Sites of Acetylation on Newly Synthesized Histone H4 Are Required for Chromatin Assembly and DNA Damage Response Signaling

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The best-characterized acetylation of newly synthesized histone H4 is the diacetylation of the NH₂-terminal tail on lysines 5 and 12. Despite its evolutionary conservation, this pattern of modification has not been shown to be essential for either viability or chromatin assembly in any model organism. We demonstrate that mutations in histone H4 lysines 5 and 12 in yeast confer hypersensitivity to replication stress and DNA-damaging agents when combined with mutations in histone H4 lysine 91, which has also been found to be a site of acetylation on soluble histone H4. In addition, these mutations confer a dramatic decrease in cell viability when combined with mutations in histone H3 lysine 56. We also show that mutation of the sites of acetylation on newly synthesized histone H4 results in defects in the reassembly of chromatin structure that accompanies the repair of HO-mediated double-strand breaks. This defect is not due to a decrease in the level of histone H3 lysine 56 acetylation. Intriguingly, mutations that alter the sites of newly synthesized histone H4 acetylation display a marked decrease in levels of phosphorylated H2A (γ-H2AX) in chromatin surrounding the double-strand break. These results indicate that the sites of acetylation on newly synthesized histones H3 and H4 can function in nonoverlapping ways that are required for chromatin assembly, viability, and DNA damage response signaling.

Each time a eukaryotic cell divides, it must duplicate not only its genomic DNA but also the chromatin structure in which it is packaged. At its most fundamental level, chromatin structure consists of ~147 bp of DNA wrapped around the histone octamer (which contains two molecules of each of the core histones, H2A, H2B, H3, and H4) to form a nucleosome. The linear strings of nucleosomes that package eukaryotic chromosomes can then form a number of successively higher-order structures to achieve the necessary level of compaction. The rapid and efficient regeneration of chromatin structure during DNA synthesis is essential for the mechanical packaging of the enormous linear length of eukaryotic chromosomes within the confines of the nucleus as well as for the physical protection of the DNA that ensures genomic integrity. Importantly, as posttranslational modifications to histones are an integral component of epigenetic regulation, the process of chromatin assembly also plays a critical role in the inheritance of distinct chromatin states.

The process of chromatin assembly actually begins in the cytoplasm, where histone proteins are produced. Analysis of these cytoplasmic histones gave the first indication that acetylation of histone NH₂-terminal tails might play a role in chromatin assembly, as it was shown that newly synthesized histone H4 was rapidly acetylated in the cytoplasm following its synthesis (1–3). Subsequently, newly synthesized histone H3 was also found to be acetylated (4). The acetylation of newly synthesized histone H4 molecules was found to occur in a specific pattern that is highly conserved across eukaryotic evolution. Newly synthesized histone H4 is primarily diacetylated with high levels of modification at lysines 5 and 12 and little or no modification on lysines 8 and 16 (4, 5). Newly synthesized histone H3 appears to be acetylated in most organisms but with different patterns of acetylation on the five NH₂-terminal tail lysine residues (at positions 9, 14, 18, 23, and 27) (4, 6).

Acetylation of lysine residues in the core domain of newly synthesized histones H3 and H4 has also recently been found.

The most well-characterized example of core domain acetylation occurs on lysine 56 of histone H3 (7–9). Lysine 56 is located at a point of contact with DNA at the entry/exit point of the nucleosome that may make this modification capable of physically altering the interaction between histone H3 and DNA. Histone H3 lysine 56 acetylation occurs on newly synthesized molecules and peaks in abundance during S phase before being removed from histones in G₂/M phase (7, 10–14). Mutations in *Saccharomyces cerevisiae* that alter H3 lysine 56 to mimic the constitutively unacetylated state (H3 K56R) result in cells that are sensitive to DNA-damaging agents and have increased levels of genome instability (7, 11, 12, 15–19). While originally thought to be limited to yeast, H3 lysine 56 acetylation may also be widespread in eukaryotic evolution, having been found in mammalian cells. However, this modification appears to be much less abundant in mammalian cells, in which its function is not well understood (13, 20–22).

The core domain of histone H4 is also acetylated on lysine 91 (23). This modification was identified on the pool of histones associated with the type B histone acetyltransferase Hat1p, consistent with the presence of this acetylation on newly synthesized molecules. This modification lies along the interface between the H3/H4 tetramer and the H2A/H2B dimers, where it forms a salt bridge with an aspartic acid residue in histone H2B (24, 25). Hence, neutralization of the positive charge of H4 lysine 91 by acetylation may function to

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regulate the process of histone octamer assembly by regulating the stability of the tetramer-dimer interaction. Histone H4 lysine 91 is also a highly conserved modification that has been observed in human, bovine, and yeast cells (26, 27). Mutations in yeast that alter H4 lysine 91 cause severe defects in silent chromatin formation and sensitivity to DNA-damaging agents. Consistent with a role for H4 lysine 91 acetylation in chromatin assembly, genetic analysis of these mutations indicates that this modification functions in common pathways with the histone chaperones Asf1 and CAF-1 in the context of DNA repair (23, 28). A histone acetyltransferase, termed HAT4, that appears to be responsible for this modification was recently isolated from mammalian cells. Interestingly, this enzyme promotes nucleosome assembly while its knockdown sensitizes cells to DNA-damaging agents (29).

While it has been known for decades that histones H3 and H4 can exist in an acetylated state during the process of chromatin assembly, the acetylation of newly synthesized histones has never been shown to be absolutely essential for histone deposition or viability in any organism. For example, mutation of any of the sites of acetylation on newly synthesized H3 or H4 results in viable cells that are capable of chromatin assembly (30, 31). In addition, none of the histone acetyltransferases that are involved solely in the acetylation of newly synthesized histones are essential for viability in yeast (17–19, 32–34). However, a number of recent studies have provided a direct functional link between the acetylation of newly synthesized histones and histone deposition. Mutations that alter histone H3 lysine 56 or deletion of *RTT109* (the histone acetyltransferase responsible for H3 lysine 56 acetylation) and *ASF1* (which is required for Rtt109p-mediated acetylation of H3 lysine 56) causes a defect in the reassembly of chromatin following the recombinational repair of a DNA double-strand break in yeast (35). Histone H3 lysine 56 acetylation has also been directly linked to replication-independent chromatin assembly (or histone exchange) (10, 36). In addition, deletion of *HAT1*, which encodes the histone acetyltransferase that is thought to generate the diacetylation pattern on newly synthesized histone H4, also results in defects in DNA repair-linked chromatin reassembly and histone exchange (37, 38).

Studies of histone H3 lysine 56 acetylation have also provided a link between the acetylation of newly synthesized histones and the DNA damage checkpoint response. The DNA damage checkpoint is activated to facilitate the repair of DNA lesions by providing time for the repair process through arrest of the cell cycle (39). In budding yeast, the recruitment of the Mec1 and Tel1 kinases to the site of a DNA lesion is believed to initiate the checkpoint cascade through phosphorylation downstream kinases, such as Rad53p and Chk1p. In addition, Mec1p and Tel1p phosphorylate histone H2A (H2AX in mammalian cells) in a large domain of chromatin near the site of a DNA double-strand break (phosphorylated histone H2A will be referred to as γ -H2AX). The phosphorylation of H2A is an early event in the DNA damage checkpoint that is important for maintaining the checkpoint and for recruiting factors to chromatin that are involved in modulating the chromatin structure during the repair process (40, 41). The mechanism of checkpoint recovery is not well understood, and the minimum requirement seems to be deactivation of Rad53 and dephosphorylation of γ -H2AX (42–44). The fact that fully repaired

DNA does not turn off the DNA damage checkpoint suggests that a proper chromatin state needs to be restored in order for cells to reenter the cell cycle. In fact, recent results have demonstrated that deactivation of the DNA damage checkpoint requires the reassembly of chromatin containing histone H3 that is acetylated on lysine 56 (13, 20, 35, 45).

In this study, we have explored the function of the acetylation sites on newly synthesized histone H4, namely, lysines 5, 12, and 91. We have found that combining a mutation that mimics the constitutively acetylated state of H4 lysine 91 (H4 K91Q) with mutations that alter both H4 lysine 5 and lysine 12 to arginine (H4 K5,12R) results in a pronounced sensitivity to DNA damage and DNA replication stress. Furthering the connection between these sites of newly synthesized histone acetylation on H4, mutations of these sites are synthetically lethal with the mutation of histone H3 lysine 56. We also use the inducible HO endonuclease system to directly demonstrate the requirement for the sites of newly synthesized histone H4 acetylation in the reassembly of chromatin structure that accompanies the recombinational repair of a DNA double-strand break. Strikingly, we also find that, while these sites of modification are not required for the phosphorylation of histone H2A, they are required for the association of this modified histone with chromatin, suggesting a role for chromatin assembly in DNA damage signaling.

MATERIALS AND METHODS

Yeast strains. Yeast culture and genetic manipulation were done by standard methods. Strains used in the phenotypic assays are derivatives of MPY302 [*MATa hhf2-hht2::LEU2 hhf1-hht1::MET15 (LYS2 CEN ARS)-HHF2-HHT2*], with *MEC1*, *MEC3*, *RAD9*, and *MRC1* deleted using PCR-mediated gene disruption with *URA3* and shuffled with *TRP1*-based plasmids carrying the corresponding histone H4 mutations. Strains used in the histone H3 and γ -H2AX chromatin immunoprecipitation (ChIP) assays are derivatives of ZGY110 [*MATa ade3::GAL10-HO hhf2-hht2::LEU2 hhf1-hht1::ADE3 (LYS2 CEN ARS)-HHF2-HHT2*] shuffled with *TRP1*-based plasmids carrying the corresponding histone H4 mutations. Tests of synthetic lethality were performed in a derivative of MPY302 that contained wild-type (WT) copies of *HHT2* and *HHF2* on a *URA3*-based plasmid (RMY200U) (46). Strains are listed in Table 1.

Yeast phenotypic assays. Yeast strains were grown overnight in rich medium with 2% glucose or raffinose. Log-phase cells were collected and concentrated to an A_{600} of 1 to plate in 10-fold serial dilutions onto rich medium or medium with 2% galactose or plates with the indicated concentration of DNA-damaging drugs.

Cell extracts, chromatin fractionation, and immunoblotting. Cell extracts were prepared by trichloroacetic acid addition as described in reference 47 and were subjected to Western blot analysis. γ -H2AX was detected by polyclonal anti-yeast γ -H2A antibody (Abcam). Yeast cell fractionation was carried out as described previously (42). Briefly, cells were collected and spheroplasts were made using Zymolyase 100T. Spheroplasts were then washed and lysed with cell lysis buffer with 0.5% Triton X-100, which was then layered over a 60% sucrose cushion and spun for 10 min in the cold room. The upper layer was taken as the cytosolic fraction that was acid extracted to isolate histones. The white glassy pellet at the bottom of the cushion was further lysed with nuclear lysis buffer with 1% Triton X-100. The nuclear lysate was spun for 10 min in the cold room, and the supernatant was taken as the soluble nuclear fraction and acid extracted to isolate histones. The pellet was the chromatin fraction and was boiled in SDS dye prior to electrophoresis.

DNA damage and repair analysis. Primers flanking the HO site in the *MAT* locus were used to determine the degree of cutting and repair of mating type by PCR amplification. Cells were grown overnight in rich

TABLE 1 Strains used in this study

Strain	Genotype	Reference or source
SQY501	<i>MATα ade2Δ::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 ade3::GAL10-HO hhf2-hht2::LEU2 hhf1-hht1::ADE3 (TRP1 CEN-ARS-HHF2-HHT2)</i>	37
ZGY104	<i>MATα ade2Δ::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 ade3::GAL10-HO hhf2-hht2::LEU2 hhf1-hht1::ADE3 asf1::URA3 (LYS CEN ARS-HHF2-HHT2)</i>	37
UCC1111	<i>MATα ade2Δ::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 adh3-URA3-TEL(VII-L) hhf2-hht2::MET15 hhf1-hht1::LEU2 (LYS2 CEN ARS-HHF2-HHT2)</i>	76
MPY302	<i>MATα ade2Δ::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 hhf2-hht2::MET15 hhf1-hht1::LEU2 (ADE2 CEN-ARS-HHF2-HHT2)</i>	This study
ZGY201	<i>MATα ade2Δ::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 hhf2-hht2::MET15 hhf1-hht1::LEU2 sml1::URA3 (ADE2 CEN-ARS-HHF2-HHT2)</i>	This study
ZGY202	<i>MATα ade2Δ::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 hhf2-hht2::MET15 hhf1-hht1::LEU2 sml1::URA3 (ADE2 CEN-ARS-HHF2-HHT2)</i>	This study
ZGY203	<i>MATα ade2Δ::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 hhf2-hht2::MET15 hhf1-hht1::LEU2 mec1::HIS3 sml1::URA3 (ADE2 CEN-ARS-HHF2-HHT2)</i>	This study
ZGY204	<i>MATα ade2Δ::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 hhf2-hht2::MET15 hhf1-hht1::LEU2 rad9::HIS3 (ADE2 CEN-ARS-HHF2-HHT2)</i>	This study
ZGY205	<i>MATα ade2Δ::hisG his3Δ200 leu2Δ0 lys2Δ0 met15Δ0 trp1Δ63 ura3Δ0 hhf2-hht2::MET15 hhf1-hht1::LEU2 mrc1::HIS3 (ADE2 CEN-ARS-HHF2-HHT2)</i>	This study

medium containing 2% raffinose. Galactose and then glucose were added to 2% at the times indicated in the figure legends. The number of PCR cycles to produce amplification in the linear range was determined empirically. PCR products were resolved by agarose gel electrophoresis. Gels were stained with ethidium bromide, and PCR products were quantitated with one-dimensional (1D) image analysis software (Kodak).

Stable isotope labeling by amino acids in cell culture (SILAC) quantitation of histones. For each pair of wild-type and mutant histone strains to be compared, the cells were grown in duplicate cultures containing either normal L-lysine or $^{13}\text{C}_6$, $^{15}\text{N}_2$ -lysine. Cells were harvested by centrifugation in mid-log phase at an A_{600} of 0.6 to 0.8. Equal weights of cells from each culture were mixed, and histones were subsequently isolated as described previously (48). Histones were digested with endoproteinase Arg-C (sequencing grade from *Clostridium histolyticum*; Roche Applied Science) at a 1:50 enzyme/substrate ratio in 100 mM Tris-HCl (pH 7.6) for 8 h at 37°C. Peptides were desalted with a C_{18} ZipTip and eluted with 0.1% trifluoroacetic acid (TFA)/50% acetonitrile (ACN). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) and data analyses were performed as described previously (49).

ChIP and real-time PCR. Cultures were grown overnight in rich medium containing 2% raffinose, diluted, and grown until the cells reached an optical density at 600 nm (OD_{600}) of approximately 0.5. Galactose and then glucose were added to 2% at the times indicated in the figure legends. Samples were taken for ChIP analysis at the time points indicated in the figure legends and were processed as described previously (37). Samples were analyzed using quantitative real-time PCR in a multiplex reaction with primers and probes designed as described previously (35). All experiments were performed with two or three biological replicates. Real-time PCR was used to quantitate amounts of DNA fragments in the immunoprecipitated (IP) samples from the ChIP analyses, using the ABI 7300 sequence detector and TaqMan PCR master mix protocol or Sybr green master mix protocol. Each PCR was performed in triplet with the following cycling conditions: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s and 60°C for 1 min. The cycle threshold (C_T) value was set so that the fluorescence signal was above the baseline noise and as low as possible in the exponential amplification phase. The amount of change compared to the SMC2 control was calculated for each IP using the standard comparative C_T method.

RESULTS

Functional redundancy in the sites of acetylation found on newly synthesized histones H3 and H4. Histone H4 lysine 91 lies in an interface between histones H4 and H2B and appears to form a salt bridge with a glutamic acid residue at position 74 of H2B (yeast). H4 lysine 91 is acetylated in the soluble fraction of H4 molecules that copurify with the nuclear Hat1p-containing complex type B histone acetyltransferase complex (NuB4). Based on the potential for modulating this H4-H2B salt bridge, acetylation of lysine 91 was proposed to influence chromatin assembly via modulating the association of H3/H4 tetramers and H2A/H2B dimers (23). To further test the model that H4 lysine 91 acetylation functions in the process of chromatin assembly, we tested whether genetic interactions exist between this site and the other sites of modification on newly synthesized histones H3 and H4.

As previously reported, H4 K91Q mutants (which may mimic the constitutive acetylation of this residue) were sensitive to DNA damage and replication stress (methyl methanesulfonate [MMS] and hydroxyurea [HU]) while H4 K91R mutants were not (Fig. 1A) (23). In addition, mutating H4 lysines 5 and 12 to arginine did not increase the sensitivity of cells to HU or MMS. However, when the H4 K91Q and H4 K5,12R mutations were combined, there was a defect in cell growth as well as a striking increase in HU and MMS sensitivity. H4 K5,12R K91R mutants did not display any of these phenotypes. The genetic interactions between sites on the H4 NH_2 -terminal tail and the H4 core domain required mutation of both H4 lysine 5 and lysine 12 (Fig. 1B). In addition, changing H4 lysines 5 and 12 to glutamine, which mimics the diacetylation pattern, slightly suppresses the HU sensitivity of the H4 K91Q mutant (Fig. 1C). These results indicate that mutations in all three sites of acetylation on newly synthesized histone H4 can cause phenotypes that are consistent with a role in chromatin assembly and that the H4 NH_2 -terminal tail is functionally redundant with H4 lysine 91.

We next tested whether the sites of newly synthesized histone acetylation on histone H4 showed genetic interactions with his-

FIG 1 The histone H4 lysines 5, 12, and 91 are functionally redundant. (A to C) Tenfold serial dilutions of cells with the indicated alleles of histone H4 were spotted on plates containing synthetic complete (SC) medium with or without the indicated concentrations of hydroxyurea (HU) or MMS. Plates were incubated at 30°C for 3 days. YPD, yeast extract-peptone-dextrose. (D) Tenfold serial dilutions of strains containing the indicated alleles of histone H3 and H4 were spotted on SC medium with or without the indicated concentrations of hydroxyurea. Plates were incubated at 30°C for 3 days. (E) Cells with the indicated alleles of histone H3 and H4 on a *TRP1*-based plasmid were grown for several passages in liquid SC medium lacking tryptophan (TRP). Tenfold serial dilutions were then plated on SC medium with or without 5-FOA. Plates were incubated at 30°C for 3 days.

tone H3 lysine 56. It has previously been shown that H4 lysine 5 and 12 mutations enhance the growth defect and DNA damage sensitivity phenotypes of H3 K56R mutants (50, 51). Therefore, we focused on potential genetic interactions between histone H4 lysine 91 and histone H3 lysine 56. Mutation of H3 lysine 56 showed an opposite pattern of phenotypes relative to H4 lysine 91 when comparing the effects of altering these residues to glutamine or arginine. As reported previously, mimicking constitutive deacetylation of H3 lysine 56 (H3 K56R) resulted in sensitivity to DNA-damaging agents and replication stress while there were no defects seen when mimicking the constitutive acetylation of this site (H3 K56Q) (Fig. 1D) (7, 11, 12, 15–19). Interestingly, mutating H4 lysine 91 to arginine (H4 K91R) suppressed the phenotypes of the H3 K56R mutant. However, the converse was not true, as

the H3 K56Q allele did not suppress the phenotypes of the H4 K91Q mutant. In addition, while both the H3 K56R and H4 K91Q alleles individually display sensitivity to DNA damage and replication stress, combining these alleles did not lead to a synthetic increase in sensitivity. These genetic interactions strongly support the hypothesis that H4 lysine 91 plays a functional role in chromatin assembly and that H4 lysine 91 and H3 lysine 56 are functioning in a common pathway.

We also sought to determine whether additional genetic interactions existed with H4 lysines 5 and 12. A plasmid shuffle assay in which cells contained both an *ADE2*-based plasmid with wild-type copies of yeast H3 and H4 genes (*HHT2/HHF2*) and a second *TRP1*-based plasmid that contained the mutated alleles of H3 and H4 (the test plasmid) was used. As the starting strain is *ade2*, only

cells that can lose the *ADE2*-based plasmid will form red colonies. Using this assay, we were unable to obtain red colonies when the test plasmid contained the H3 K56R, H4 K91Q, and H4 K5,12R mutations. This suggested that the yeast cannot survive with the mutant forms of H3 and H4 as the sole copies of these histones. To confirm this synthetic decrease in viability, a separate strain of yeast that contained wild-type copies of the *HHT2* and *HHF2* genes on a plasmid that contained a *URA3* selectable marker was transformed with a second plasmid that contained either the wild type, the H4 K5,12R K91Q allele, or the H4 K5,12R K91Q H3 K56Q alleles (*TRP1* marker). These cells were grown extensively in medium that selected for the test plasmid (medium lacking tryptophan) but in the absence of selection for the *URA3* plasmid. Serial dilutions were then plated on synthetic medium with and without 5-fluoroorotic acid (5-FOA). The 5-FOA is lethal to cells that are expressing the *URA3* enzyme (52). Therefore, cells that are not able to retain viability following the loss of the *URA3*-based plasmid that contains wild-type H3 and H4 genes will not be able to grow on medium containing 5-FOA. As expected, cells containing a test plasmid with wild-type H3 and H4 genes showed full viability on 5-FOA medium (Fig. 1E). The H4 K91Q K5,12R allele supported moderate growth on 5-FOA, consistent with the growth defect seen with this combination of mutations. However, combining the H4 K91Q, H4 K5,12R, and H3 K56R mutations resulted in a nearly complete loss of viability on 5-FOA medium. This indicated that the sites of newly synthesized histone acetylation on both H3 and H4 are functionally redundant and important for viability in yeast.

Histone H4 lysines 5, 12, and 91 are involved in DNA repair-linked chromatin reassembly. One important aspect of the DNA damage response is the reassembly of chromatin after the completion of DNA repair (35). The repair of an HO-induced DNA double-strand break has provided a powerful model system for the study of chromatin dynamics during DNA repair (53). By expressing HO from a regulated promoter, a double-strand break can be initiated at a specific place (the *MAT* locus) and at a specific time, allowing for the use of chromatin immunoprecipitation (ChIP) to measure histone occupancy near the break site during the course of repair (54). This system was used to show that the Rtt109p- and Asf1p-mediated acetylation of histone H3 lysine 56 was involved in the reassembly of chromatin structure that accompanies the recombinational repair of a DNA double-strand break (35). We have employed the inducible HO system to determine whether the sites of acetylation on newly synthesized histone H4 are also involved in DNA repair-linked chromatin reassembly.

We first tested whether the H4 mutants were sensitive to a single double-strand break at the *MAT* locus (55). Plasmids containing various combinations of histone H4 mutations were introduced into strains containing a galactose-inducible HO gene integrated into the genome. Serial dilutions of equal numbers of cells from each strain were spotted on synthetic medium containing either glucose or galactose (Fig. 2A). The H4 K5,12R, H4 K91R, and H4 K5,12R,91R alleles did not show sensitivity to a single double-strand break, as reflected in the ability to grow on galactose-containing medium. The H4 K91Q allele showed a slight decrease in viability in the presence of galactose. However, combining the H4 K5,12R and K91Q mutations resulted in a pronounced decrease in viability following induction of HO that was similar to that seen with an *asf1Δ* mutant strain. These results again demonstrate the functional redundancy between the NH₂-

terminal tail and core domain acetylation sites on newly synthesized histone H4 and are consistent with a role for these sites of modification in chromatin assembly.

To directly test whether these histone H4 mutations cause a defect in DNA repair-linked chromatin reassembly, we monitored chromatin structure near the site of the HO-mediated double-strand break by ChIP. For these experiments, galactose was added at time zero to induce expression of HO and then glucose was added at 2 h to repress HO expression and allow for DNA repair. The introduction of the HO-induced double-strand break and its subsequent repair were detected by a PCR that spanned the HO cut site and that generates distinct fragments from *MATa* and *MATα* cells (Fig. 2B). The kinetics of DNA double-strand break formation and repair were similar in all of the strains examined (Fig. 2C).

In a wild-type strain, following induction of HO, levels of histone H3 found at a point ~600 bp from the break site began to decrease. During the course of repair, the level of histone H3 was restored (Fig. 3A). The clear defect in histone restoration in the absence of the key histone chaperone Asf1 indicates that chromatin assembly is required for this process (Fig. 3A) (35). We found that while the H4 K91R mutant did not show any defect in chromatin reassembly, H4 K5,12R and H4 K5,12,91R mutants displayed a level of reassembly that is intermediate between those seen in wild-type cells and *asf1Δ* mutant cells. Interestingly, this level of reassembly is similar to that seen in the absence of Hat1p, which is thought to be responsible for the acetylation of H4 lysines 5 and 12 (37). In the presence of the H4 K91Q allele, chromatin reassembly near the double-strand break was similar to that seen in the *asf1Δ* mutant cells. Combining the H4 K5,12R and H4 K91Q mutations (H4 K5,12R K91Q) resulted in a further decrease in chromatin reassembly.

Replication-coupled chromatin assembly is not easily visualized by ChIP, as the sites at which assembly occurs are continuously changing. Therefore, we used a surrogate assay to determine whether the sites of acetylation on newly synthesized histones are important for generalized chromatin assembly. The levels of soluble histones are typically very low, as the synthesis of histones is tightly linked to DNA synthesis. However, a disruption in chromatin assembly caused by a block to DNA replication results in an increase in the level of non-chromatin-associated histones (56, 57). Therefore, mutations that decrease the rates of chromatin assembly would be predicted to increase the soluble pools of histones. We analyzed the levels of soluble histones in the H4 K91Q and H4 K5,12R K91Q cells. Soluble cytosolic and nuclear fractions were visualized by SDS-PAGE and Western blotting for histone H4. As seen in Fig. 3B, the H4 K91Q allele caused a significant increase in soluble histone H4 and this increase was enhanced by the H4 K5,12R mutation. In addition, treatment of these cells with the DNA-damaging agent MMS further accentuated the levels of soluble histones.

To determine whether the mutations in the sites of acetylation on newly synthesized histone H4 caused a global change in histone abundance, we used a mass spectrometry-based approach with stable isotope labeling (SILAC) to quantitate the levels of the core histones in each of the mutants (49). Yeast with WT histones was grown in synthetic medium supplemented with normal lysine (referred to as "light"), while yeast containing the H4 WT, H4 K5,12R, H4 K91Q, and H4 K5,12R K91Q alleles of histone H4 was grown in synthetic medium supplemented with heavy-isotope-labeled lysine (¹³C₆, ¹⁵N₂-lysine). The heavy-isotope-labeled lysine

FIG 2 Chromatin assembly and the repair of an HO-induced DNA double-strand break. (A) Tenfold serial dilutions of cells with the indicated genotype, containing a galactose-inducible HO gene integrated into the genome, were spotted onto plates containing either glucose or galactose as a carbon source. Plates were incubated at 30°C for 3 days. (B) Schematic diagram depicting the *MAT* locus and the silent mating loci (*HML α* and *HMR α*). The site of HO cutting is indicated by the red triangle. The repair status of the *MAT* locus was assayed by a PCR that spanned the HO digestion site and generates a longer fragment from a *MAT α* cell than from a *MAT α* cell (black lines). For ChIP experiments, PCRs amplified fragments that were 0.6 kb, 10 kb, and 20 kb from the HO cut site, as indicated. (C) Galactose was added to cultures containing the indicated alleles of histone H4 at the 0-h time point, and glucose was added at the 2-h time point. Reaction products were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining. *MAT α* and *MAT α* products are indicated, and the *RAD27* locus was used as a control. (D) Quantitation of double-strand break (DSB) formation and repair. Stained agarose gels were photographed, and the *MAT α* , *MAT α* , and *RAD27* bands were quantitated using 1D image analysis software (Kodak). *MAT α* and *MAT α* bands were normalized relative to the 0-h or 5-h time point.

has a mass increase of 8 Da relative to normal lysine. Cultures were harvested in mid-log phase, and equal weights of cells grown in light and heavy media were mixed. Cells were lysed, and histones were purified by acid extraction. Histones were digested with en-

doproteinase Arg-C, and peptides were analyzed by LC-MS/MS. "Matched pairs" of peptides that appear as doublets with a difference of 8 Da per lysine residue were identified. By determining the light/heavy ratio of these matched pairs, the relative abundance of

FIG 3 The histone H4 lysines 5, 12, and 91 function in chromatin assembly. (A) Cultures of strains with the indicated genotypes, containing a galactose-inducible HO gene integrated into the genome, were grown in raffinose, and galactose was added at the 0-h time point to induce expression of HO. HO expression was then repressed by the addition of glucose at 2 h. DNA repair of a single double-strand break introduced at the *MAT* locus. Galactose was added at the 0-h time point, and glucose was added at the 2-h time point. ChIP analysis was used to determine histone H3 levels 600 bp from the DSB at the *MAT* locus. The bar graph shows a comparison of the indicated histone H4 mutant to a wild-type strain as well as an *asf1*Δ mutant strain. Histone H3 levels were normalized to H3 levels at the SMC2 locus at each time point. Subsequent time points were then normalized to the 0-h time point. (B) (Top) Western blots of cytosolic and soluble nuclear extracts of the indicated strains. Cultures were treated with 0.03% MMS as indicated. (Bottom) Cytosolic and nuclear extracts were resolved by SDS-PAGE, transferred to nitrocellulose, and stained with Ponceau red.

that peptide in the two strains can be calculated. The total abundance of each of the core histones was determined by quantitating the levels of matched pairs that have not been shown to be subject to any posttranslational modifications. As seen in Fig. 4A, none of the histone H4 alleles significantly influenced the abundance of the core histones.

Acetylation of histone H3 lysine 56 has previously been shown to be required for proper reassembly of chromatin following repair of an HO-mediated double-strand break. Therefore, we also used the SILAC technique to determine whether the defects seen with the histone H4 mutations were an indirect effect resulting

from a decrease in the level of H3 lysine 56 acetylation. The Arg-C peptide pairs representing the light- and heavy-labeled peptides containing H3 lysine 56 [FQ⁵⁶K(Ace₀₋₁)STELLIR] were identified and quantitated. By determining the ratio of light to heavy for each peptide, the effect of each histone H4 allele on the level of H3 lysine 56 acetylation was determined (49). As seen in Fig. 4B, there was no significant decrease in H3 lysine 56 acetylation in any of the histone H4 mutants. Taken together, our results are direct evidence that sites of acetylation on newly synthesized histone H4 are involved in chromatin assembly and are consistent with the genetic evidence that they function in nonoverlapping ways.

FIG 4 The histone H4 lysine 5, 12, and 91 mutations do not alter the abundance of the core histones or H3 lysine 56 acetylation. (A) SILAC-derived protein ratios for the core histones in mixtures in which the WT was grown on light (L) medium and mutants (H4 K5,12R, K91Q and K5,12R/91Q, as indicated) were grown on heavy (H) medium. Each ratio is the average of three replicates. Error bars correspond to two standard deviations. N.S., not significant. (B) SILAC ratios for the H3 K56 peptide FQ⁵⁶K(Ace₀₋₁)STELLIR across the indicated mutants. To compare the peptide levels between mutants, raw H/L ratios of each peptide are normalized using the filtered robust linear regression normalization factor. To correct the labeling bias, the normalized ratios are further normalized to the ratio of unmodified H3 K56 peptide. Error bars correspond to 2-fold of the sample-based standard deviation.

Sites of newly synthesized histone H4 acetylation are required for normal DNA damage response signaling. Mutations that block the acetylation of histone H3 lysine 56 have a significant impact on the DNA damage response. In the absence of H3 lysine 56 acetylation, the DNA damage checkpoint is activated normally, but following the completion of repair, the checkpoint is not deactivated (13, 35, 45). This suggests that assembly of chromatin that contains H3 acetylated at lysine 56 is an essential step for recovery from the DNA damage checkpoint.

To determine whether the acetylation sites on newly synthesized histone H4 have a similar effect, we monitored the status of the DNA damage checkpoint during DNA repair. Phosphorylated histone H2A is detected soon after DNA damage (within 1 h in budding yeast around an HO-mediated double-strand break at the *MAT* locus) and is found to span a large region (up to 100 kb in yeast cells) (58, 59). ChIP assays were carried out to monitor the γ -H2A levels at 10 kb from the *MAT* locus. We found that the kinetics and abundance of γ -H2AX in wild-type cells were similar

to those seen with the H4 K5,12R, H4 K91R, H4 K91Q, and H5 K5,12R K91R mutants. However, there was a dramatic loss of γ -H2AX specifically in the H4 K5,12R K91Q cells (Fig. 5A). We also observed a similar decrease in γ -H2AX levels at 20 kb from the break in the H4 K5,12R K91Q cells (Fig. 5B). These results indicated that the sites of acetylation on newly synthesized histone H4 function redundantly in regulating the DNA damage response.

Previous results have shown that the RSC ATP-dependent chromatin remodeler is required to maintain normal γ -H2A levels on chromatin and that the enrichment of Mec1p and Tel1p at the DNA lesion is reduced in *rsc2Δ* mutant cells (60). To test the hypothesis that DNA damage checkpoint kinase recruitment was impaired in the H4 K5,12R K91Q mutant, we monitored the chromatin localization of Ddc2p. Ddc2p is the binding partner of Mec1p, and its absence impairs Mec1p recruitment to DNA double-strand breaks and the formation of γ -H2AX domains (61–63). As assayed by ChIP utilizing a Ddc2p-myc fusion, we found that in a wild-type strain, Ddc2p accumulation near the break site peak

FIG 5 Newly synthesized H4 acetylation site mutants are defective in the formation of γ -H2AX domains near a double-strand break. (A) ChIP analysis of γ -H2A levels 10 kb from the DSB. Each bar graph shows a comparison of the indicated histone H4 mutant to a wild-type strain. Galactose was added at the 0-h time point, and glucose was added at the 2-h time point. γ -H2A levels are normalized to the internal control SMC2 and to the 0-h time point. (B) ChIP analysis of γ -H2A levels 20 kb from the DSB was performed as described in panel A.

begins following induction of the HO endonuclease and then decreases as repair proceeds (Fig. 6A). In all of the histone H4 mutants tested, including the H4 K5,12R K91Q mutant, we observed that Ddc2p was recruited with similar kinetics and to similar levels. This suggests that a defect in Mec1p recruitment was not responsible for the loss of chromatin-associated γ -H2AX in the H4 K5,12R K91Q mutant.

If DNA damage checkpoint kinase recruitment is not altered in the H4 K5,12R K91Q mutant, another explanation for the loss of γ -H2AX is that a specific modification state or structure of chromatin may be necessary for the catalytic activity of the checkpoint kinases. To test this model, we isolated total histones from WT, H4 K91Q, and H4 K5,12R K91Q cells before and after treatment with MMS. As expected, MMS treatment resulted in an increase in γ -H2AX in WT cells (Fig. 6B). In addition, we saw identical increases in γ -H2AX in the histone H4 mutant cells. This indicated that the defect in localization of γ -H2AX near DNA double-strand breaks was not due to an inability to generate this phosphorylated form of H2A.

As the defect in the formation of a γ -H2AX chromatin domain

FIG 6 Sites of acetylation of histone H4 are not necessary for the formation of γ -H2AX. (A) ChIP analysis of Ddc2-myc levels at 600 kb from the DSB. Galactose was added at the 0-h time point, and glucose was added at the 2-h time point. Ddc2 levels were normalized to Ddc2 levels at the SMC2 locus at each time point. Subsequent time points were then normalized to the 0-h time point. (B) Indicated strains were grown to log phase, and 0.03% MMS was added. Whole-cell extracts were resolved by SDS-PAGE and analyzed by Western blots probed with α -H2A and α - γ -H2A antibodies. (C) Cytosolic extracts were made from the indicated strains treated or not with 0.03% MMS. Proteins were resolved by SDS-PAGE and analyzed by Western blotting probed with α - γ -H2AX antibody. Extracts are identical to those shown in Fig. 3B.

is not due to a decrease in total γ -H2AX in the cell, another possibility is that the H4 K5,12R K91Q mutation is influencing either the assembly of γ -H2AX into chromatin or the stability of γ -H2AX in chromatin. Either scenario would predict an increase in soluble γ -H2AX in these cells. Therefore, we purified soluble histones from cytosolic extracts from wild-type, H4 K91Q, and H4 K5,12R K91Q cells. In both the wild type and the H4 K91Q mutant, there were undetectable levels of γ -H2AX in the cytosol in the absence of DNA damage (MMS treatment). Following MMS treatment, there was a similar increase in soluble γ -H2AX in both strains. However, in the H4 K5,12R K91Q mutant, there was a significant increase in the level of γ -H2AX in untreated cells that was increased further by MMS treatment (Fig. 6C). This indicated that the H4 K5,12R K91Q mutation specifically caused an increase in the soluble pool of γ -H2AX, consistent with a defect in either γ -H2AX assembly or stability.

Histone H4 lysine 91 and lysines 5 and 12 affect different aspects in the DNA damage response. As seen above, the H4

FIG 7 The redundant function of newly synthesized histone H4 acetylation sites in the NH₂-terminal tail and core domains requires Mec1p. Strains containing the indicated genotype and alleles of histone H4 were plated in 10-fold serial dilutions on SC medium containing the indicated concentrations of HU or MMS. Plates were incubated at 30°C for 3 days.

K5,12R and H4 K91Q mutations display a pronounced synthetic increase in sensitivity to DNA damage and DNA replication stress. In addition, these mutations display defects in chromatin assembly and in the chromatin localization of γ -H2AX. We used a series of genetic assays to determine whether the DNA damage sensitivity of the H4 K5,12R K91Q mutant is due primarily to the defect in γ -H2AX chromatin localization. First, alleles that contain combinations of mutations in the acetylation sites on newly synthesized histone H4 were combined with a deletion of *MEC1*. Similar to what was previously reported for an H4 K91A allele, the H4 K91Q allele increased the DNA damage sensitivity of a *mec1* Δ mutant strain (23). We did not detect an increase in sensitivity to DNA replication stress at the low levels of HU that are necessary for use with the *mec1* Δ mutant cells. In addition, the H4 K5,12R allele also increased the DNA damage sensitivity of the *mec1* Δ mutant strain. However, there was no further increase in sensitivity to MMS when the H4 K5,12R and H4 K91Q mutations were combined in the absence of Mec1p (Fig. 7A). One interpretation of these results is that the sites of acetylation on newly synthesized histone H4 impact multiple aspects of the cellular response to DNA damage. One role for these acetylation sites is outside the DNA damage checkpoint and is likely to involve the process of chromatin assembly. A second function in DNA repair, which H4 lysines 5 and

12 and H4 lysine 91 impact in a functionally redundant way, requires the function of Mec1p.

Mec1p (and Tel1p) phosphorylates a number of proteins in addition to histone H2A in response to DNA damage. To determine whether the interactions observed between the sites of acetylation on newly synthesized histone H4 and Mec1p are restricted to the γ -H2AX pathway or also include other Mec1p-activated pathways, we combined the same set of histone H4 alleles with deletions of key factors involved in other aspects of the DNA damage checkpoint. When the H4 alleles were introduced into a *rad9* Δ , *mec3* Δ , or *mrc1* Δ mutant strain, there was a synthetic increase in sensitivity to both MMS and HU when the H4 K5,12R and H4 K91Q alleles were combined (Fig. 7B to D). This suggests that these sites of acetylation on histone H4 influenced the formation of γ -H2AX but not other pathways activated by Mec1p phosphorylation.

Our current study makes a number of advances in our understanding of the function of the acetylation of newly synthesized histones. First, the acetylation sites in the NH₂-terminal tail and globular core domain of histone H4 act in a functionally redundant manner. This is evidenced by synthetic increases in sensitivity to DNA damage and DNA replication stress. Second, the sites of acetylation on newly synthesized histone H4 are essential for via-

bility in the absence of histone H3 lysine 56 acetylation. Third, we provide direct evidence that the sites of acetylation on newly synthesized histone H4 participate in chromatin assembly. Finally, these sites of acetylation are required for the formation of γ -H2AX domains on chromatin surrounding the sites of DNA double-strand breaks through facilitating either the assembly or stability of this modified histone.

DISCUSSION

The diacetylation of the newly synthesized histone H4 on lysines 5 and 12 was the first pattern of posttranslational modification to be identified on a histone. Despite this, the functional significance of this modification pattern is not known (64, 65). Initial genetic studies in yeast using mutations that altered H4 lysines 5 and 12 to arginine indicated that the absence of this modification was well tolerated (30, 31, 66, 67). More-recent studies have shown that the H4 K5,12R allele can have a significant effect on cell viability in strain backgrounds that have mutations in the Pob3p subunit of the yeast FACT (facilitates chromatin transcription) complex (28, 68). These studies suggest that FACT may be involved in nucleosome assembly but, given the multifunctional nature of the FACT complex, do not shed light on the specific function of H4 lysine 5/12 acetylation. However, experiments in *Physarum polycephalum* and HeLa cell systems suggest that the acetylation of H4 lysines 5 and 12 may influence the nuclear import of histone H4 (69, 70).

The results presented here suggest that one reason for the minor phenotypes associated with the loss of the H4 lysine 5/12 acetylation pattern in yeast is functional redundancy with other sites of modification on newly synthesized histones H3 and H4. In particular, while combining the H4 K5,12R mutations with a H4 K91Q mutation caused a modest decrease in cell viability/growth, it also produced a pronounced sensitivity to DNA-damaging agents and DNA replication stress. Importantly, the observation that mutations of H4 lysine 91 and H3 lysine 56 are synthetically lethal with the H4 K5,12R allele suggests that the evolutionarily conserved diacetylation of the H4 NH₂-terminal tail may, in fact, have an essential function in the cell.

While the sites of acetylation on newly synthesized histones appear to play essential and functionally redundant roles, it is still an open question whether this essential function is chromatin assembly. As with any alterations to the histones, these mutations may impact the regulation of transcription in such a way as to negatively impact viability. However, the idea that the sites of acetylation on newly synthesized H3 and H4 function primarily through histone deposition is supported by a number of observations. First, these modifications are found on soluble histones that are in the process of chromatin assembly (7, 12, 14, 23, 71–73). Second, histone H3 lysine 56 has been directly shown to participate in chromatin assembly and can influence the binding of histones to histone chaperones that are involved in chromatin assembly (10, 35, 51). Third, this study provides a demonstration of a role for H4 lysines 5, 12, and 91 in DNA repair-linked chromatin reassembly. Finally, the accumulation of soluble histones when these sites are mutated strongly suggests that chromatin assembly has been globally altered.

Studies of the DNA damage checkpoint pathway have made the interesting observation that the regions of chromatin with which the checkpoint kinases Mec1p and Tel1p associate do not overlap the domains of chromatin that contain γ -H2AX (59).

FIG 8 Model for the involvement of chromatin assembly or histone exchange in DNA damage response signaling. The formation of domains of γ -H2AX is proposed to be a two-step process. The first step involves the phosphorylation of the soluble histone H2A by Mec1p or Tel1p. Chromatin assembly or histone exchange then incorporates the phosphorylated H2A into chromatin near the break site.

Tel1p is recruited to unprocessed double-strand breaks by the MRX complex, while Mec1p is recruited to resected double-strand breaks through interactions with Ddc2p and the single-strand binding complex replication protein A (RPA). The recruitment of these kinases to chromatin is necessary for the subsequent phosphorylation of histone H2A (39). One explanation for these unusual patterns is that the generation of large domains of chromatin that contain γ -H2AX may be the result of spreading of the kinases from their initial point of recruitment. Another possibility is that chromatin looping brings distal nucleosomes into contact with Mec1p/Tel1p located at the break, which allows for long stretches of chromatin to be modified. Our results suggest another possibility. Mutations in the sites of acetylation on histone H4 do not affect the production of γ -H2AX but do have a profound effect on the localization of γ -H2AX to chromatin. This suggests that the formation of γ -H2AX domains may be a multistep process in which localization of γ -H2AX to chromatin is a distinct step in the DNA damage checkpoint pathway (Fig. 8).

There are a number of models that can explain the involvement of sites of acetylation on newly synthesized histone H4 in the chromatin localization of γ -H2AX. The first is a model in which chromatin assembly or histone exchange plays a critical role in the chromatin localization of γ -H2AX. In this model, the recruitment of Mec1p and Tel1p to chromatin brings them into contact with soluble pools of histones that are concentrated in close proximity to chromatin. Mec1p and Tel1p then phosphorylate soluble histone H2A, which is then incorporated into nearby chromatin through chromatin assembly or histone exchange. The incorporation of γ -H2AX may require concomitant assembly of H3/H4, which would be facilitated by the proper modification state of histone H4. Conversely, the mutations in the sites of acetylation on newly synthesized histone H4 might actually increase the rate of histone exchange, leading to a loss of γ -H2AX from chromatin.

An alternative model is that combining the H4 K5,12R and H4 K91Q alleles destabilizes nucleosomes such that γ -H2AX is lost from chromatin. Indeed, it has been demonstrated that a mutation that eliminates the ability of H4 lysine 91 to form a salt bridge with glutamic acid 74 of histone H2B leads to a destabilized histone octamer (23). However, the crystal structure of a nucleosome that contains a mutation of histone H4 lysine 91 to glutamine

shows that, while the salt bridge is lost, there are no significant structural alterations to the nucleosome (74). In addition, γ -H2AX localization is not affected by the H4 K91Q allele alone. Therefore, the mutation at H4 lysines 5 and 12 must be contributing to the structural destabilization of the nucleosome. As these residues are not visible in nucleosome crystal structure, it is not clear whether they would alter nucleosome stability through a direct or indirect mechanism (75).

The involvement of histone exchange/chromatin assembly in the generation of γ -H2AX domains would be analogous to the mechanisms that have been identified as being responsible for the removal of H2A phosphorylation from chromatin in yeast. Pph3p is the phosphatase that is responsible for the dephosphorylation of γ -H2AX. However, in the absence of Pph3p, γ -H2AX is still lost from chromatin with normal kinetics (42). This indicates that γ -H2AX is removed from chromatin, perhaps by histone exchange, prior to dephosphorylation. Hence, modulation of the phosphorylation state of histone H2A may occur on soluble histones and chromatin assembly and histone exchange pathways may control the presence of γ -H2AX on chromatin.

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