9]. The role of Rad5 in TLS appears to be structural as neither its ubiquitin ligase activity nor its ATPase activity is required [37–39]. Some specific DNA structures, rather than mono-ubiquitination of PCNA, have been suggested to be required for recruiting Rad5 to damaged sites [77]. Compromised nucleosome disassembly and/or reassembly caused by the LRS mutations (Figure 8) may affect the generation of the specific DNA structures required for recruiting Rad5 and associated TLS polymerases. Again, future studies are needed to test this hypothesis.

In short, we discovered that the nucleosomal LRS plays an important role in TLS and UV mutagenesis. The discovery may set a foundation for future studies regarding how TLS takes place in the context of chromatin.

CRediT authorship contribution statement

Kathiresan Selvam: Conceptualization, Data curation. **Sheikh Arafatur Rahman:** Conceptualization, Data curation. **Derek Forrester:** . **Adam Bao:** . **Michael Lieu:** . **Shisheng Li:** Conceptualization, Investigation.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.dnarep.2020.102959.

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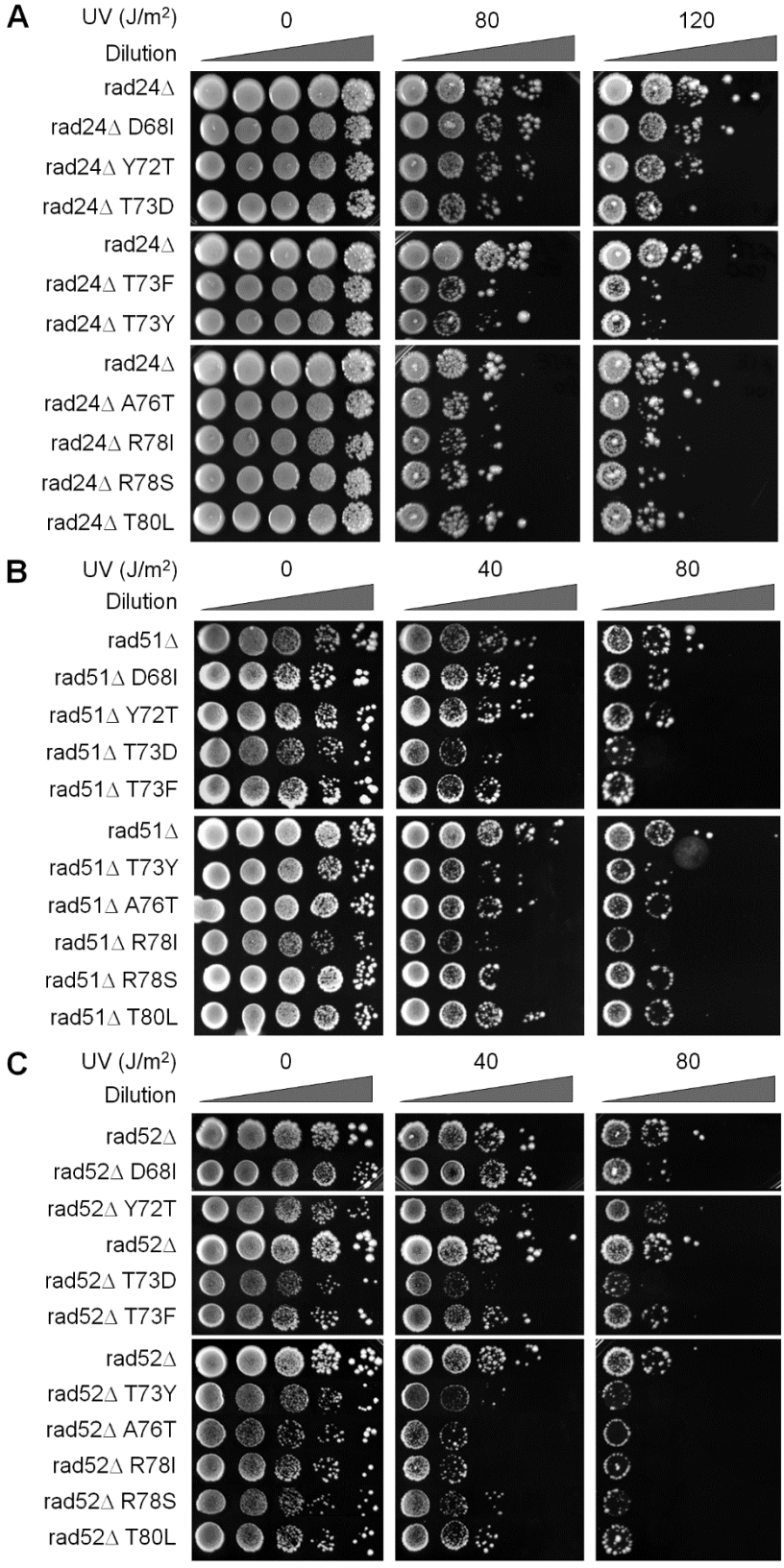
Supplementary materials for Selvam et al entitled "Histone H4 LRS mutations can attenuate UV mutagenesis without affecting PCNA ubiquitination or sumoylation".

Supplementary Table S1. Plasmids used.

Plasmid	**Description**	**Source**
pRS414	Single-copy empty vector with *TRP1* as election marker	(1)
pRS416	Single-copy empty vector with *URA3* as election marker	(1)
p3FLAG-KanMX	For 3' end tagging of a genomic gene with 3×FLAG-KanMX, and for deletion of a gene using KanMX as selection marker	(2)
p6FLAG-KanMX	As p3FLAG-KanMX, but with 6×FLAG replaced the 3×FLAG	This study
pDM9	pRS416 with 1.8 kb *HHT1-HHF1* gene pair (encoding wild type histones H3 and H4) inserted at the HindIII-XmaI site	(3)
pHTF2	pRS414 with 2.1 kb *HHT2-HHF2* gene pair (encoding wild type histones H3 and H4) inserted at the XhoI-SacII site	(4)
pHTF2_R67V	As pHTF2, but with *HHF2* codon R67V mutation	(4)
pHTF2_D68I	As pHTF2, but with *HHF2* codon D68I mutation	(4)
pHTF2_T71I	As pHTF2, but with *HHF2* codon T71I mutation	(4)
pHTF2_Y72T	As pHTF2, but with *HHF2* codon Y72T mutation	(4)
pHTF2_T73D	As pHTF2, but with *HHF2* codon T73D mutation	(4)
pHTF2_T73F	As pHTF2, but with *HHF2* codon T73F mutation	(4)
pHTF2_T73Y	As pHTF2, but with *HHF2* codon T73Y mutation	(4)
pHTF2_E74M	As pHTF2, but with *HHF2* codon E74M mutation	(4)
pHTF2_A76T	As pHTF2, but with *HHF2* codon A76T mutation	(4)
pHTF2_R78I	As pHTF2, but with *HHF2* codon R78I mutation	(4)
pHTF2_R78S	As pHTF2, but with *HHF2* codon R78S mutation	(4)
pHTF2_T80L	As pHTF2, but with *HHF2* codon T80L mutation	(4)

Supplementary Table S2. Yeast strains expressing wild type histones used.			
Strain	**Genotype**	**Plasmid**	**Source**
Y452	*MATα ura3-52 his3-1 leu2-3 leu2-112*		(5)
YBL574	*MATa, leu2Δ1, his3Δ200, ura3-52, trp1Δ63, lys2-128Δ, (hht1-hhf1)Δ::LEU2, (hht2-hhf2)Δ::HIS3 Ty912Δ35-lacZ::his4* (aka FY2162)	pDM9	(3)
DL28	As Y452, but with *rtf1::KanMX*		(6)
DL68	As Y452, but with *dot1::KanMX*		(6)
AR197	As YBL574, but with *rad51::KanMX*	pDM9	This study
AR198	As YBL574, but with *rad52::KanMX*	pDM9	This study
KS109	As YBL574, but with *rad18::KanMX*	pDM9	This study
KS110	As YBL574, but with *rad30::KanMX*	pDM9	This study
KS111	As YBL574, but with *rev3::KanMX*	pDM9	This study
KS113	As YBL574, but with *rad24::KanMX*	pDM9	This study
KS107	As YBL574, but with *rad5::KanMX*	pDM9	This study
KS108	As YBL574, but with *rad6::KanMX*	pDM9	This study
KS281	As YBL574, but with *siz1::KanMX*	pDM9	This study
KS292	As KS107, but with *KanMX* deleted	pDM9	This study
KS293	As KS108, but with *KanMX* deleted	pDM9	This study
KS294	As KS281, but with *KanMX* deleted	pDM9	This study
KS337	As YBL574, but with *POL30* tagged with 3×FLAG	pDM9	This study
KS338	As KS292, but with *POL30* tagged with 3×FLAG	pDM9	This study
KS339	As KS293, but with *POL30* tagged with 3×FLAG	pDM9	This study
KS340	As KS294, but with *POL30* tagged with 3×FLAG	pDM9	This study
KS644	As YBL574, but with *RAD30* tagged with 3×FLAG	pDM9	This study
KS969	As YBL574, but with *REV7* tagged with 3×FLAG	pDM9	This study
KS970	As YBL574, but with *REV1* tagged with 3×FLAG	pDM9	This study
KS971	As YBL574, but with *RAD18* tagged with 3×FLAG	pDM9	This study
KS972	As YBL574, but with *RAD6* tagged with 3×FLAG	pDM9	This study
KS1005	As YBL574, but with *REV3* tagged with 6×FLAG	pDM9	This study

Supplementary Figure 1. Histone H4 LRS mutations that attenuate UV mutagenesis additively increase the UV sensitivities of *rad24Δ* (A), *rad51Δ* (B) and *rad52Δ* (C) cells.



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