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Role of Histone H4 Mutations in DNA Repair Pathways

Sheikh Arafatur Rahman

Louisiana State University and Agricultural and Mechanical College

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ROLE OF HISTONE H4 MUTATIONS IN DNA REPAIR PATHWAYS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

Veterinary Medical Sciences through
the Department of Comparative Biomedical Sciences

by

Sheikh Arafatur Rahman
DVM, Bangladesh Agricultural University, 2005
MS, Bangladesh Agricultural University, 2007
August 2016

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ABSTRACT

Histone H3K79 methylation has proven to play roles in different DNA repair pathways. Histone H4 residues serine 64 to threonine 80 surround histone H3K79 residue. We have analyzed the effect of mutation of the residues on UV sensitivity, H3K79 methylation, nucleotide excision repair, chromatin state, and homologous recombination. We found that the mutation of residues 64 to 72 causes resistance to killing by UV, whereas the mutation of residues 73 to 80 causes sensitivity to killing by UV compared to the wild type. In general, we found that the mutations make nucleotide excision repair more proficient at the constitutively active RPB2 loci. We found that global genomic repair occurs more quickly in most of the mutants except H75E. Transcription-coupled repair is normal in most of the mutants except Y72T. In mutant H75E, Rad26-independent transcription-coupled repair is also defective. The mutations T73D, T73F, and T73Y affect the mono, di, and trimethylation of H3K79, but they experience faster or normal nucleotide excision repair. We also found that these histone mutations make chromatin more accessible to micrococcal nuclease. UV-sensitive histone mutants have normal or faster nucleotide excision repair. The methyl methane sulfonate (MMS) sensitivity test and Rad14 and Rad52 epistasis analysis suggest that UV-sensitive histone H4 mutants could play a role in homologous recombination repair pathway. Taken together, the results imply that the histone mutations remodel the chromatin that helps to recruit nucleotide excision repair factors for efficient repair.

CHAPTER 1

INTRODUCTION

Molecules present in the cellular environment can alter the structure of DNA. In human cells, at body temperature and pH, DNA depurination and deamination occur spontaneously. Additionally, oxygen free radicals, the byproduct of metabolism, react with DNA to change or destroy the coding information of bases. Methylating agents (e.g., S-adenosylmethionine) react with DNA to methylate the bases. During DNA replication, errors are also incorporated (Lindahl and Nyberg 1972, Lindahl and Nyberg 1974, Rydberg and Lindahl 1982, Frederico, Kunkel et al. 1990, Nakamura, Walker et al. 1998).

External sources of DNA damages exists. UV radiation from the sun causes DNA damage like cyclobutane pyrimidine dimers and 6,4, - photoproducts. Radioactive materials in the earth produce ionizing radiation that causes DNA strand breaks. DNA strand breaks also occur due to X-rays, radiation therapy and some forms of chemotherapy (Jackson and Bartek 2009).

Even though a number of endogenous and exogenous DNA-damaging agents exist, mutation is rare due to DNA repair. About 130 human genes have proven to play a role in DNA repair. If DNA is repaired accurately, cells survive. Inability to repair may lead to cell death. Misrepair causes genomic instability and the development of cancer (Christmann, Tomicic et al. 2003).

DNA repair can be done mainly in three ways: first, direct reversal of base damage; second, excision of damaged, mispaired or incorrect bases known as excision repair; and third, strand break repair. Damaged bases can be removed from the genome as free bases in the base excision repair pathway and as nucleotides in the nucleotide excision repair pathway. Nucleotide excision repair can be subdivided into transcription-coupled repair and global genomic repair. Transcription-coupled repair removes lesions from the transcribed strand of active genes, whereas global genomic repair removes lesions from silent regions of

the genome including non-transcribed strand of transcriptionally active genes (Li, Selvam et al. 2014). Another form of excision repair is called mismatch repair (MMR), which corrects errors introduced during DNA replication (Jackson and Bartek 2009). In addition, double strand breaks cause the breakdown of the sugar phosphate backbone of DNA and threaten cell viability. Single and double strand breaks can be repaired by homologous recombination and non-homologous end joining mechanism (Lieber 2010, Krejci, Altmannova et al. 2012).

There are biological responses that do not remove damage from DNA. They are called DNA damage tolerance mechanism. Multiple strategies exist for tolerating base damage to DNA: recombinational repair, template switching and translesion DNA synthesis (Ghosal and Chen 2013). In addition to these mechanisms, DNA damage and/or arrested replication can activate cell cycle checkpoints that lead to arrested cell cycle progression, thereby providing more time to repair or damage tolerance (Ishikawa, Ishii et al. 2006).

Chromatin structure is essential for DNA organization and cellular functionality in eukaryotic cells (Luger 2006). Chromatin is composed of highly conserved eukaryotic histone proteins. Four core histone proteins, H2A, H2B, H3, and H4, assemble as heterodimers to form a histone octamer. Each octamer is wrapped with 146 base pairs of DNA (Dai, Hyland et al. 2008). Histone proteins undergo posttranslational modifications (Kouzarides 2007). Some of these modifications lead to chromatin remodeling to enable repair proteins to recognize and access damaged DNA (Thoma 2005). The histone H3K79 can undergo methylation by Dot1 methyltransferase. H3K79 methylation plays a role in nucleotide excision repair, cell cycle checkpoint and homologous recombination (Nguyen and Zhang 2011). Additionally, histone H4 residues R78 to T80 are located in the LRS (Loss of ribosomal DNA silencing) domain. Mutation of these residues causes the LRS phenotype (Park, Cosgrove et al. 2002, Norris, Bianchet et al. 2008, Fry, Norris et al. 2006). Studies have shown the effect of histone mutations in DNA repair. A single amino acid change in

histone H4R45C (a sin mutant) alters the chromatin and may influence the accessibility of DNA repair factors. Mutant yeast cells are more resistant to UV and have a higher rate of nucleotide excision repair (Nag, Gong et al. 2008). Seventeen histone H4 residues, serine 64 to threonine 80, surround each of the two H3K79 residues (Figure 1, Table 1) (White, Suto et al. 2001). We hypothesized that as these residues surround histone H3K79, mutation of the residues are likely to influence H3K79 methylation and DNA repair.

Table 1. List of Histone H4 residues used in the study

S64	R67	V70	T73	A76	K79
V65	D68	T71	E74	K77	T80
I66	S69	Y72	H75	R78	Total=17

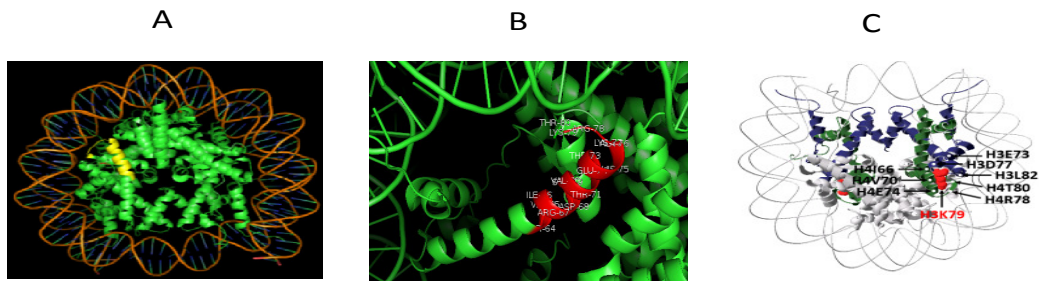


Figure 1. Histone H4 residues Serine 64 to Threonine 80. (A) The histone residues are shown in yellow. (B) Seventeen histone H4 residues from Serine 64 to Threonine 80 are shown in red. (C) Histone H4 residues along with histone residue H3K79. Based on PDB file 1ID3

Little is known about the role of histone residues (H4S64-H4T80) located in the LRS domain and neighboring H3K79 in DNA repair. It is necessary to study the effect of the histone mutations on regulating H3K79 methylation and DNA repair pathways as well as the mechanisms. As the histone proteins and DNA repair mechanisms are highly conserved among eukaryotes, the finding of the study is likely to be applicable to humans.

Our lab carried out a high throughput mutational screening of all the 17 residues. On the basis of UV sensitivity, we selected 33 mutations. Given the fact that UV-induced DNA

damages are repaired by nucleotide excision repair, homologous recombination repair and post replication repair pathways (Nguyen and Zhang 2011), the objectives of the present study are to analyze:

1. The role of histone H4 mutations in nucleotide excision repair pathways:
 - a. The role of mutations in global genomic repair pathway
 - b. The role of mutations in transcription-coupled repair pathway
 - c. The role of mutations in Rad26-independent transcription-coupled repair pathway
2. The role of histone H4 mutations in homologous recombination repair pathway

CHAPTER 2

LITERATURE REVIEW AND BACKGROUND

In the late 1950s, a research group at York University discovered an *E. coli* strain that was UV sensitive (Hill 1958). In the mid-1960s, Paul Howard Flanders and coworkers at Yale University and another group at Oak Ridge National Laboratory separately identified that *E. coli* can remove small DNA pieces containing pyrimidine dimers after UV irradiation (Boyce and Howard-Flanders 1964, Setlow and Carrier 1964). Howard Flander's group also discovered three different genes *uvrA*, *uvrB*, and *uvrC* that play a role in the repair of UV-induced DNA damage in *E. coli*. At the same time, UV radiation-induced DNA damage and excision repair was discovered in mammalian cells (Rasmussen and Painter 1964). Nucleotide excision repair is one of the most versatile DNA repair mechanisms. It removes helix-distorting DNA lesions including CPDs and 6,4-PPs induced by UV irradiation. The nucleotide excision repair is subdivided into transcription-coupled repair and global genomic repair pathways. The initial damage recognition steps are different but the later steps are similar in the pathways (Li, Selvam et al. 2014).

In eukaryotes, UV-induced DNA damage in the non-transcribed strand and heterochromatin are recognized by Rad7-Rad16 complex in yeast and by XPC-Rad23 A/B complex in humans (Evans, Moggs et al. 1997, Sugasawa, Okamoto et al. 2001, Volker, Mone et al. 2001, Riedl, Hanaoka et al. 2003, Tapias, Auriol et al. 2004). Rad7-Rad16 complex is an ATP dependent motor that travels along the damaged DNA (Guzder, Sung et al. 1998). In yeast, Elc1 is an ubiquitin ligase and a global genomic repair specific factor besides Rad7-Rad16 (Lejeune, Chen, et. al. 2009).

In transcription-coupled repair, damage recognition is initiated by physical blockage of RNA polymerase II stalled at a lesion. This stalled RNA polymerase II triggers the recruitment of transcription coupled repair machinery (Svejstrup 2002). Rad 26 (a homolog of the human CSB protein) and Rpb9 (a non-essential subunit of RNA polymerase II)

mediate two subpathways of transcription-coupled repair (Li 2015). The role of Rad26 in transcription-coupled repair has been shown by antagonizing the actions of RNA Pol II-asso-

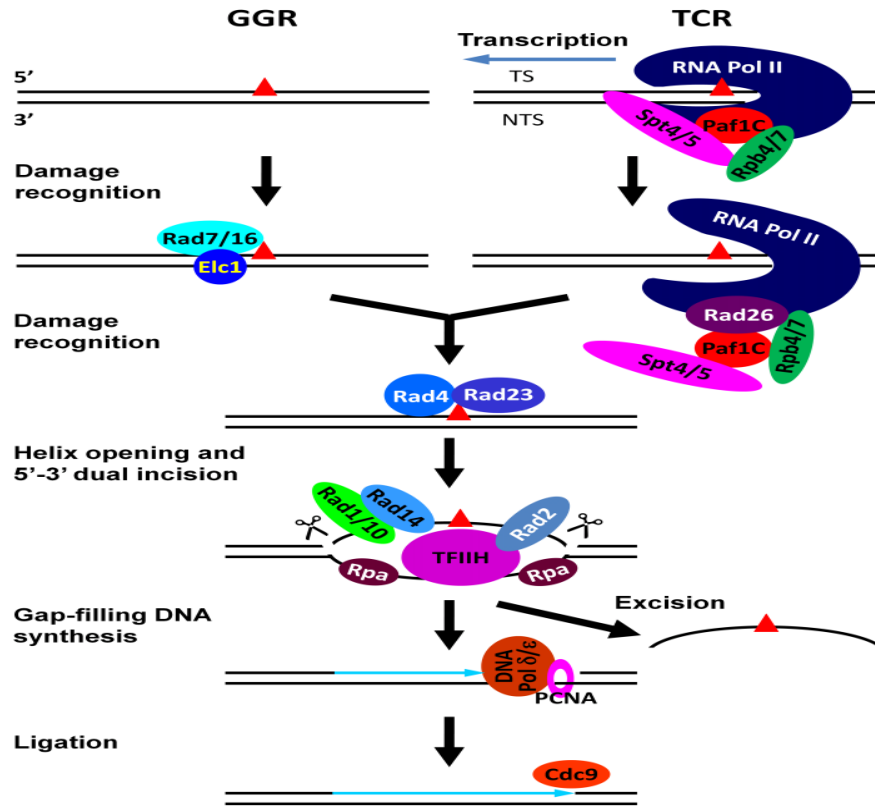


Figure 2. Nucleotide Excision Repair (GGR and TCR) in *S. cerevisiae*. Red triangle denotes a DNA lesion. From Tatum and Li, 2011

ciated transcription-coupled repair suppressors. At least three complexes or sub complexes suppress transcription coupled repair in the absence of Rad26: Rpb4/Rpb7, Spt4/Spt5 and the Paf1 complex (Paf1C) (Jansen, den Dulk et al. 2000, Ding, LeJeune et al. 2010, Li and Smerdon 2002, Tatum, Li et al. 2011). After damage recognition, TFIIH (Transcription factor IIH in *S. cerevisiae* is required for transcription by RNA polymerase II and for nucleotide excision repair of damaged DNA), a helicase is recruited. Two subunits of THIIH, Rad 3 (XPD in humans) and Rad 25 (XPB in humans) translocate in the 5'-3' and 3'-5' direction (Prakash and Prakash 2000). Rad14 (a homolog of human XPA) then binds with the damaged

DNA. Rad 14 along with Replication protein A (RPA), THIIIH, Rad 2 and Rad1-Rad10 forms a preincision complex. Rad1-Rad10 and Rad2 are endonucleases that cut in the 5' and 3' end of damaged DNA. Later, DNA pol δ/ϵ and DNA ligase seal the gap (Prakash and Prakash 2000) (Figure 2).

DNA double strand breaks are lethal DNA lesions, which are repaired by homologous recombination (HR) and non-homologous end joining (NHEJ) pathways. For a lesion to be repaired by homologous recombination there should be a 5'-3' processing of broken DNA strands. 5'-3' resection is a complex process. About one hundred (100) nucleotide removal is performed by Mre11-Rad50-Xrs2 along with Sae2 protein (Huertas, Cortes-Ledesma et al. 2008). In addition, Exo1, an exonuclease removes mononucleotides, and Sgs1-Top3-Rmi1-Dna2 is a helicase/endonuclease that removes short oligonucleotides from the 5' end (Huertas, Cortes-Ledesma et al. 2008, Mimitou and Symington 2008, Zhu, Chung et al. 2008, Cejka, Cannavo et al. 2010, Niu, Chung et al. 2010). Ku70-Ku80 are DNA double strand break binding proteins that inhibits Exo1 to facilitate non-homologous end joining (Balestrini, Ristic et al. 2013). A similar mechanism exists in mammalian cells where CtIP (an ortholog of Sae2) and BLM1 helicase (an ortholog of Sgs1) work with EXO1 and DNA2 (Sartori, Lukas et al. 2007, Nimonkar, Genschel et al. 2011, Sun, Lee et al. 2012). After DNA processing, Replication Protein A (RPA) binds to ssDNA overhangs to take out kinks and secondary structures. RPA is then replaced by Rad51 recombinase with the help of Rad52 mediator protein. Upon Rad51 binding, it stretches ssDNA within the Rad51 nucleofilament (Chen, Yang et al. 2008). RPA is replaced with the recombination mediator proteins Rad52 in yeast and BRCA2 in vertebrates and Rad51 paralog proteins (Rad55, Rad57 in yeasts and Rad51B, Rad51C, Rad51D, XRCC2, XRCC3 in vertebrates) (Daley, Gaines et al. 2014, Morrical 2015). Following strand invasion, new DNA is synthesized by polymerase δ/ϵ using the 3' invading end as the primer (Mehta and Haber 2014).

Most studies have focused on the role of chromatin in regulating transcription. Recently, an emphasis on the role of the chromatin in the DNA damage response has emerged. Two common chromatin-remodeling mechanisms are known, chromatin remodeling complexes and post-translational modifications (PTMs) (Mendez-Acuna, Di Tomaso et al. 2010). Histone proteins undergo posttranslational modifications, which take part in many cellular processes including DNA repair. One of the most well-known histone PTMs is γ H2AX phosphorylation that is considered as a marker for homologous recombination repair. When a double strand break is formed, γ H2AX, a histone variant is phosphorylated by ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) kinases (Mendez-Acuna, Di Tomaso et al. 2010).

Another important histone PTM is histone H3 Lysine 79 methylation. The H3K79 residue can be mono, di, or trimethylated. H3K79 methylation plays a role in cell cycle checkpoint, homologous recombination and nucleotide excision repair (Nguyen and Zhang 2011). Evans et al. (2008) analyzed four histone mutants, H3L70S, H3E73D, H3Q76R, and H3T80A, which are H3K79 neighboring residues. They found that these mutations are sensitive to UV and that each mutation effects H3K79 methylation states. These mutations act through a distinct subset of DNA damage response pathways including nucleotide excision repair, checkpoint, post replication repair and recombinational repair (Evans, Bostelman et al. 2008). Histone H4 tail deletions lacking residues 17 to 23 completely abolish H3K79 dimethylation (Dai, Hyland et al. 2008). In addition, some of the residues we analyze lie in the LRS (Loss of Ribosomal DNA Silencing) domain. The LRS domain is composed of amino acids 72 to 83 of histone H3 and 78 to 81 of histone H4 (Park, Cosgrove et al. 2002, Norris, Bianchet et al. 2008). In LRS mutant alleles, loss of repression of genes occurs in transcriptionally silent regions of the genome (e.g. ribosomal DNA locus, telomeres) (Fry, Norris et al. 2006).

Limited literature is available regarding the role of histone mutations in DNA repair. Recently, a study of all the core histone proteins was conducted in yeast (Dai, Hyland et al. 2008). They systematically substituted each residue with alanine and changed all alanine residues to serine. They tested the DNA damage response in the presence of UV irradiation, methyl methane sulfonate (MMS), hydroxyurea (HU) and camptothecin (CPT). According to their study, histone H4 residues S64 to T80 substitutions were not sensitive to UV. They found that two mutations of histone H4 Y72F and T80A were sensitive to MMS (Dai, Hyland et al. 2008).

This study is a mutational analysis of 17 histone H4 residues to analyze the role of each of the 20 possible mutations in different DNA repair pathways in *Saccharomyces cerevisiae*. Before I started the project, the lab conducted a mutational screening of all 17 histone H4 residues. We mutated histone H4 residues, serine 64 to threonine 80 to all possible 20 amino acids in a single copy centromeric plasmid. We transformed the mutated plasmid pool in the YBL574 strain and shuffled out the wild type plasmid using a technique known as plasmid shuffling. In the wild type yeast strain YBL574, the genomic HHT1-HHF1 and HHT2-HHF2 genes were deleted and complemented with a centromeric URA3 plasmid bearing the wild type HHT1-HHF1 genes. In yeast, the HHT1 and HHT2 genes encode histone H3, and the HHF1 and HHF2 genes encode histone H4 (Dollard, Ricupero-Hovasse et al. 1994). The mutant plasmid has a TRP1 and the wild type a URA3 selection marker specifically designed for plasmid shuffling.

We irradiated the selected cells and grew them to ten generations. We isolated plasmids from unirradiated and irradiated cells. We then took plasmids from the original mutated pool, unirradiated and irradiated cells for next-generation sequencing. On the basis of sequencing reads, we found that six mutants were $\geq 5X$ UV resistant than their respective

wild type and 12 mutants were $\geq 5X$ UV sensitive than their respective wild type. On the basis of UV sensitivity, we selected 33 mutations for further analysis.

CHAPTER 3

MATERIALS AND METHODS

Yeast Strains and Plasmids

All yeast strains we used in this study derive from the wild type yeast strain YBL574 [MATa, leu2 Δ 1, his3 Δ 200, ura3-52, trp1 Δ 63, lys2-128 Δ , (hht1-hhf1) Δ LEU2 (hht2-hhf2) Δ ::HIS3 Ty912 Δ 35-lacZ::his4]. We introduced histone H4 mutations in the plasmid pRS414 which contained tryptophan (TRP) as a selection marker using site-directed mutagenesis. We confirmed the mutations by sequencing after screening. We transformed the histone H4 mutant plasmids into the YBL574 wild type strain and the YBL574 strain with other backgrounds. We shuffled out the wild type plasmid containing a functional URA3 gene using a technique described previously (Boeke, LaCroute et al. 1984).

We detected Rad7, Rad14, Rad26, Rad52 and Rad7-Rad26 by making deletion cassette containing a 50 base pair homology at the 5' and 3' ends within the ORF of each gene and containing the kanamycin gene as a selection marker in the middle. We amplified the deletion cassette using a p3Flag-KanMx plasmid. We transformed the specific deletion cassette into the wild type yeast strain for homologous recombination, cassette exchange and gene deletion. To create double knockouts, we removed the kanamycin gene by transforming a plasmid pSH63 expressing Cre recombinase. We created the second knockout using the process described above. We confirmed the deletions using PCR. We tagged the Rad1, Rad2, Rad3, Rad10 and Rad23 genes with three consecutive Flags at the 3' end of each gene using a process described previously (Gelbart, Rechsteiner et al. 2001). We confirmed the three Flag tagging of each gene using PCR.

UV and Methyl Methane Sulfonate (MMS) Sensitivity Tests

We grew the yeast cells in appropriate liquid media at 30°C until saturation. For UV sensitivity tests, we serially diluted the saturated cells were ten times and spotted them onto appropriate agar plates. We irradiated the spotted plates with 254 nm of ultraviolet C (UVC)

light for different time periods. We incubated the irradiated plates at 30°C for 3 to 5 days before photographing them. For the MMS sensitivity test, we added different concentrations of MMS into liquid YPD media to make YPD MMS plates. We serially diluted saturated cells ten times and spotted them onto the YPD MMS plates, and incubated them at 30°C for 3 to 5 days before photographing them.

Detection of H3K79 Methylation by Western Blotting

We grew the histone mutant yeast strains to late log phase ($A_{600} \approx 1.0$). We prepared whole yeast extracts from the cells as described previously (Kushnirov 2000). We ran the yeast extracts on SDS-PAGE gel (12% gels). We transferred the gels to PVDF membrane (Millipore). We detected monomethyl, dimethyl and trimethyl H3K79 bands using H3K79 monomethyl, dimethyl, or trimethyl antibodies, respectively, which were purchased from Abcam. We treated the blots with Super Signal West Femto Maximum sensitivity substrate (Pierce) and scanned them with ChemiDoc[™] XRS+ System (Bio-Rad).

Micrococcal Nuclease (MNase) Accessibility Assay

We performed the MNase accessibility assay using a method described previously (Kent, Bird et al. 1993). We harvested late log phase yeast cells ($A_{600} \approx 1.0$). For the irradiated and unirradiated sample, we separated an equal amount of yeast cells. We irradiated 45 ml of cells with 100 J/m² of UVC (254 nm) and incubated them at 30°C for 1 hour. We pelleted and resuspended the unirradiated and irradiated cells with 5 ml zymolyase buffer and treated them with 50 units of zymolyase (Zymoresearch) followed by 30°C incubation for 40 mins to complete the spheroplasting. We aliquoted the spheroplasts six times for a five point three fold MNase dilution series starting from 4000 units and permeabilized by NP-40. We performed the MNase digestion at 37°C for 10 min. We stopped the reaction using an MNase stop buffer (6% SDS, 200 mM EDTA). We disrupted the cells with incubation at 65°C for 2 to 3 hours. We cooled the samples to room temperature. We extracted DNA twice using the

phenol chloroform method and DNA from each MNase dilution point dissolved into TE, pH 8.0 and ran them into 0.8 % agarose gel.

Repair Analysis of UV Induced Cyclobutane Pyrimidine Dimers (CPDs)

We performed genomic DNA isolation and repair analysis of UV induced CPDs using a method described previously (Li, Waters et al. 2000, Li and Smerdon 2002). We grew yeast cells at 30°C in selective media to late log phase ($A_{600} \approx 1.0$). We irradiated the harvested cells with 100 J/m² of UVC light (254 nm) and incubated at 30°C for different time points in the dark. We isolated genomic DNA samples from different time points. We cut the isolated DNA with DraI to release fragment of RPB2 gene and incised with T4 endonuclease V. We fished out the transcribed and non-transcribed strands with appropriate biotinylated oligonucleotides. The DNA strands were 3' end labeled with [α -³²P] dATP, eluted and ran on a sequencing gel. We exposed the gels to phosphorimager screens.

CHAPTER 4

RESULTS

The Histone H4 Residues 64 to 72 Mutations Are More Resistant to UV and Residues 73 to 80 Mutations Are More Sensitive to UV

Nucleotide excision repair pathways mainly repair UV-induced DNA damages (Rastogi, Richa et al. 2010). Our lab previously performed a high throughput mutational screening. This screening suggested that 33 mutations are more sensitive or resistant to UV than that of the wild type strain. To confirm this initial screening data, we introduced the 33 selected mutations in a centromeric plasmid containing TRP (tryptophan) as a selection marker. We transformed the mutant plasmids into wild type yeast strains in which genomic HHT1-HHF1 and HHT2-HHF2 genes were deleted and complemented with a centromeric URA3 plasmid bearing the wild type HHT1-HHF1 genes. We shuffled out the wild type plasmid containing the wild type HHT1-HHF1 genes to construct the 33 mutant yeast strains (Figure 3). On those 33 selected histone H4 mutants, we conducted UV sensitivity tests, testing each strain three times. We found that 11 mutants are more resistant to UV from residues 64 to 72 and 13 mutations are more sensitive to UV from residues 73 to 80 as compared to the wild type strain. We selected these 24 mutations for DNA repair analysis (Table 2, Figure 4).

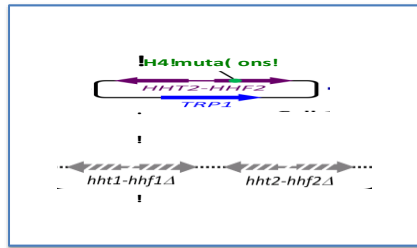


Figure 3. Histone H4 mutant strains

Table 2. UV sensitivity test result of the 24 selected mutants

Histone H4 mutations	UV sensitivity	Histone H4 mutations	UV sensitivity
S64G	Resistant: 2-3x	T73D	Sensitive: 3-4x
S64I	Resistant: 2-3x	T73F	Sensitive: 10x
V65T	Resistant: 10x	T73Y	Sensitive: 100x
V65Y	Resistant: 3x	E74M	Sensitive: 10x
R67A	Resistant: 10x	H75E	Sensitive: 10x
R67D	Resistant: 10x	A76P	Sensitive: 2x
R67S	Resistant: 2x	A76T	Sensitive: 5x
R67V	Resistant: 2x	R78I	Sensitive: 50x
D68I	Sensitive: 10x	R78S	Sensitive: 10x
D68Y	Resistant: 3x	T80F	Sensitive: 10x
T71I	Resistant: 2x	T80I	Sensitive: 5x
Y72T	Slightly Resistant/similar to WT	T80L	Sensitive: 5x

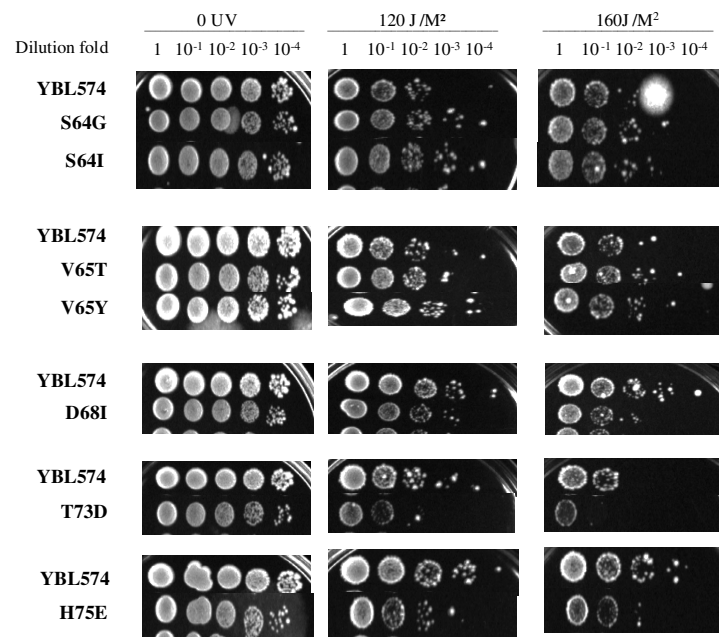


Figure 4 (A). UV sensitivity test result of selected histone H4 mutants. Spot plate assay showing the UV sensitivities of YBL574 wild type and histone H4 mutant yeast cells.

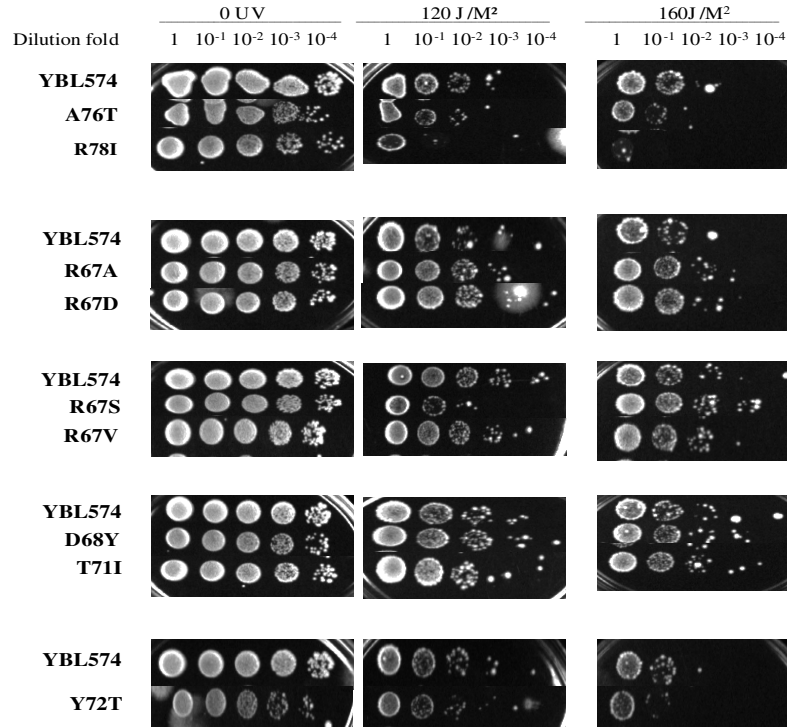


Figure 4 (B). UV sensitivity test result of selected histone H4 mutants. Spot plate assay showing the UV sensitivities of YBL574 wild type and histone H4 mutant yeast cells.

The Histone H4 Mutations T73D, T73F and T73Y Affect H3K79 Methylation and Other Mutations Have Normal Level of H3K79 Methylation

Studies have shown that histone mutations alter H3K79 methylation status (Dai, Hyland et al. 2008, Evans, Bostelman et al. 2008). We hypothesized that as these residues surround H3K79, the mutations may have altered the level of the H3K79 methylation state and that each specific methylation state would dictate repair. To test this hypothesis, we conducted western blotting analysis using monomethyl, dimethyl and trimethyl H3K79 antibody to see each state of H3K79 methylation (mono, di, and trimethylation) in those mutants. Among the mutants, the H4T73F and H4T73Y mutations affected trimethylation, the H4T73D, H4T73F and H4T73Y mutations affected dimethylation and the H4T73D mutation affected monomethylation. In other mutants, we observed normal states of mono, di, and trimethylation (Figure 5, 6).

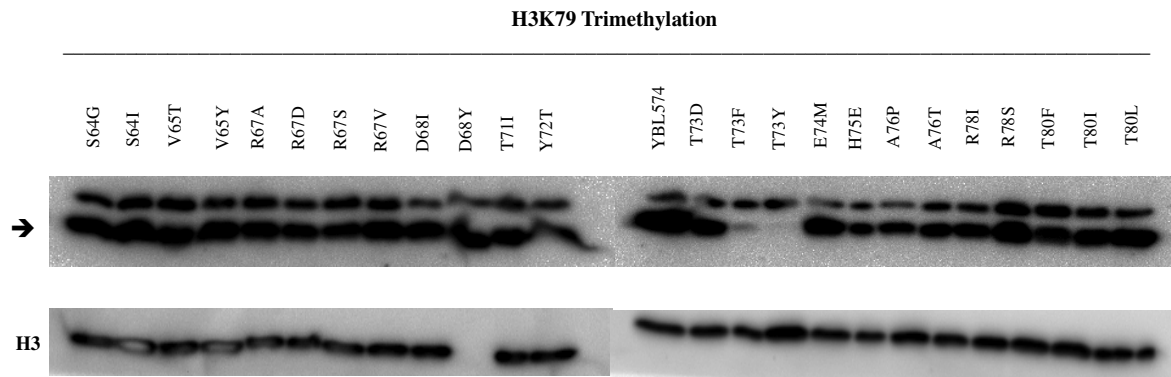


Figure 5. H3K79 trimethylation status of wild type YBL574 and selected histone H4 mutants. Histone H3 was used as a loading control.

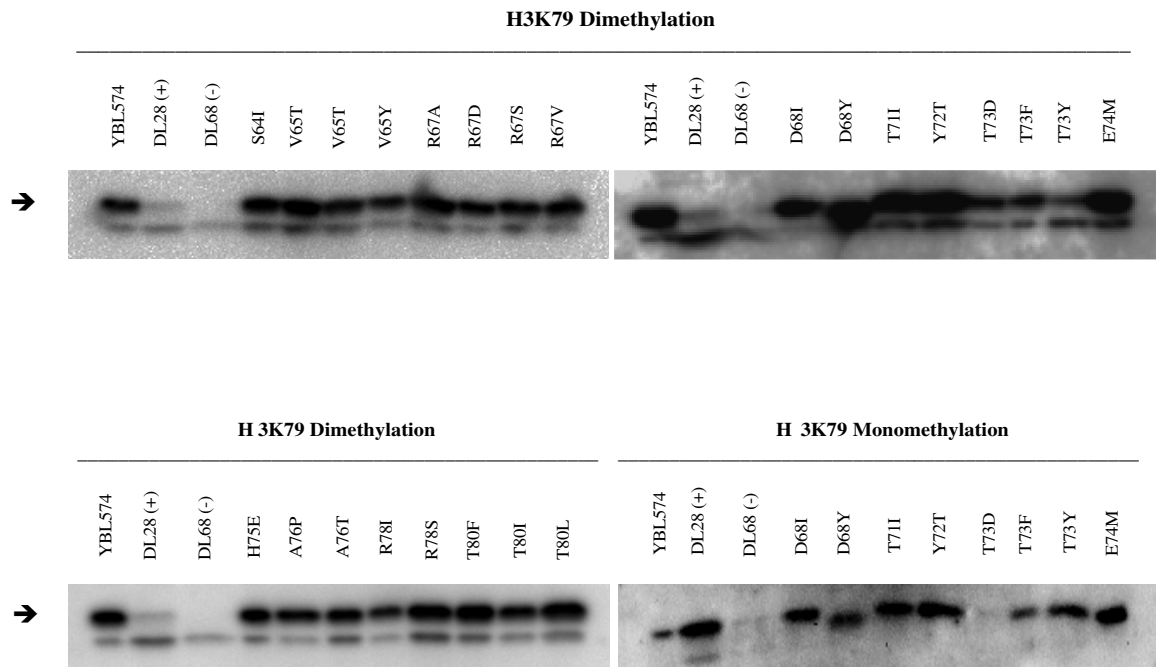


Figure 6. H3K79 dimethylation and monomethylation status of selected histone H4 mutants. DL28 and DL68 strains were used as positive and negative controls, respectively. Histone H3 was used as a loading control as shown in Figure 5.

The Histone H4 Mutations Make Nucleotide Excision Repair More Proficient Except H75E

Previous studies have shown that histone mutations make nucleotide excision repair more proficient (Nag, Gong et al. 2008). But researchers have not studied the role of histone residues S64 to T80 mutations in DNA repair. To examine the role of the mutations in nucleotide excision repair, we analyzed the nucleotide excision repair rate in the RPB2 gene, which is the second largest subunit of RNA Polymerase II. We conducted the transcription-coupled repair and global genomic repair analysis in the transcribed and non-transcribed strands in the RPB2 gene in a sequencing gel using radioactive DNA labeling. Using this technique, we can analyze different sub-pathways of nucleotide excision repair at the nucleotide level at different sites of a DNA fragment. We found that most of the mutants have faster global genomic repair than that of the wild type. In one mutant, H4H75E, we observed limited global genomic repair (Figure 7).

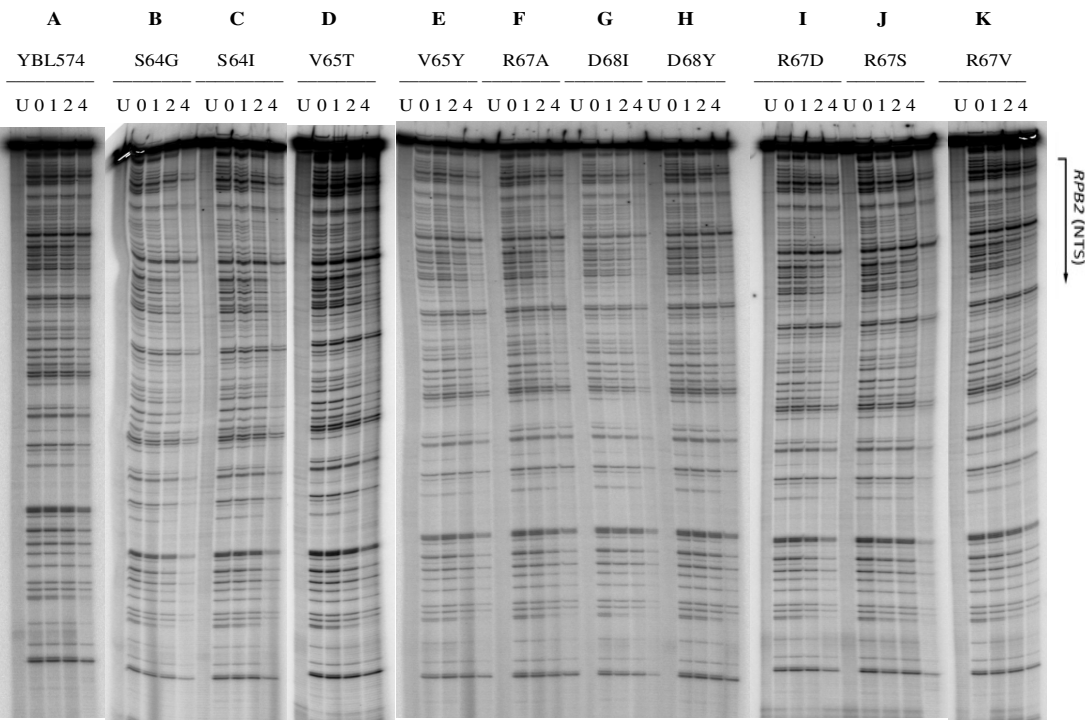


Figure 7 (A). Gels showing repair of the CPDs in the nontranscribed strand of the RPB2 gene in wild type YBL574 strain and histone H4 mutants. U denotes unirradiated. 0, 1, 2 and 4 denotes hours of repair incubation. The arrow indicates the transcription start site.

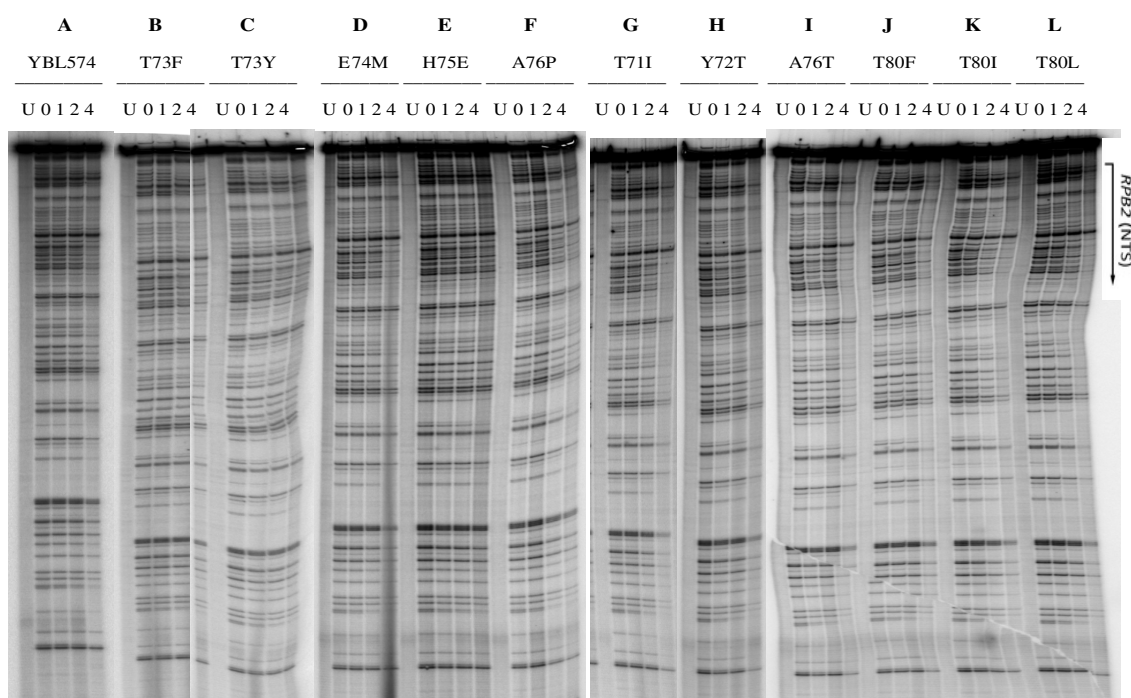


Figure 7 (B). Gels showing repair of the CPDs in the nontranscribed strand of the RPB2 gene in wild type YBL574 strain and histone H4 mutants. U denotes unirradiated. 0, 1, 2 and 4 denotes hours of repair incubation. The arrow indicates the transcription start site.

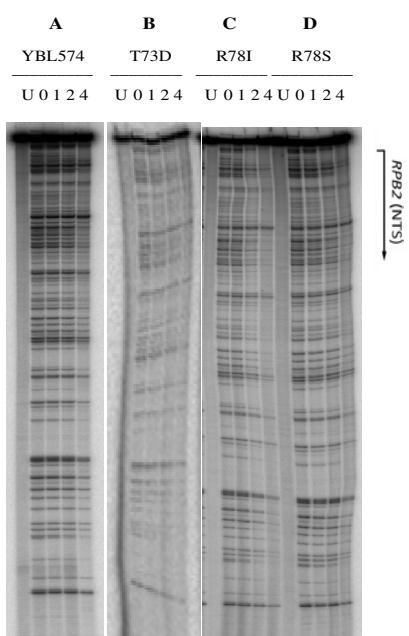


Figure 7 (C). Gels showing repair of the CPDs in the nontranscribed strand of the RPB2 gene in wild type YBL574 strain and histone H4 mutants. U denotes unirradiated. 0, 1, 2 and 4 denotes hours of repair incubation. The arrow indicates the transcription start site.

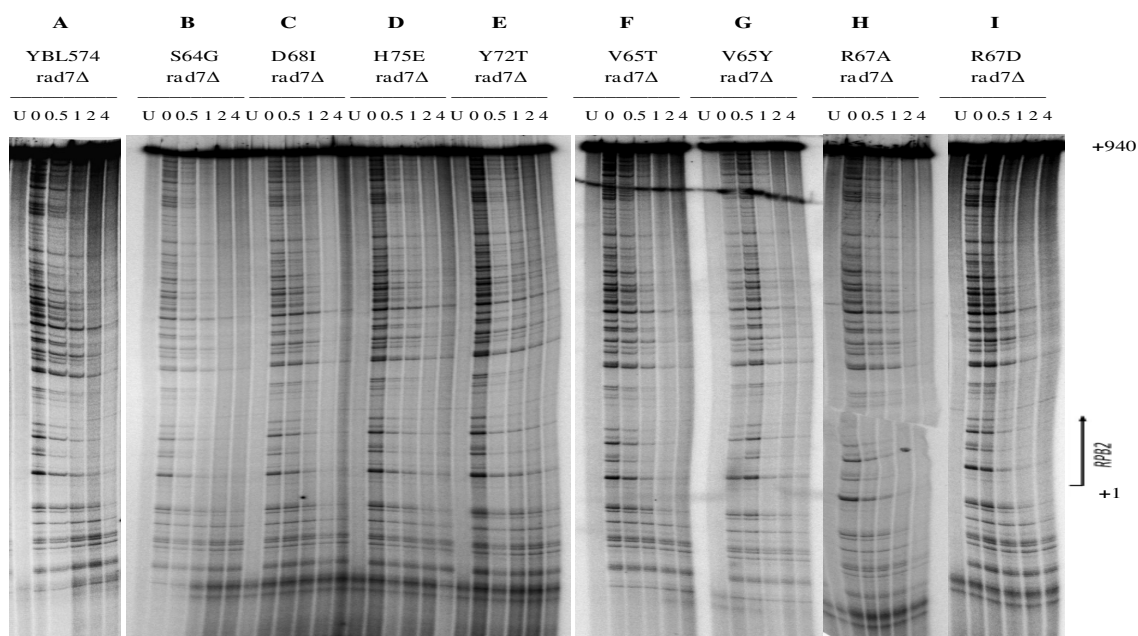


Figure 8 (A). Gels showing repair of the CPDs in the transcribed strand of the RPB2 gene in wild type YBL574 strain and histone H4 mutants in rad7Δ background. U denotes unirradiated. 0, 0.5, 1, 2 and 4 denotes hours of repair incubation. The arrow indicates the transcription start site. The numbers on the right indicate nucleotide positions relative to transcription start site.

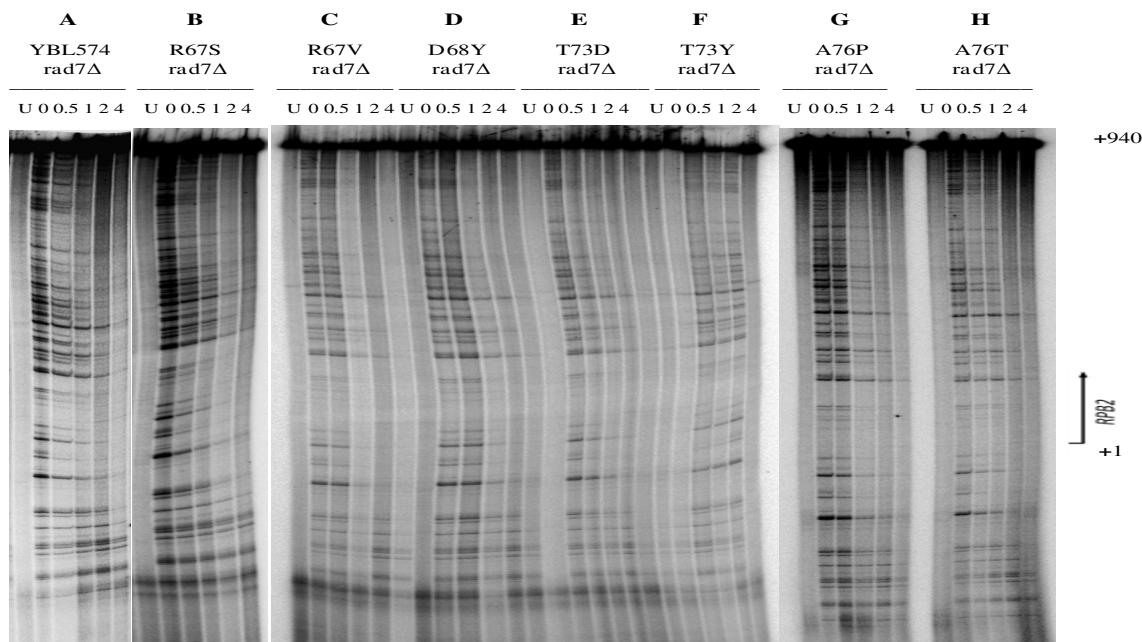


Figure 8 (B). Gels showing repair of the CPDs in the transcribed strand of the RPB2 gene in wild type YBL574 strain and histone H4 mutants in rad7Δ background. U denotes unirradiated. 0, 0.5, 1, 2 and 4 denotes hours of repair incubation. The arrow indicates the transcription start site. The numbers on the right indicate nucleotide positions relative to transcription start site.

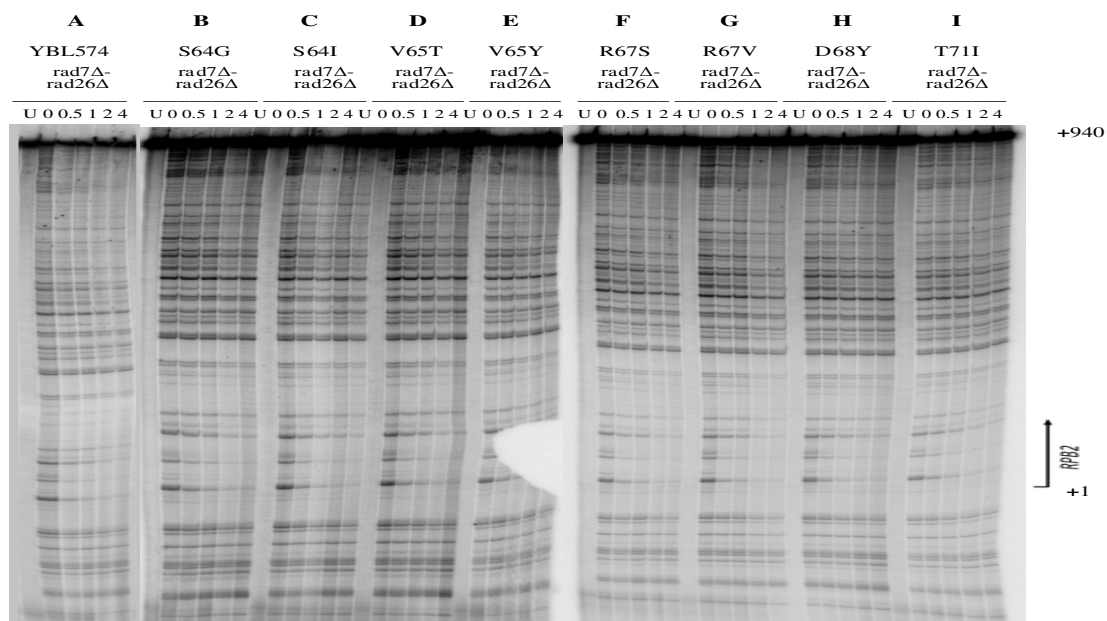


Figure 9 (A). Gels showing repair of the CPDs in the transcribed strand of the RPB2 gene in wild type YBL574 strain and histone H4 mutants in rad7Δ-rad26Δ background. U denotes unirradiated. 0, 0.5, 1, 2 and 4 denotes hours of repair incubation. The arrow indicates the transcription start site. The numbers on the right indicate nucleotide positions relative to transcription start site.

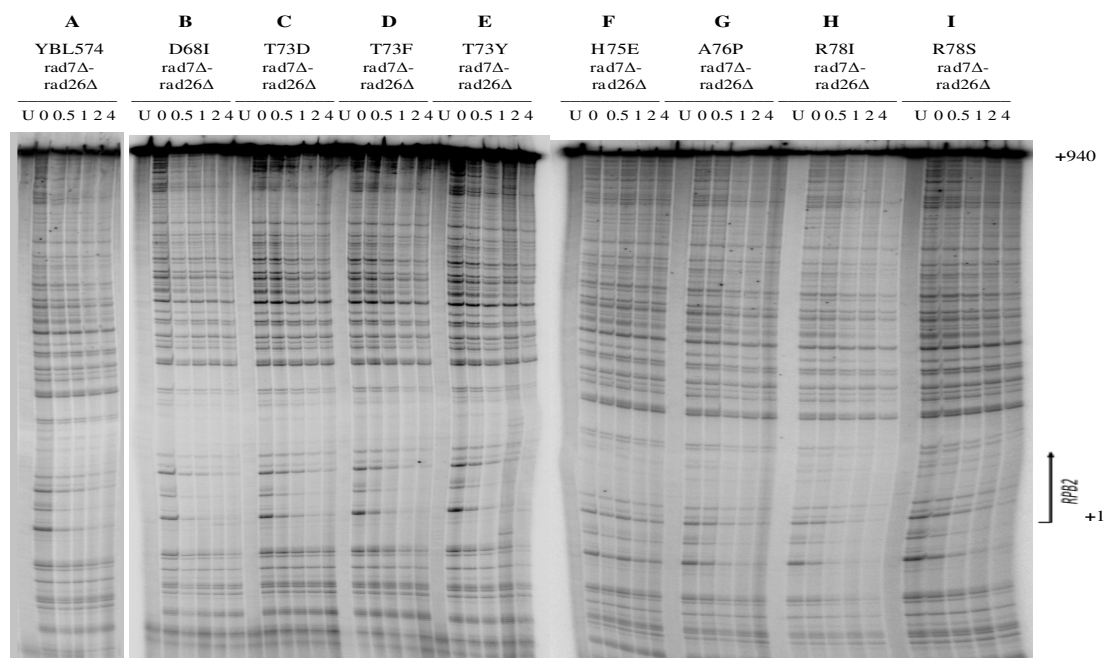


Figure 9 (B). Gels showing repair of the CPDs in the transcribed strand of the RPB2 gene in wild type YBL574 strain and histone H4 mutants in rad7Δ-rad26Δ background. U denotes unirradiated. 0, 0.5, 1, 2 and 4 denotes hours of repair incubation. The arrow indicates the transcription start site. The numbers on the right indicate nucleotide positions relative to transcription start site.

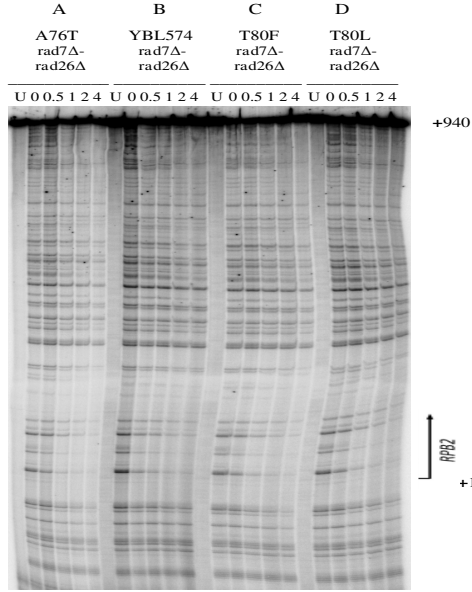


Figure 9 (C). Gels showing repair of the CPDs in the transcribed strand of the RPB2 gene in wild type YBL574 strain and histone H4 mutants in rad7 Δ -rad26 Δ background. U denotes unirradiated. 0, 0.5, 1, 2 and 4 denotes hours of repair incubation. The arrow indicates the transcription start site. The numbers on the right indicate nucleotide positions relative to transcription start site.

We performed transcription-coupled repair analysis of those mutants in rad7 Δ background in the transcribed strand of the RPB2 gene with the same technique. Rad7 along with Rad16 forms a heterodimeric complex that binds preferentially to UV-damaged DNA. Rad7 is absolutely required for GGR (Prakash and Prakash 2000). We found that transcription-coupled repair was not affected in those mutants except one: H4Y72T (Figure 8).

In yeast, Rpb9 and Rad26 mediate two subpathways of transcription-coupled repair (Li 2015). To determine whether the mutations play any role in Rad26-independent transcription-coupled repair, we performed TCR analysis of those mutants in rad7 Δ -rad26 Δ background. Rad26 is an ATPase of the SWI2/SNF2 family of the chromatin-remodeling complex that facilitates transcription-coupled repair. In the rad7 Δ -rad26 Δ background, we ob-

served only residual transcription-coupled repair (Lee, Yu et al. 2002, Li 2015). We found that Rad26-independent transcription-coupled repair was not affected in those mutants except mutation H4H75E (Figure 9).

Our initial hypothesis was that the UV sensitivity of those histone mutants could be due to nucleotide excision repair deficiency. But the mutants are nucleotide excision repair proficient, except mutant H4H75E. Nucleotide excision repair and H3K79 methylation data imply that nucleotide excision repair is not dependent on the H3K79 methylation signal, as the H3K79 methylation-deficient mutants have faster or normal nucleotide excision repair.

Global Chromatin Becomes More Accessible to Micrococcal Nuclease in the Histone H4 Mutants

To further investigate the mechanism behind the faster or slower nucleotide excision repair in those mutants, we conducted MNase accessibility assay. MNase is a micrococcal nuclease that cleaves DNA in the linker region connecting two nucleosomes (Chung, Dunkel et al. 2010). This assay is an indication of the state of chromatin at global level. To study the effect of the mutations on chromatin state we used unirradiated cells and to examine the chromatin state in the early event of repair we did a 1-hour repair incubation after UV irradiation. MNase digestion produces more digested material in the histone H4 mutants compared to that in the wild type YBL574 strain, indicating that chromatin becomes open in those mutants (Figure 10, Table 3). So the faster level of nucleotide excision repair in those mutants could be due to the open state of chromatin, which helps recruit nucleotide excision repair machinery to the damaged DNA. The MNase accessibility assay explains the faster nucleotide excision repair in the H4 mutants except mutant H75E.

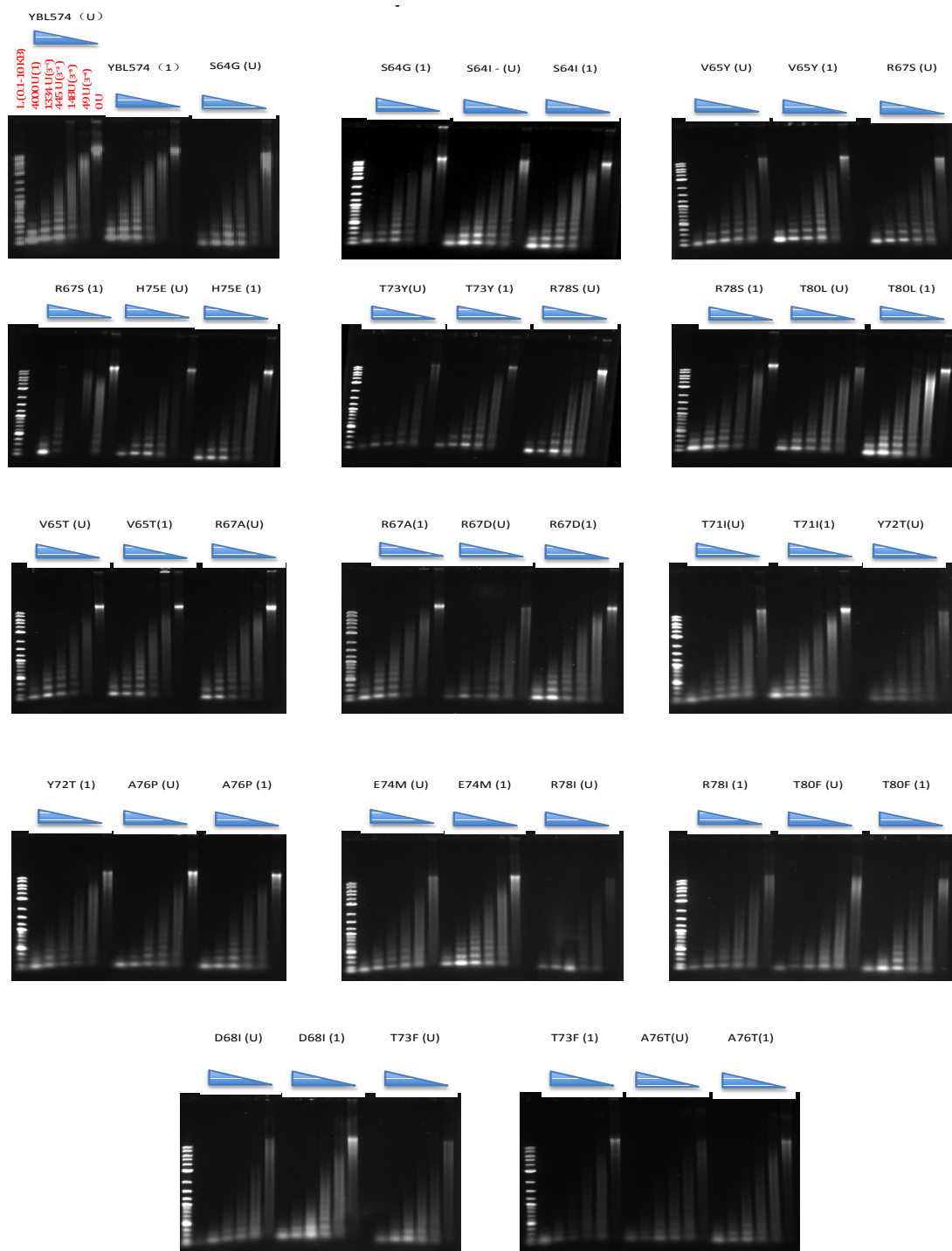


Figure 10. MNase accessibility assay of the histone H4 mutants. All samples were treated with decreasing amount of MNase as shown in the red panel. In parentheses, U denotes unirradiated cells and 1 denotes 1 hour of incubation after irradiation.

Table 3. MNase accessibility assay results for the 24 selected mutants

Histone H4 mutations	Chromatin state compared to wild type strain	Histone H4 mutations	Chromatin state compared to wild type strain
S64G	Open	T73D	Open
S64I	Open	T73F	Open
V65T	Open	T73Y	Open
V65Y	Open	E74M	Open
R67A	Open	H75E	Open
R67D	Open	A76P	Open
R67S	Open	A76T	Open
R67V	Open	R78I	Open
D68I	Open	R78S	Open
D68Y	Open	T80F	Open
T71I	Open	T80I	Open
Y72T	Open	T80L	Open

Rad14 and Rad52 Epistasis Analysis and MMS Sensitivity Test Suggest that the UV Sensitive Histone H4 Mutants Could Play Role in Homologous Recombination Repair Pathway

Previous reports have suggested that UV-induced DNA damages are repaired by nucleotide excision repair, homologous recombination and post-replication repair pathways (Nguyen and Zhang 2011, Rastogi, Richa et al. 2010). Nucleotide excision repair proficiency in most of the H4 mutants suggests that UV sensitivity is not due to nucleotide excision repair deficiency. These mutants could impair some other DNA repair pathways, including homologous recombination and post-replication repair pathways. To investigate the role of the mutants other than the nucleotide excision repair pathway, we conducted epistasis analysis of the histone mutants with Rad14. Rad 14 is a homolog of human XPA that binds damaged DNA and proteins in a pre-incision complex. Rad14 is absolutely required for nucleotide excision repair (Prakash and Prakash 2000). We found that most of the sensitive mutants were more sensitive than Rad 14 cells suggesting that the mutants are on pathways other than nucleotide excision repair. The mutant H4H75E is epistatic to Rad14, suggesting that the mutation causes a defect in nucleotide excision repair pathway (Table 4, Figure 11).

Table 4. Epistasis analysis of the UV-sensitive histone H4 mutants with Rad14

Histone H4 mutations	UV sensitivity (rad14-H4 mutations)
D68I	Sensitive: 10x
T73D	Sensitive: 3-5x
T73F	Sensitive: 10x
T73Y	Sensitive: 5x
H75E	Similar to rad14
A76T	Sensitive: 10x
R78S	Sensitive: 100x
T80F	Sensitive: 10x
T80L	Sensitive: 10x

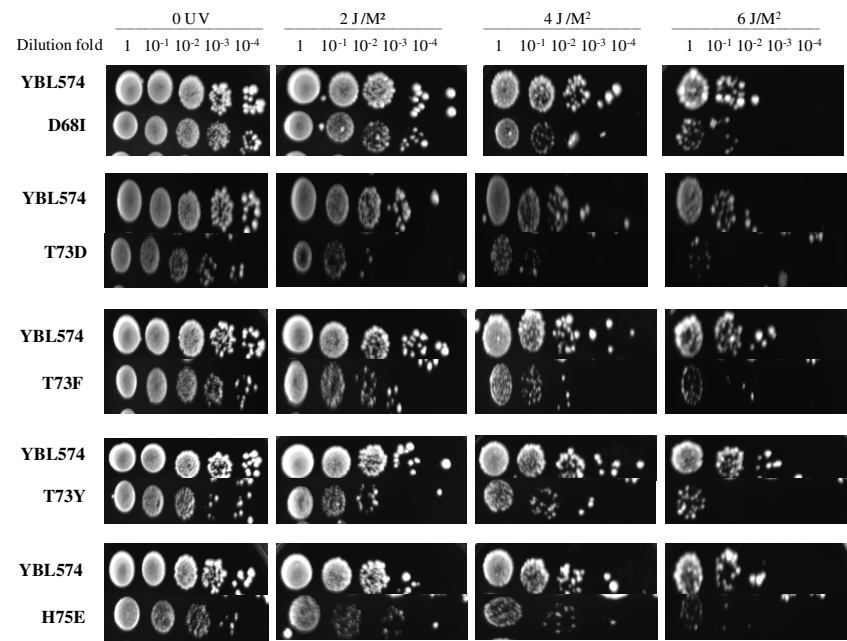


Figure 11. Epistasis analysis of the histone mutants with Rad14. Spot plate assay showing the UV sensitivities of YBL574 wild type and histone H4 mutant yeast cells in rad14 background.

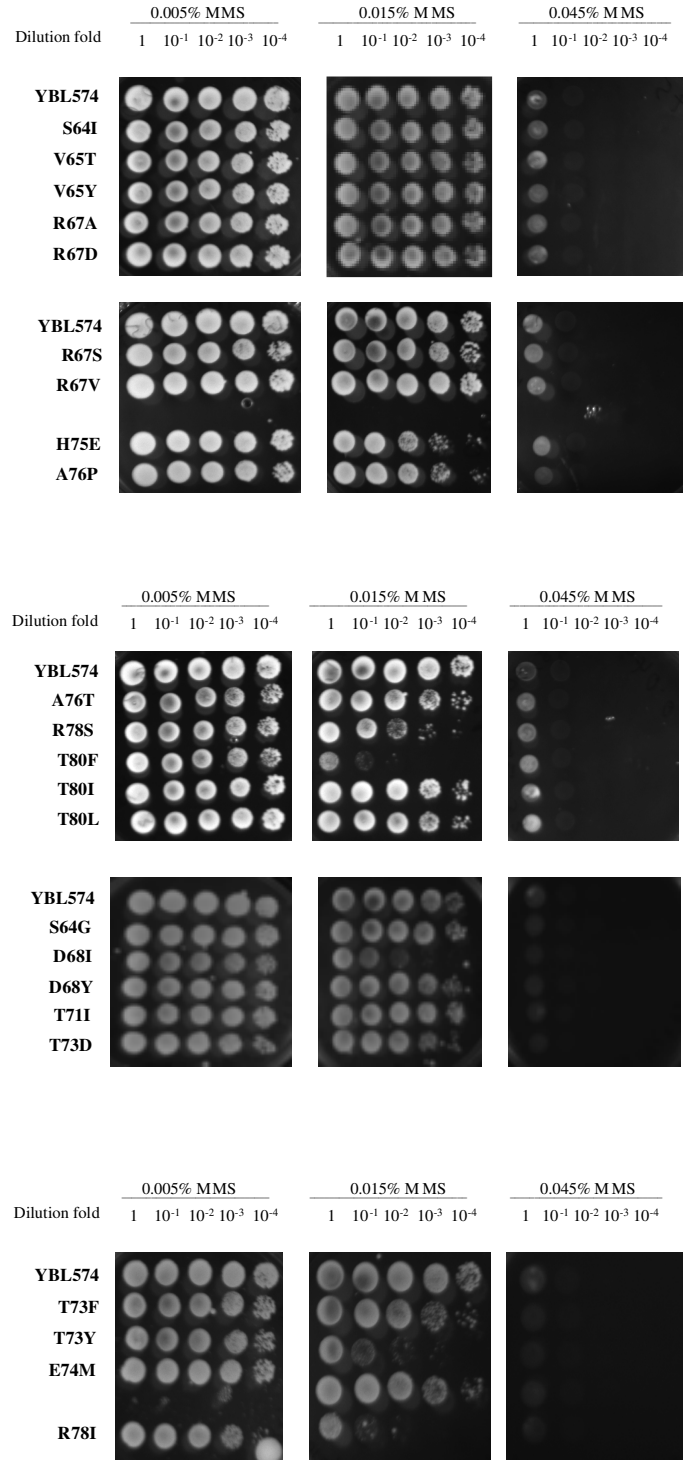


Figure 12. MMS sensitivity test result of the histone H4 mutants. Spot plate assay showing the MMS sensitivities of YBL574 wild type and histone H4 mutant yeast cells.

Table 5. MMS sensitivity assay results for UV-sensitive histone H4 mutants

Histone H4 mutations	MMS sensitivity
D68I	100X sensitive
T73D	2X sensitive
T73F	5X sensitive
T73Y	1000X sensitive
H75E	10X sensitive
A76P	10X sensitive
A76T	3X sensitive
R78I	1000X sensitive
R78S	100X sensitive
T80F	1000X sensitive
T80L	3X sensitive

Table 6. Epistasis analysis of the selected histone H4 mutants with Rad52

Histone H4 mutations	UV sensitivity (rad52-H4 mutations)	MMS sensitivity (rad52-H4 mutations)
D68I	Similar to rad52	Similar to rad52
T73D	Similar to rad52	Similar to rad52
T73F	Similar to rad52	Similar to rad52
T73Y	Sensitive: 5x	Sensitive: 5x
H75E	Slightly sensitive than rad52	Similar to rad52
A76T	Sensitive: 10x	Similar to rad52
R78I	Sensitive: 10x	Similar to rad52
R78S	Similar to rad52	Similar to rad52
T80F	Similar to rad52	Similar to rad52
T80L	Similar to rad52	Similar to rad52

To further analyze the role of mutants in homologous recombination, we conducted methyl methane sulfonate (MMS) sensitivity assay. MMS is a DNA alkylating agent, which methylates DNA bases. The three pathways responsible for the removal of MMS-induced DNA damages are recombination repair, post-replication repair and base excision repair (Chang, Bellaoui et al. 2002). We found that most of the UV sensitive mutants were sensitive to MMS suggesting that they could be in homologous recombination pathway (Table 5, Figure 12). We performed an epistasis analysis of the UV-sensitive histone H4 mutants with

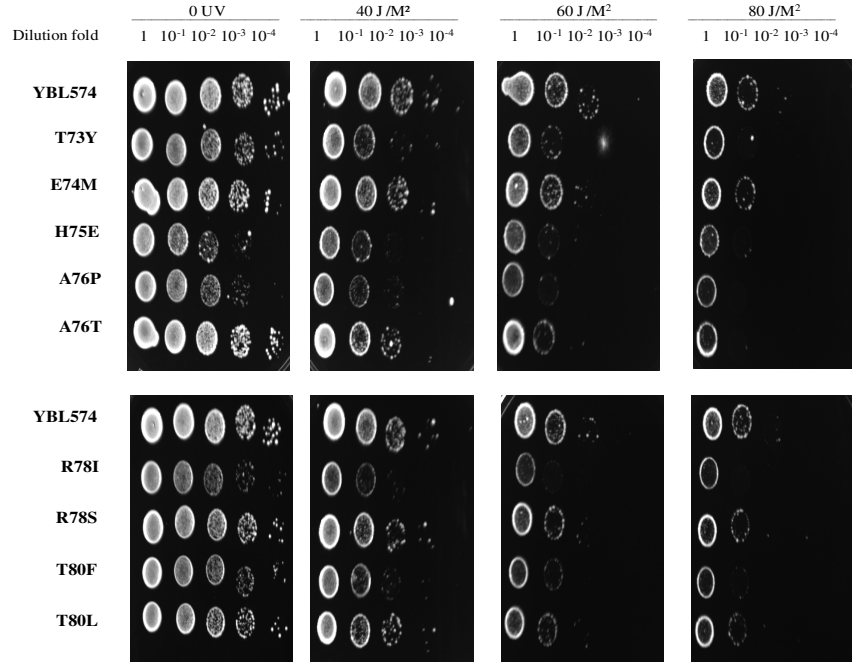


Figure 13 (A). Epistasis analysis of the histone mutants with Rad52. Spot plate assay showing the UV sensitivities of YBL574 wild type and histone H4 mutant yeast cells in rad52 background.

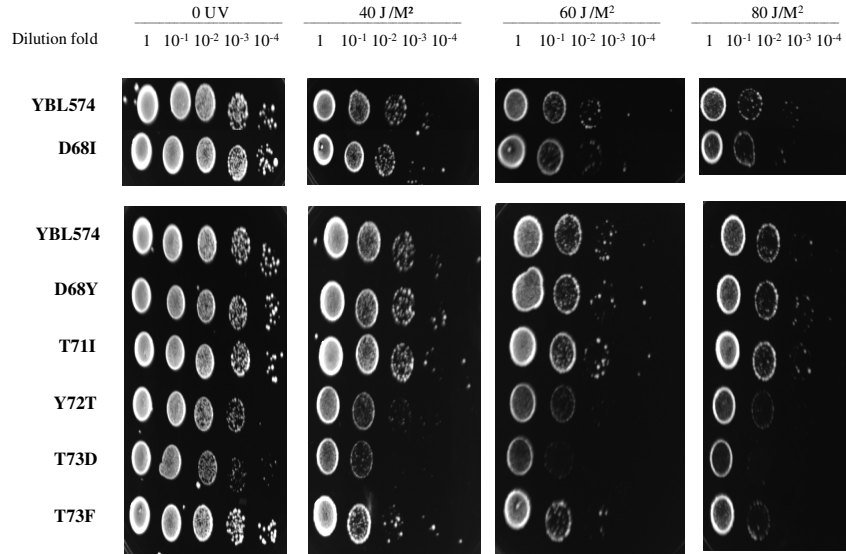


Figure 13 (B). Epistasis analysis of the histone mutants with Rad52. Spot plate assay showing the UV sensitivities of YBL574 wild type and histone H4 mutant yeast cells in rad52 background.

Rad52. Rad52 is a homologous recombination mediator protein, which is the most essential gene in budding yeasts for homologous recombination (Mehta and Haber 2014, Altmannova et al. 2012). Most of the sensitive mutants were epistatic to Rad52, suggesting that these mutants could play role in homologous recombination pathway. T73Y mutation is more UV and MMS sensitive and the mutations A76T and R78I are more UV sensitive than Rad52 cells, suggesting their role in pathways other than homologous recombination. (Table 6, Figure 13).

CHAPTER 5

DISCUSSION

In this study, we found that histone H4 mutations of the residues 64 to 72 are more resistant to UV, whereas mutations of the residues 73 to 80 are more sensitive to UV. We also showed that histone H4 mutations of the residues serine 64 to threonine 80 make nucleotide excision repair more proficient and that one mutation (i.e. H75E) makes nucleotide excision repair defective. Limited literature is available regarding the role of the histone mutations in DNA repair. Recently, researchers conducted a functional study of all the core histone proteins in yeast (Dai, Hyland et al. 2008). They systematically substituted each residue with alanine and changed all alanine residues to serine. They tested the DNA damage response in the presence of UV irradiation, methyl methane sulfonate (MMS), hydroxyurea (HU) and camptothecin (CPT). According to their study, the residues serine 64 to threonine 80 substitutions were not sensitive to UV. Two mutations R67A and T73D overlapped with our study. We found that R67A is ten times more resistant to UV and T73D is four times more sensitive to UV than that of wild type. This could be due to the differences in the strain. They found that two mutations (i.e. Y72F and T80A) are sensitive to MMS (Dai, Hyland et al. 2008). But we did not consider these two mutations in our study as we selected the mutants on the basis of UV sensitivity.

Histone H4 residues T73D, T73F and T73Y alter H3K79 mono-, di-, and trimethylation states and these mutations are sensitive to UV but have faster or normal nucleotide excision repair. S. Chaudhury et al. (2009) found that H3K79R mutation in *Saccharomyces cerevisiae* has normal nucleotide excision repair in the constitutively expressed RPB2 and transcriptionally repressed Gal10 gene (Chaudhuri, Wyrick et al. 2009). This agrees with our study. Evans et al. (2008) analyzed four histone mutants, H3L70S, H3E73D, H3Q76R, and H3T80A, which are H3K79 neighboring residues. They found that these mutations are sensitive to UV and that each of the mutation has an effect on H3K79

methylation states. These mutations act through a distinct subset of DNA damage response pathways including nucleotide excision repair, checkpoint activation, post-replication repair and recombinational repair (Evans, Bostelman et al. 2008). Their result agrees with ours that H3K79 methylation-deficient mutants are sensitive to UV. Our study makes clear that H3K79 methylation-deficient mutants do not impair nucleotide excision repair. Future research should study the role of the mutants in homologous recombination, post-replication repair and checkpoint responses to reach a conclusion.

In this study, micrococcal nuclease accessibility assay indicated that the global state of chromatin becomes open in histone mutants. Previous studies have also shown that histone mutations affect chromatin state. Nucleosome remodeling after DNA damage permits the entrance of TFIIH, XPC, and other nucleotide excision repair factors to remove damaged strands and initiate gap filling DNA synthesis and ligation reactions (Ura, Araki et al. 2001, Hara and Sancar 2003, Gong, Fahy et al. 2006, Teng, Liu et al. 2008, Zhao, Wang et al. 2009). A single amino acid change in histone H4R45C (a sin mutant) alters the chromatin landscape, which may influence the accessibility of DNA repair factors. The mutant yeast cells are more resistant to killing by UV and have a higher rate of nucleotide excision repair (Nag, Gong et al. 2008). In our histone H4 mutant strains we also observed an open chromatin state that might lead to efficient nucleotide excision repair.

We wanted to determine whether the H4 mutants affect the expression and recruitment of nucleotide excision repair factor(s) to the chromatin, which lead to increased or decreased nucleotide excision repair. To examine the expression and recruitment of nucleotide excision repair proteins in those mutants, we added three consecutive Flag tags to the Rad1, Rad2, Rad3, Rad4, Rad7, Rad10, Rad14, Rad16, Rad23 and Elc1 genes in the YBL574 strain. It is necessary to analyze the expression level of the proteins with the mutant

backgrounds. It is expected to see a higher or lower level of expression of those nucleotide excision repair factors in the mutants. If the expression of the proteins is higher in the mutants with faster nucleotide excision repair, one can conclude that the faster nucleotide excision repair is due to increased expression of the nucleotide excision repair proteins. If the expression of one or more protein(s) in mutants with slower nucleotide excision repair is lower, one can conclude that the slower nucleotide excision repair is due to the reduced expression of nucleotide excision repair protein(s). If the level of nucleotide excision repair protein expression in the mutants with faster nucleotide excision repair is normal, one can conclude that faster nucleotide excision repair is due to the open state of chromatin, which helps to recruit more nucleotide excision repair factors to the damaged DNA to facilitate efficient repair.

In the mutant H4H75E, we noted slower global genomic repair and Rad26-independent transcription-coupled repair. In this mutant, the global state of chromatin is open but the nucleotide excision repair is slower which lead us to speculate that the expression or recruitment of one or more nucleotide excision repair factor(s) is lower. A reduced level of expression of one or more nucleotide excision repair protein(s) can explain the mechanism. Alternatively, if future researchers observe the normal expression of nucleotide excision repair proteins, they can examine the recruitment of Rad4, Rad14 and Rad16 proteins to the chromatin. These three proteins are unique to nucleotide excision repair and mediate three important steps in the nucleotide excision repair process.

Saccharomyces cerevisiae has a few heterochromatin regions of the genome: silent mating type loci (HML and HMR), rDNA (encoding ribosomal RNA), and sub-telomeric regions. In those heterochromatin regions, global genomic repair mainly fixes UV-induced DNA damages (Struhl 2007). It might be necessary to characterize the state of chromatin in

one of the heterochromatin regions. We created a probe specific for HML α locus to examine the chromatin state at HML α locus using southern blotting. The state of chromatin in this heterochromatin region will give a broader idea about the effect of the mutants in heterochromatin.

To directly analyze homologous recombination, we used a system of continuous HO endonuclease induction in a mating type locus. In yeast, the two mating types are MAT α and MAT a . The haploid yeast strain has either MAT α or MAT a . HO endonuclease is expressed during cell division and causes a double strand break in the MAT α or MAT a gene. Homologous sequences HML α and HMR α are present in the same chromosome. By homologous recombination MAT α is converted to MAT a and MAT a is converted to MAT α . This phenomenon is known as mating type switching (Haber 2012). We performed spot plate assay to see the role of the mutants in homologous recombination and cell survival in the YBL574 strain. We used a single copy plasmid pGAL-HO where the HO gene is under the control of an inducible Gal 1-10 promoter for continuous HO induction. We could see sensitivity in the UV and MMS-sensitive H4 mutants (data not shown). To better analyze the homologous recombination, we constructed WY121 strain with a multicopy plasmid pESC-HO. In WY121 strain, there are genomic deletion of the HHF1-HHT1 and HHT2-HHF2 genes and complementation with a plasmid pJL001 containing the HHT2-HHF2 genes. Spot plate assay might be done in WY121 strain to see the effect of the mutations in repairing double strand breaks and cell survival. Then in the sensitive mutants, direct analysis of double strand breaks repair in the MAT locus using Southern blotting might be carried out.

CHAPTER 6

SUMMARY AND CONCLUSIONS

We identified 24 UV-sensitive or UV-resistant histone H4 mutations surrounding histone H3K79. In 22 mutations, we observed faster or normal nucleotide excision repair. In one mutation (i.e. Y72T), we noted defective transcription coupled repair and in another mutation (i.e. H75E), defective global genomic repair and Rad26-independent transcription-coupled repair. Three mutations, T73D, T73F and T73Y, altered H3K79 methylation but they had normal or faster level of nucleotide excision repair. Increased chromatin accessibility may explain the faster nucleotide excision repair in the H4 mutants. Faster nucleotide excision repair in the UV sensitive mutants suggest their role in pathways other than nucleotide excision repair. Preliminary studies have indicated that the UV-sensitive mutants could be in homologous recombination repair pathway. In this study, histone H4 mutation H75E blocked the global genomic repair and Rad26-independent transcription-coupled repair pathway. This is the first report of a single histone residue mutation can block nucleotide excision repair. Further study is necessary to elucidate the expression and recruitment of nucleotide excision repair proteins to the mutant backgrounds. Repair analysis of the role of the mutations in homologous recombination and post replication repair might explain the UV sensitivity of the histone H4 mutants.

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VITA

Sheikh Arafatur Rahman was born in Dhaka, Bangladesh in 1981. After completing higher secondary school, he started college at Bangladesh Agricultural University, in the Faculty of Veterinary Science, Bangladesh. He received a Doctor of Veterinary Medicine degree in 2005 and a Master of Science degree in 2007 from the same institution. After graduation, he began working as a lecturer in the Department of Pathology and Parasitology, in the Faculty of Veterinary and Animal Science, at Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh in 2009. In 2012, he joined the Department of Pathobiology, in the Faculty of Veterinary Medicine and Animal Science, at Bangabandhu Sheikh Mujibur Rahman Agricultural University, in Gazipur, Bangladesh as a lecturer. In August 2012, he started graduate study in the Department of Comparative Biomedical Sciences, at Louisiana State University under the supervision of Dr. Shisheng Li. He studied the role of histone H4 mutations in DNA repair in *Saccharomyces cerevisiae*.