Histone H3 Lys79 methylation is required for efficient nucleotide excision repair in a silenced locus of *Saccharomyces cerevisiae*

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ABSTRACT

Methylation of specific histone lysine residues regulates gene expression and heterochromatin function, but little is known about its role in DNA repair. To examine how changes in conserved methylated residues of histone H3 affect nucleotide excision repair (NER), viable H3K4R and H3K79R mutants were generated in *Saccharomyces cerevisiae*. These mutants show decreased UV survival and impaired NER at the transcriptionally silent *HML* locus, while maintaining normal NER in the constitutively expressed *RPB2* gene and transcriptionally repressed, nucleosome loaded *GAL10* gene. Moreover, the *HML* chromatin in these mutants has reduced accessibility to *Micrococcal* nuclease (MNase). Importantly, chromatin immunoprecipitation analysis demonstrates there is enhanced recruitment of the Sir complex at the *HML* locus of these mutants, and deletion of the *SIR2* or *SIR3* genes restores the MNase accessibility and DNA repair efficiency at this locus. Furthermore, following UV irradiation expression of NER genes in these mutants remains at *wild type* levels, with the exception of *RAD16* which decreases by more than 2-fold. These results indicate that impaired NER occurs in the silenced chromatin of H3K79R and H3K4,79R mutants as a result of increased binding of Sir complexes, which may reduce DNA lesion accessibility to repair enzymes.

INTRODUCTION

In eukaryotic cells, chromatin acts as the 'platform' for various nuclear processes including replication, recombination, repair and transcription. The dynamic structure of chromatin and its ability to adopt numerous conformations acts as a control mechanism for different processes that target DNA. The fundamental unit of chromatin is the nucleosome which is comprised of a nucleoprotein core of 147 bp of DNA wrapped 1.65 times around an octamer of the core histones H2A, H2B, H3 and H4 (1). These nucleosome core particles are further assembled into arrays of oligonucleosomes to give the increasingly compact structural hierarchy of chromatin, which is inhibitory to protein factors that interact with DNA. Based on the compaction and accessibility to nuclear machinery, the eukaryotic genome is organised into active regions known as euchromatin and inactive regions known as heterochromatin. Unlike higher eukaryotes, the majority of chromatin in budding yeast, *Saccharomyces cerevisiae*, is euchromatic in nature. Only a few regions, such as telomeres, the silent mating type loci (HM), and ribosomal RNA genes (rDNA), are packaged into transcriptionally silent heterochromatin-like structures (2). Formation of silent chromatin at HM loci and telomeres is governed by the binding of silencing proteins Sir2, Sir3 and Sir4 (which form the Sir complex) to specific DNA sequences (called silencers) (2,3)

Packaging of chromatin into silenced regions is also affected by post-translational modification of histone tails, including acetylation and methylation of the ε-amino groups of specific lysine residues (4). It has been observed that lysines in the tails of histone H3 and H4 are generally 'hyperactelyated' in active chromatin and 'hypoacetylated' in silenced chromatin, the latter facilitating binding of the Sir complex. Of particular importance is H4K16, which is the direct target of Sir2-mediated deacetylation (2,5). Apart from acetylation, Set1 and Dot1 (histone methyltransferases) methylate histone H3 at K4 and K79, respectively, and this 'signature' is important for preventing heterochromatin formation (6–9). Indeed, methylation of H3K4 and H3K79 is extremely low in heterochromatin, and it has been suggested that Sir

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proteins predominantly associate with nucleosomes that are hypomethylated at H3K79 (10). In addition, formation of the Sir complex inhibits methylation of K79 by Dot1 (9,10).

Nucleotide excision repair plays a key role in removing bulky, helix distorting DNA damage such as DNA photoproducts produced by UV-light (11–13). It appears that the rate-limiting step in nucleotide excision repair (NER) is recognition of DNA lesions in different chromatin 'landscapes'. Several *in vitro* studies have demonstrated that chromatin is a barrier for efficient repair of various NER substrates including UV-induced DNA lesions (14–16). *In vivo*, however, such lesions are efficiently repaired despite being assembled into nucleosomes because of alterations in nucleosome structure during NER. A popular model for NER in chromatin is the 'access-repair-restore' model, which proposes that chromatin structure is altered during repair thereby exposing damage sites to repair factors (17,18). Following repair, the site is restored to its original state (17). Recent studies have indicated that modifications in histones assist in the recognition and accessibility of DNA repair sites (14,15,19). For example, histone H3 is hyperacetylated *in vivo* following UV irradiation, which may aid in damage recognition and NER (20). Furthermore, the human Gcn-5 containing HAT (histone acetyltransferase) complex TFTC has been shown to contain a DDB1 like protein, SAP130 (21). The DDB1 protein is a component of the UV-DDB heterodimer, which binds to UV lesions *in vivo* (17,21).

Unlike histone acetylation, histone methylation does not appear to be induced by DNA damage (22). However, methylation of histones appears to play a role in checkpoint control and methylated histones interact with checkpoint proteins following DNA damage (19,23). For example, the checkpoint protein in *Schizosaccharomyces pombe* Crb2, and its human homolog 53BP1, interacts with methylated histone H4K20 following ionizing radiation-induced DNA damage (22,24,25). Although p53BP1 also has an affinity for methylated H3K79 (22), the biological significance of this interaction remains to be determined. Similar observations were made in budding yeast where Dot1p, which methylates H3K79, is required for the activation of rad53 checkpoint control following UV damage (26). In addition, a recent study shows that Dot1 null mutants and H3K79 point mutants are sensitive to UV radiation (27). Finally, epitasis analysis between dot1 and various UV repair genes indicates that H3K79 methylation plays overlapping roles in NER, post replication repair, and Rad9-mediated checkpoint function (27).

Since histone proteins can undergo a number of different modifications at various residues, it is unclear how these modifications work in concert in the cell. Therefore, to examine the role of these modifications in DNA repair in *intact* cells, we have begun exploring site-specific mutation of histone modification sites in yeast chromatin that preserve cell viability. In the present study, we examined how histone methylation in both the core domain and tail region affect DNA repair of UV-induced cyclobutane pyrimidine dimers (CPDs).

The following histone H3 methylation mutants were used: H3 K4R, H3K79R and H3K4,79R where lysines 4, 79 or 4 and 79, respectively, are mutated to an arginine. Our results indicate that change at the methylation site in the core domain of histone H3 alters cell survivability following UV irradiation and impairs the rate of CPD removal from transcriptionally silent loci by enhancing binding of the Sir complex. In contrast, change in the methylation site in the tail domain of histone H3 alone does not exert an effect of similar magnitude on the UV damage sensitivity of the cell.

MATERIALS AND METHODS

Cell growth and UV irradiation

For DNA repair studies, yeast cells were grown in YPD medium at 30°C until early log phase (A_{600} ~0.6), harvested, washed and resuspended in ice-cold PBS (phosphate buffered saline). The resuspended cells were irradiated at $100 \, \text{J/m}^2$ UV light (254 nm), measured with a Spectroline DM-254N short wave ultraviolet meter (Spectronics Corp.), and incubated in pre-warmed YPD medium in the dark at 30°C. After various time periods, cells were harvested and the DNA isolated using the glass-bead method described previously (28). For UV sensitivity assays, cells were diluted to different concentrations, spread onto YPD-containing agarose plates and irradiated with different UV doses, measured as described above. Colonies were counted after 48 h of incubation in the dark at 30°C.

Locus-specific repair

Gene-specific repair analyses at the *HML*, *RPB2* and *GAL10* chromatin loci were performed as described in Nag *et al.* (29). Briefly, equal amounts of restriction-digested DNA were treated with or without a saturating concentration of T4 endonuclease V for 120 min at 37°C. The samples were then electrophoresed on 1% alkaline agaorse gels, transferred to Hybond N^+ membranes (Amersham), and hybridized with gene specific ($\alpha^{32}P$)dATP-labeled DNA probes. The number of CPDs in these restriction fragments was determined by methods using Poisson analysis, as described in detail elsewhere (30,31). The level of repair was then calculated as a function of the number of CPDs remaining per fragment (32).

RT–PCR and western blot

Cells were grown to early log phase (A_{600} ~0.6) under the same conditions as for the repair experiments. Total RNA was isolated from each sample as described previously (28), and 5 µg of RNA was reverse transcribed using Superscript III RT enzyme (Invitrogen), as per manufacturer's instructions. The resultant cDNA was PCR amplified for 30 cycle using primers specific for the *HMLα1*, *RPB2*, *GAL10* gene and the NER genes listed in Supplementary Table 1. The mRNA levels of the *ACT1* gene were used as a loading control.

For western blot, total cellular protein was isolated using a trichloroacetic acid precipitation method as

described previously (33). Both anti-Sir2 (Santa Cruz Biotechnology, sc-6666) and anti-tubulin antibodies (Abcam, ab-6160) were used.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described by Kuo and Allis (34). Mid-log phase yeast cells were crosslinked by 1% formaldehyde, lysed in buffer (50 mM Hepes–KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin A) using glass beads (425–600 µm, Sigma), followed by sonication. Aliquots of 500 µg of protein from each sample were immunoprecipitated with 10 µl of anti-Sir2 antibody (Santa Cruz: sc-6666) overnight at 4°C. The immunoprecipitated complex was purified using Protein A sepharose beads (50% slurry). Chromatin was then eluted from the beads with elution buffer (1% SDS, 0.1 M NaHCO$_3$) and crosslinks reversed by incubation at 65°C overnight. The *HML* locus was amplified using the primer sets 5′-AGTTTTCGGCACGGACTTATTTGG-3′ and 5′-TCGTCTAATACAAGTTTGAATGACG-3′ for the *HML-E* silencer region and 5′-GATGCAATTTATT GCTTCCC-3′ and 5′-CATATTGTGAATGTCGTC-3′ for the fragment adjacent to the *HMLα2* transcription start site.

Chromatin accessibility assay

Micrococcal nuclease digestion was performed using methods described previously (35,36) with modifications described in Nag *et al.* (29). Briefly, spheroplast were isolated from mid-log phase ($\sim 1.0 \times 10^7$ to 2.0×10^7 cells/ml) cells using Zymolyase. The spheroplasts were suspended in spheroplast digestion buffer [1 M Sorbitol, 50 mM NaCl, 10 mM Tris–HCl, pH 7.5, 5 mM MgCl$_2$, 1 mM CaCl$_2$, 1 mM β-mercaptoethanol, 0.5 mM spermidine and 0.075% (v/v) NP-40]. 200 µl aliquots of spheroplasts were digested with varying concentrations of micrococcal nuclease (MNase) (Worthington) for 10 min at 37°C. The reactions were terminated with 0.1 X vol of stop solution (5% SDS, 250 mM EDTA) followed by Proteinase-K (50 µg/ml) treatment for 2 h at 55°C. After isolation, the DNA was electrophoresed on 1.2% agarose gels, transferred to Hybond N$^+$ membranes via the alkaline transfer method and hybridized to probes specific for either the *HML* locus (i.e. 518 bp probe to the *HMLα1* ORF, and 900 bp probe to the *HMLα1* promoter and *HMLα2* ORF region) or *RPB2* gene. The blots were quantified using ImageQuant 5.2 software and expressed as the ratio of mono- to tri-nucleosome as a function of MNase concentration.

RESULTS

Histone H3K79 mutants are more sensitive to UV irradiation

Histone methylation mutants H3K4R, H3K79R and H3K4,79R were generated by plasmid shuffling into the wild-type (*wt*) yeast strain WY121 (37,38). Both H3K79R and H3K4,79R mutants show enhanced sensitivity to UV-irradiation, compared to *wt* cells (Figure 1),
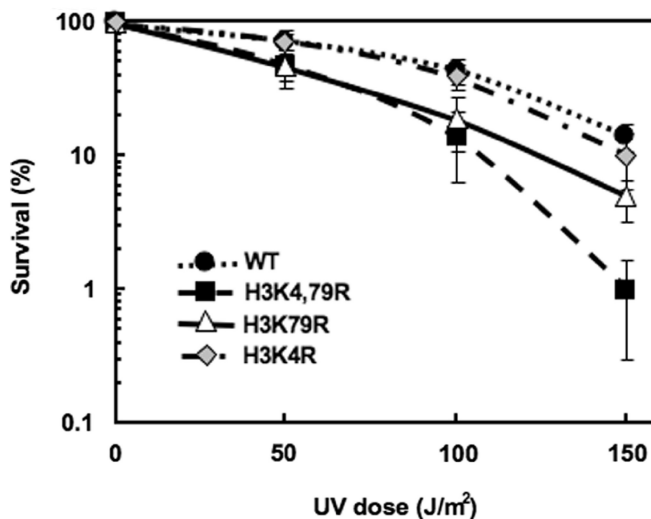

Figure 1. H3K79R and H3K4,79R cells are more sensitive to UV radiation than *wt* cells. Cells were diluted to appropriate concentrations, spread on YPD plates and irradiated at different UV doses. Colony forming ability following UV radiation was monitored and expressed as percent survival relative to unirradiated cells.

with the double mutant H3K4,79R being the most sensitive. For example, following a UV dose of 150 J/m^2, H3K4,79R cells were over 10 times more sensitive than *wt* cells, while H3K79R cells were about 3 times more sensitive and H3K4R cells showed almost no sensitivity (Figure 1). As increased UV sensitivity can reflect a deficiency in DNA repair, we examined NER of UV damage to the DNA of H3K79R and H3K4,79R mutants.

Mutation of Histone H3K79 leads to impaired NER in the HML locus

Repair of CPDs was examined in three different chromatin loci in yeast: (i) *HML*, a nucleosome-loaded, transcriptionally silent mating-type locus; (ii) *RPB2*, a constitutively expressed gene encoding the second largest subunit of RNA Pol II and (iii) *GAL10*, a nucleosome loaded inducible gene under transcriptionally repressed conditions. For repair experiments, cells were irradiated with 100 J/m^2 UV light (predominately 254 nm) and incubated for different times following irradiation. As shown in Figure 2, both of the methylated lysine mutants showed a significant decrease in the rate of CPD removal from a 2.3 kb *Bsp*1286I fragment containing the *HML* locus compared to *wt* cells (Figure 2A and B). On the other hand, only a small difference was observed when CPD removal was examined in a 3.4 kb *Nru*I fragment containing the *RPB2* locus (Figure 2C and D). Furthermore, in a 2.2 kb *EcoR*1-*EcoR*V fragment containing the transcriptionally repressed *GAL10* gene, the rate of CPD removal was again almost identical between *wt* and mutant cells (Figure 2E and F). In addition, RT-PCR analysis of the expression of *HMLα*, *GAL10* and *RPB2* genes in the presence of glucose indicates there is no leaky expression of either the *HMLα* or *GAL10* genes of these mutants, while expression of the *RPB2* gene remains high in both *wt* and
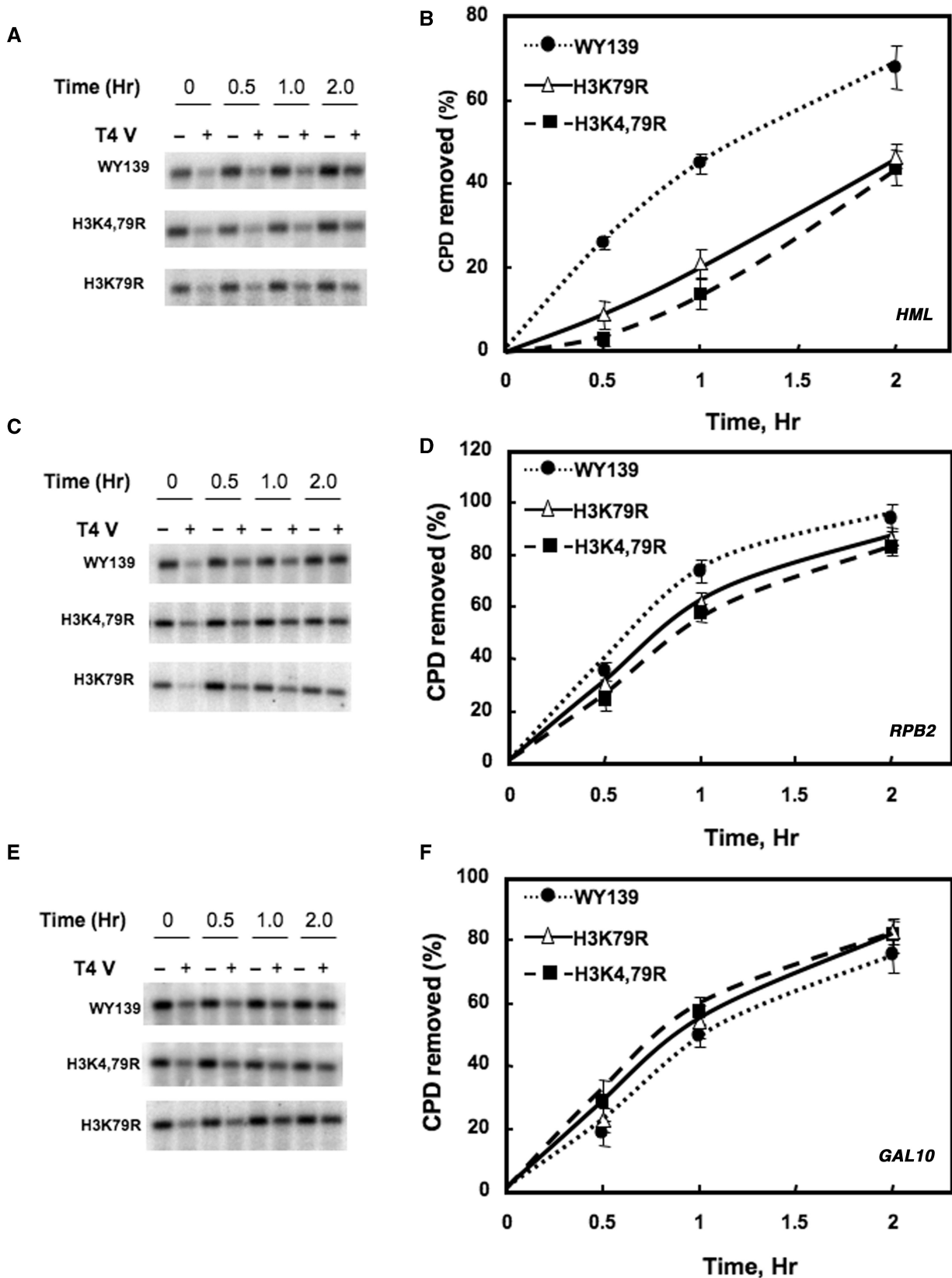
Figure 2. NER of the *HML*, *RPB2* and *GAL10* loci in H3 methylation mutants. Cells were irradiated with $100 \, J/m^2$ UV light and allowed to repair in the dark at 30°C for various times. Genomic DNA was isolated, digested with appropriate restriction enzyme(s) and subjected to T4 endonuclease V digestion. Southern analysis was performed to determine CPD removal using radioactive probes to a 2.3 kb *HML* fragment (**A**), a 3.4 kb *RPB2* fragment (**C**) and a 2.2 kb *GAL10* fragment (**E**). The time course of CPD removal for each strain was plotted for *HML* (**B**), *RPB2* (**D**) and *GAL10* (**F**) as the mean ± 1 SD for three independent experiments.

mutant cells (Figure S1A). This indicates that neither repair nor transcription is altered at the *RPB2* and *GAL10* genes of H3K79R and H3K4,79R cells. Moreover, the enhanced repair of the *HML* locus in *wt* cells, compared to the mutants, is not due to the additional participation of transcription coupled repair (TCR) at these loci in *wt* cells.

Mutation of Histone H3K79 renders chromatin less accessible to micrococcal nuclease

An explanation for the reduced NER efficiency in the *HML* locus in H3K79R cells is that the H3K79 mutation affects the local chromatin structure and reduces the accessibility of nucleosome DNA to repair enzymes. Therefore, we examined the MNase accessibility of both bulk chromatin and chromatin at the *HML*, *RPB2* and *GAL10 loci* of these mutants. Spheroplasts isolated from *wt* and mutant strains were treated with increasing concentrations of MNase and the resulting DNA fragments separated on agarose gels (Figure 3). As shown in Figure 3A and D, chromatin from mutant cells is more resistant to MNase digestion compared to *wt*. However, the bulk DNA banding patterns are similar (Figure 3A, left panel), indicating there is no significant difference in overall nucleosome repeat length between *wt* and mutant cells. Furthermore, Southern blot analysis indicates that the *HML* chromatin of H3K4,79R mutant is also less accessible to MNase than that of *wt* cells (Figure 3A, second panel from left).

Quantitative analysis of the blots indicate that MNase digestion is reduced at the *HML* chromatin locus in the mutant as there is less of an increase in the ratio of mono- to tri-nucleosome formation with increased digestion, compared to *wt* (Figure 3B). In support of this, scans of lanes from equivalent digestion conditions show a relatively high yield of di- and mono-nucleosomes from *wt* chromatin, while higher order nucleosome persist for the *HML* chromatin of the mutant cells (Figure 3C). MNase accessibility at the *RPB2* and *GAL10* loci also shows a difference between *wt* and H3K4,79R mutants, but this difference was less pronounced compared to silenced *HML* chromatin (Figure 3B). Like H3K4,79R, the H3K79R mutant also showed a lower digestion level with MNase and relatively low yield of di- and mono-nucleosome populations at the *HML* locus under different enzyme concentrations compared to *wt* (Figure 3D and E). Again, the difference in MNase accessibility at the *RPB2* and *GAL10* loci of H3K79R is less significant compared to *wt* cells (Figures 3D and S2). This suggests that lysine to arginine mutations of histone H3K4,79 predominantly affects silenced chromatin.

Deletion of Sir2 or Sir3 increases repair of the HML locus

From the above results, it seems likely that nucleosome DNA in the *HML* locus is less accessible to exogenous enzymes in H3K79 mutants. To examine if this is due to enhanced binding of the Sir complex to *HML* nucleosomes, the effect of *Sir2* and *Sir3* deletion on NER was investigated. As shown in Figure 4A and B, deletion of these genes from *wt* and mutant H3K4,79R cells

significantly increases the rate of CPD removal at the *HML* locus. Furthermore, the rate of repair in *Sir* deleted H3K4,79R cells is comparable to *wt* cells having *Sir2* or *Sir3* deleted (Figure 4, compare open symbols in panels A and B). The CPD removal was also measured at the *RPB2* locus in *Sir2* deleted strains. The results show there is no significant difference in the repair rate between *wt* and H3K4,79R, and their corresponding *Sir2* deletion strains (Figure 4C). This suggests that decreased repair at the *HML* locus of H3K4,79R mutant cells is primarily due to enhanced binding of Sir proteins.

The accessibility of nucleosome DNA was also mapped in the *HML* locus of *Sir2Δ* mutants. As shown in Figure 5A, we found that deletion of *Sir2* from both *wt* and H3K4,79R renders *HML* chromatin more accessible to MNase. While a nucleosome ladder consisting of six to seven repeats could be easily detected in *wt* and H3K4,79R chromatin, formation of dinucleosomes is more prominent in the respective *Sir2* deletion strain. Moreover, quantitative analysis of the MNase digestion pattern clearly shows the increase of mono- to tri-nucleosome DNA ratio with increasing concentration of MNase in the *Sir2* deletion strains of both *wt* and H3K4,79R compared to the respective parental strains (Figure 5B). When tested for MNase accessibility in the *RPB2* locus, we observed little change in chromatin structure between *wt* and H3K4,79R, and their corresponding *Sir2* deletion strain (Figure S3B). Moreover, the Sir2 protein level in both *wt* and H3K4,79R cells is comparable (Figure S3A), indicating that the difference in nucleosome organization between these two strains is not due to differences in SIR2 protein levels.

The results discussed above indicate that the suppression of NER at the *HML* locus in H3K4,79R mutants is due to the presence of Sir complexes. To directly explore this possibility, ChIP analysis was performed. As shown in Figure 5C, the amount of Sir2 bound at the *HML*-E silencer is greater in H3K4,79R mutant cells compared to *wt* cells. A small increase in Sir proteins is also observed at the distal *HML* α2 gene suggesting that there may be an increase in spreading of Sir complex at the *HML* locus in the mutant compared to *wt* cells. Chromatin from Sir2 deletion strains were used as control in the ChIP analysis (Figure 5C, right-hand panels).

Loss of H3K4 or H3K79 methylation affects expression of repair genes

Another explanation for the reduced repair efficiency in H3K79 mutants compared to *wt* is there may be a change in the expression of repair genes. To test this possibility, we measured the expression levels of a number of NER genes between H3K4,79R mutant and *wt* cells under normal growth conditions using RT–PCR. As shown in Supplementary Table 1 and Figure 6A, the H3K4,79 mutations do not affect expression of the repair genes tested. However, when these cells were subjected to UV irradiation, we found that among these NER genes, there is an ~50% *decrease* in the expression of *RAD16* in H3K4,79R cells rather than the ~2-fold *increase* observed in *wt* cells (Figure 6B).
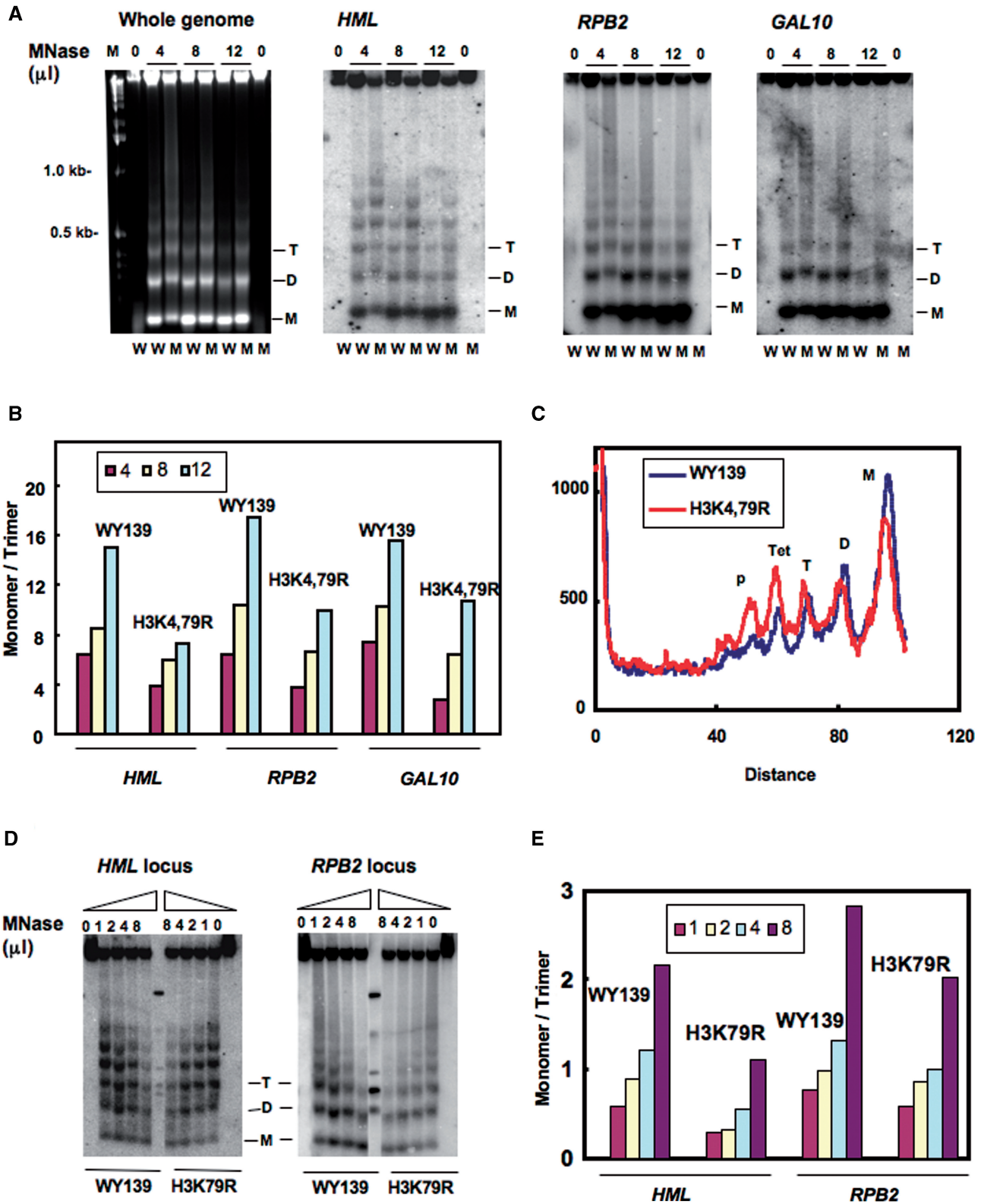
Figure 3. Nucleosome DNA is less accessible to MNase in H3 methylation mutants. Spheroplasts were isolated from WY139 (*wt*) and methylation mutants, treated with different concentrations of MNase (10 U/μl stock solution) and genomic DNA was isolated, electrophoresed on agarose gels, stained with ethidium bromide, blotted and hybridized with a probe specific for the *HMLα1*, *RPB2* and *GAL10* ORF. (**A**) MNase digestion patterns are shown for both bulk chromatin, *HML*, *RPB2* and *GAL10* chromatin. W: WY139 and M: H3K4,79R. (**B**) Quantitative analysis of MNase accessibility at the three loci. Data is expressed as the ratio of mono- to tri-nucleosome signal at different concentrations of MNase. (**C**) Comparative scans of the 4 μl MNase lanes for the *HML* locus in WY139 and mutant H3K4,79R cells. (**D**) MNase digestion patterns for *HML* and *RPB2* chromatin of WY139 and H3K79R. (**E**) Quantitative analysis of MNase accessibility at *HML* and *RPB2*. Data is expressed as the ratio of mono- to tri-nucleosome formation at different concentration of MNase.
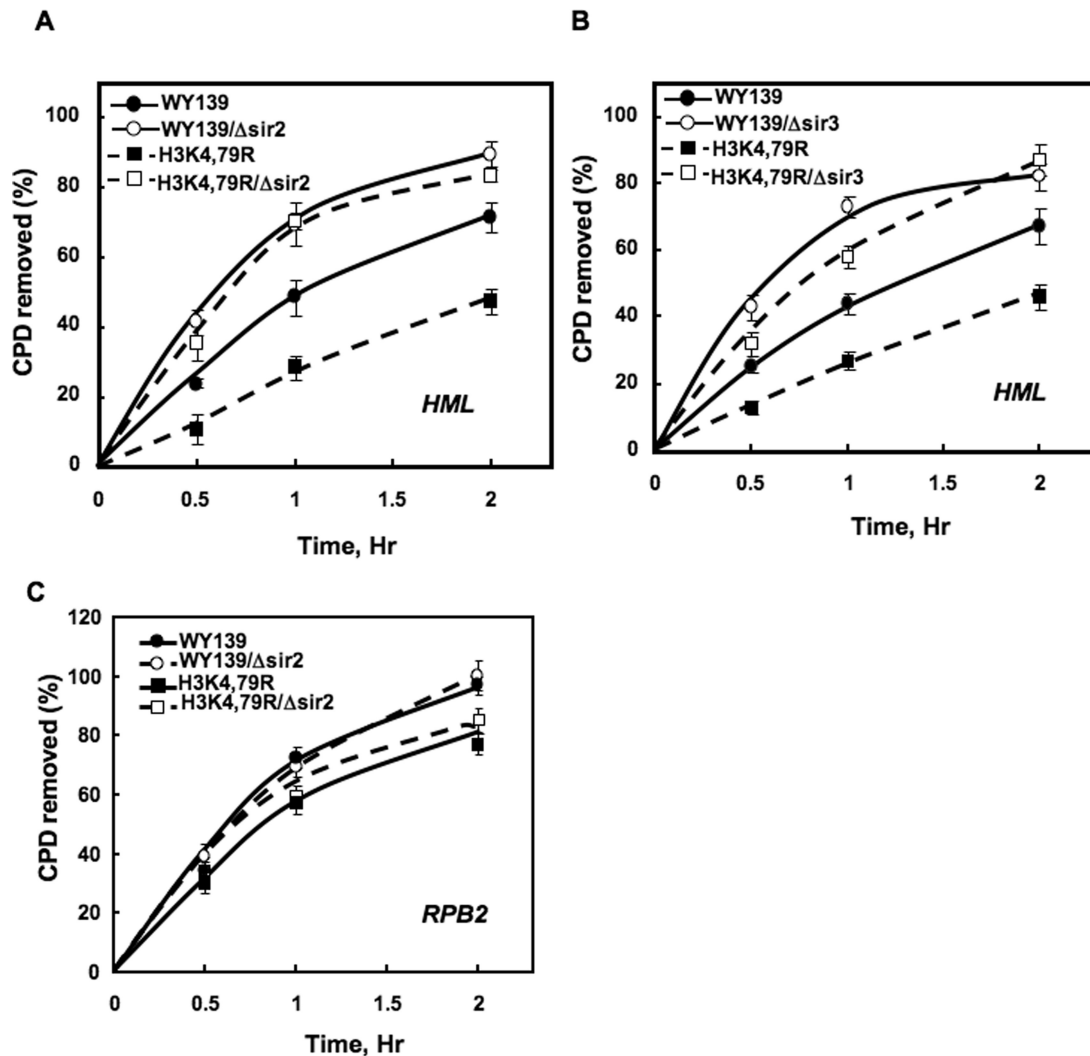
Figure 4. Deletion of *SIR* genes increases repair in the H3K4,79R mutant. The *Sir2* and *Sir3* genes were deleted from WY139 (*wt*) and H3K4,79R mutants using gene replacement, and CPD removal was measured at the *HML* and *RPB2* loci after exposure to $100 \, J/m^2$ UV light. The time courses of CPD removal are shown for the 2.3 kb *HML* fragment for *Sir2* and *Sir3* deletion mutants in (**A**) and (**B**), respectively, and the time course of CPD removal from the 3.4 kb *RPB2* fragment for the *Sir2* deletion mutant is shown in (**C**). Data represent the mean $\pm 1 \, SD$ for three independent experiments.

DISCUSSION

In eukaryotes, the packaging of DNA in chromatin not only governs DNA damage formation but also restricts DNA repair machinery from accessing these damage sites (12,13). The accessibility to damaged DNA can be achieved by altering histone–histone or histone–DNA interactions in chromatin through the action of chromatin remodelers or histone modification enzymes (39). In the present study, we have focused on methylation of two conserved lysine residues, K4 and K79, which are located in the tail and histone fold domain, respectively, of histone H3. These residues were mutated to arginine and the mutants were tested for both UV sensitivity and NER efficiency. The H3K79 mutants were found to be more sensitive to UV irradiation compared to *wt* cells (Figure 1), while H3K4R mutants showed nearly the same sensitivity as *wt* (at least for UV doses $\leq 150 \, J/m^2$).

This suggests that H3K79 methylation plays a major role in cell survival following UV irradiation, in agreement with Thompson and colleagues (27,40). Interestingly, in our case, the double mutant H3K4,79R is more sensitive to UV radiation than either of the single mutants. It is possible that H3K79 methylation may (at least partially) compensate for the effect caused by the loss of H3K4 methylation, or vice versa. Alternatively, the combined methylation states of H3K4 and H3K79 may have a cumulatively greater effect on UV sensitivity than either residue individually. It has also been shown previously that H3K79 methylation not only plays a role in UV induced RAD9-mediated checkpoint function but also affects nucleotide excision repair and RAD5 post-replication repair pathways (27). Therefore, collectively these effects in combination can cause hypersensitivity to UV radiation. Moreover, lysine 79 methylation can also affect the structure of chromatin (7,9,10,41), but how it
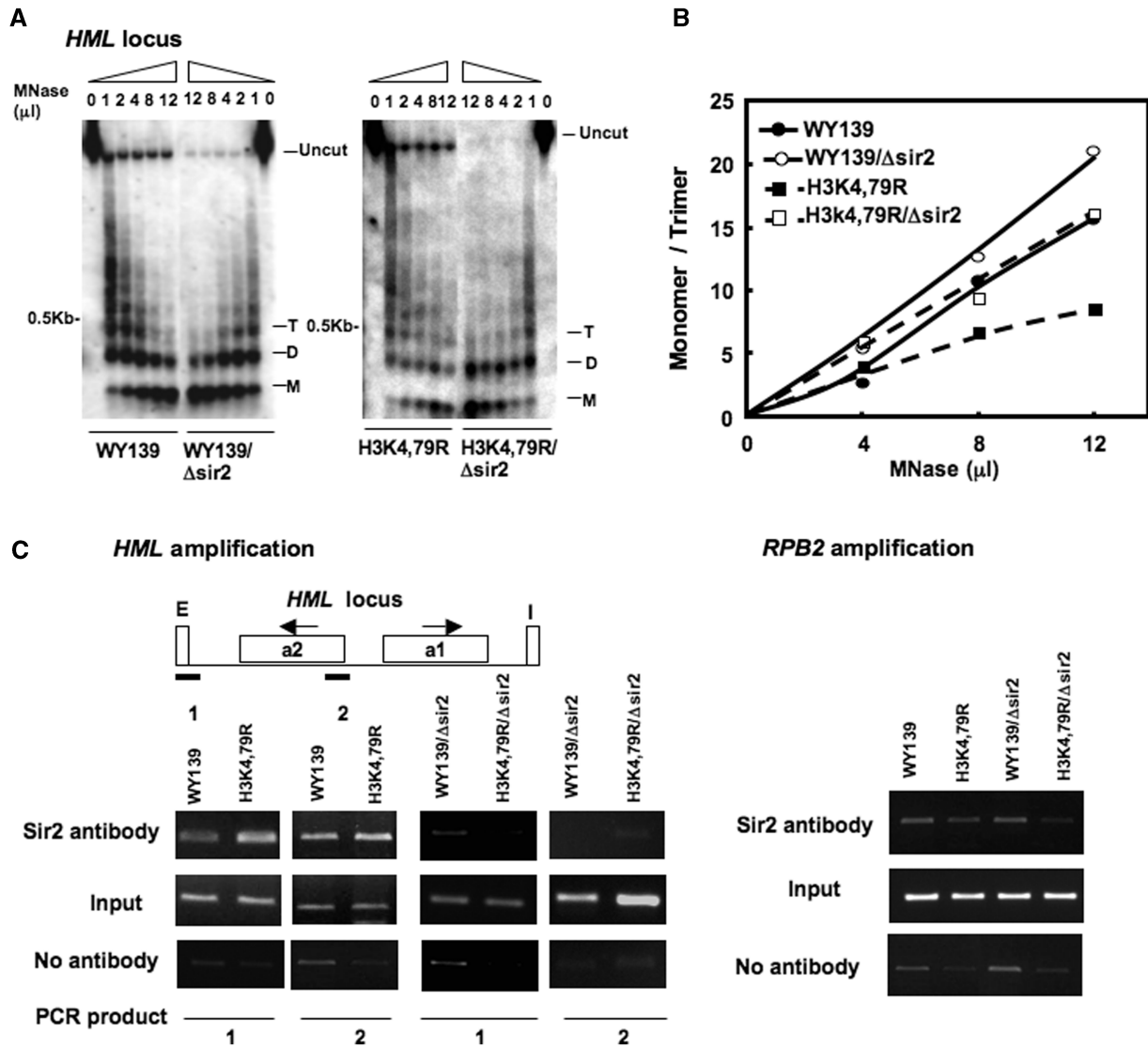
Figure 5. MNase accessibility increases in *Sir2* deletion strains. Spheroplasts were isolated from the *Sir2* deletion strains of WY139 (*wt*) and H3K4,79R, treated with different concentrations of MNase, blotted and hybridized with a *HMLα*1 ORF-specific probe, as described in legend to Figure 3. (**B**) Quantitative analysis of MNase accessibility at the *HML* locus. Data is expressed as the ratio of mono- to tri-nucleosome formation at different concentration of MNase. (**C**) ChIP analysis, using Sir2 antibody and *HMLα*1 ORF primers, showing recruitment of Sir proteins at the *HML* locus. Chromatin immunoprecipitated from Sir2 deleted strains was used as control (right-hand panels). Each experiment was repeated three times and the data shown are for a single representative experiment. Right panel shows amplification of *RPB2* gene from the ChIP DNA, which served as a control.

can influence the repair dynamics especially during NER is not known. In this study, we have addressed this aspect of histone modification by studying the efficiency of removal of CPDs in H3K79 methylation mutants.

We tested NER efficiency of the different mutants in three different, well-characterized chromatin loci of yeast. Compared to *wt* cells, all three methylation mutants exhibited reduced NER of UV-induced CPDs at the transcriptionally silenced *HML* locus, with H3K4,79R being the least efficient at carrying out CPD removal. On the other hand, there was no significant difference between these mutants and *wt* cells in repair of the transcriptionally active *RPB2* locus and the transcriptionally repressed *GAL10* locus (Figure 2). Importantly, the amount of

CPDs formed in each of the loci tested was similar for each strain (data not shown), indicating that these mutations do not affect CPD yields in these different loci.

Mutation of H3 K4,79 affects chromatin accessibility at a silenced locus

The crystal structure of the nucleosome indicates that Lys79 of the two histone H3 proteins are located at the top and bottom surfaces of the nucleosome disk and most likely regulate interactions with exogenous proteins, like Dot1p (9,42). Therefore, it is likely that any change in this residue can affect the accessibility of nucleosome DNA to nuclear proteins. In agreement with this hypothesis,
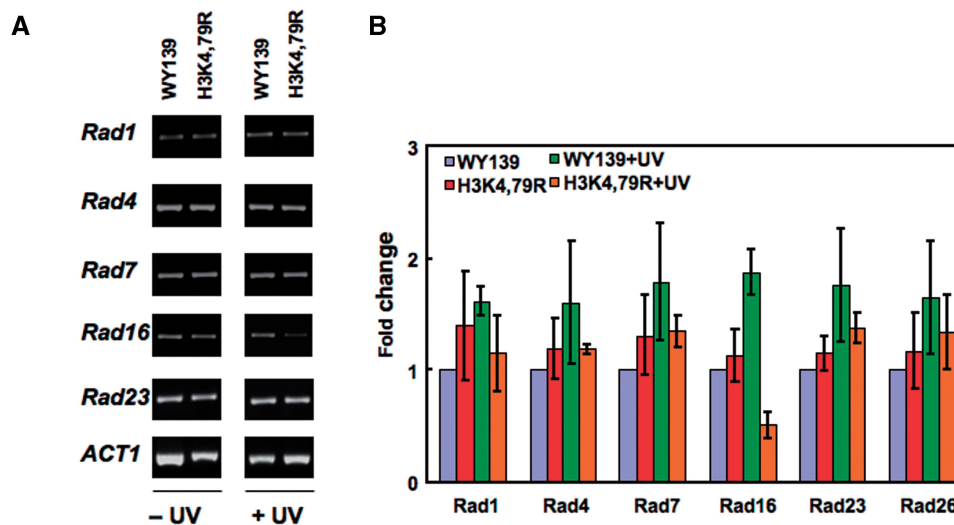
Figure 6. Expression of NER genes in H3 methylation mutants after UV irradiation. Both *wt* and H3K4,79R cells were allowed to repair for 20 min after exposure to 100 J/m² UV radiation. Total RNA was isolated and RT-PCR was performed using gene-specific primers. Cells not treated with UV radiation served as controls. (**A**) Gel showing the RT-PCR products. (**B**) Fold change in RT-PCR product for each of the NER genes shown in (A). Data represent the mean ± 1 SD for three independent experiments.

we found that DNA in the *HML* locus of H3K79R mutants was distinctly less accessible to MNase, compared to *wt* (Figure 3). The *HML* locus is distinct from the other two loci examined in our study in that it is a transcriptionally silent locus that contains positioned nucleosomes along with bound Sir complexes. Although the exact mechanism of transcriptional silencing in the *HML* locus is not clear, various studies have proposed the 'steric hindrance model' of reduced chromatin accessibility to DNA binding proteins in the silenced chromatin region (2). Our observation that deletion of Sir2 increases accessibility of MNase to nucleosome DNA and increases NER efficiency at the *HML* locus (Figures 4 and 5) supports this model. It is worth noting that deletion of Sir2 causes increased transcription of the *HML* gene (Figure S1B), indicating TCR could also play a role in repair of this locus, in addition to GGR, in Sir2 deletion cells. This situation, however, should be present in both *wt* and H3K4,79R cells, and the addition of TCR to the overall repair of *HML* would not be expected to change the NER response *between* the two cell types, as observed.

Dot1-mediated methylation of Lys79 plays an important role in establishing the transcriptional silencing and Sir protein association with heterochromatin (7,9,10). It has been found that ~10% of the yeast genome is heterochromatic and is hypomethylated at H3K79, whereas the remaining ~90% of the genome is hypermethylated at H3K79 (9). It has been proposed that Sir proteins prefer to bind to the core nucleosome surface that have hypomethylated Lys79 and maintain a stable heterochromatin condition by further preventing Dot1 mediated methylation of this residue (10). Indeed, our ChIP analysis shows that in the H3K4,79R mutant more Sir2 protein is recruited to the *HML* locus (Figure 5C) than in *wt* cells. This is also in agreement with previous studies that have shown that the LRS mutant H3K79R has enhanced silencing at telomeres and mating-type loci (41) due to

increased recruitment of Sir proteins (43). Furthermore, it has been suggested that the Lys79 to Arg79 mutation mimics the hypomethylation state of H3K79 (37), which would imply that nucleosome cores of H3K4,79R cells have a permanent modification state that results in stronger Sir binding compared to *wt* cells and hinders accessibility of repair factors. Our results support this hypothesis, and was further strengthened by testing NER in H3 K4,79G and Dot1 mutant cells. The H3K79G mutation disrupts the interaction between Sir protein and the nucleosome core, leading to loss of Sir-mediated silencing, while deletion of Dot1 leads to random binding of Sir proteins throughout the genome (9). In both mutant types, we found that NER at the *HML* locus was similar to *wt* cells (Figure S4A and S4C). Indeed, a small but consistent increase was observed in the overall repair rate of these mutants compared to *wt* cells. Furthermore, MNase digestion patterns show little difference in the chromatin accessibility of the *HML* locus between these mutants and *wt* cells (Figure S4B and S4D).

To date, only a few reports have appeared on the effect of Sir proteins on NER *in vivo*. Initially, it was reported that the silenced *HML* locus is repaired more slowly by NER than the active *MATα* locus, which could be explained by TCR in the active *MAT* locus (44,45). Another study showed that Sir3 protein interacts with NER protein Rad7 *in vitro* and that deletion of the *SIR3* gene rescues ~25% of the UV sensitivity caused by deletion of Rad7 (46). It was suggested that this interaction may play a role in damage recognition during NER in transcriptionally silent chromatin (46). More recently, Thoma and colleagues examined repair of CPDs by UV photolyase and NER in yeast strains containing the *URA3* gene inserted near a telomere end of chromosome V, where *URA3* was transcriptionally active in *sir3* mutants, partially silenced in *SIR3* cells, or completely silenced by overexpression of *SIR3* (47). These authors observed

(i) efficient repair of active *URA3* by both pathways, (ii) reduced NER in partially silenced *URA3* and (iii) considerably reduced repair by both pathways in completely silenced *URA3*. Our results are in agreement with these studies, showing that increased binding of Sir proteins at the *HML* locus decreases UV survivability and reduces NER of the *HML* locus, while having no effect on NER in the active *RPB2* and repressed *GAL10* genes, in yeast cells.

An interesting observation in this report is that expression of *RAD16* decreases after UV irradiation in H3K4,79R cells compared to *wt* (Figure 6). It has been shown that Rad16 and Rad7 form an ATP-dependent DNA damage sensing complex that is needed for efficient NER of silenced chromatin (48,49). Furthermore, it was reported that these proteins can interact with autonomously replicating sequence binding factor Abf1 to generate superhelicity in DNA, possibly required for nucleosome reloading after NER (50). In addition, it was recently reported that Rad16 mediates UV-induced acetylation of histone H3 in yeast, necessary for efficient NER of non-transcribed DNA (51). Therefore, Rad7 and Rad16 proteins may play a role in *both* early and late steps of NER (52), and the increased expression of *RAD16* after UV irradiation (53) is required for the later stages of NER. In our case, whether the decreased expression of Rad16 further retards the NER efficiency at the *HML* locus in H3K4,79R cells, is not clear and requires further investigation. It seems likely, however, that the presumed ATP-dependent chromatin remodelling activity of RAD16 is involved in NER of nucleosome bound regions, suggesting that low *RAD16* expression may indeed reduce the NER rate of silenced loci in H3K4,79R mutants.

In summary, we have shown that H3K79R methylation mutants have a constitutively altered nucleosome structure in the transcriptionally silent *HML* region. This altered chromatin structure arises from enhanced recruitment of Sir proteins to this locus, even though there is no apparent change in the overall level of Sir protein in these cells. These results indicate that increased recruitment of Sir proteins to the *HML* locus results in a 'less flexible' chromatin structure, one in which NER is significantly impaired. Although subtle changes in chromatin structure of Sir independent (devoid/depleted) loci, like *RPB2* and GAL10, were also observed the exact reason for these changes is not clear at present. However, it is possible that exchange of lysine to arginine in H3 may cause some irregular binding of Sir proteins in these loci, which could, at least slightly, impede the access to nucleosome DNA. Nevertheless, these differences are minor compared to those observed for the *HML* locus. Thus, changes in the histone methylation pattern can promote abnormal silencing in chromatin and impair removal of DNA lesions in chromatin, which can enhance rates of mutagenesis in intact cells.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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