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Phosphorylation of Histone H4 Serine 1 during DNA Damage Requires Casein Kinase II in *S. cerevisiae*

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Summary

Distinct patterns of posttranslational histone modifications can regulate DNA-templated events such as mitosis, transcription, replication, apoptosis, and DNA damage [1–5], suggesting the presence of a "histone code" in these nuclear processes [6, 7]. Phosphorylation of histone H2A S129 at sites of DNA double-strand breaks (DSBs) has been implicated in damage repair in yeast [8, 9]. Here, we describe another phosphorylation event on serine 1 (S1) of histone H4; this event is also associated with MMS- or phleomycin-induced DSBs but not with UV-induced DNA damage. Chromatin-immunoprecipitation (ChIP) studies of an HO-endonuclease-inducible strain show that S1 phosphorylation is specifically enhanced 20- to 25-fold in nucleosomes proximal to the DSB. In addition, we show that casein kinase II (CK2) can phosphorylate H4 S1 in vitro and that null or temperature-sensitive CK2 yeast mutants are defective for induction of H4 S1 phosphorylation upon DNA damage in vivo. Furthermore, H4 S1 phosphorylation and CK2 play a role in DSB re-joining as indicated by a nonhomologous end-joining (NHEJ) plasmid assay. CK2 has been implicated in regulating a DNA-damage response; our data suggest that histone H4 S1 is one of its physiological substrates. These data suggest that this modification is a part of the DNA-repair histone code.

Results and Discussion

Previous reports have documented a cell-cycle (S and M phases)-dependent increase in H4 S1 phosphorylation in cells of organisms ranging from worms and flies to mammals [10, 11], although the connection to DNA damage was not investigated. Preliminary experiments in yeast failed to detect an increase in H4 S1 phosphorylation in the *cdc20-1* mutant arrested in mitosis [12] (Figure S1 in the Supplemental Data available with this article online). However, treatment with methyl methane sulphonate (MMS) induces significant phosphorylation of H4 S1 (Figures 1A and S2). Whole-cell or nuclear extracts from yeast treated with 0.1% MMS and collected at successive time points were probed with an H4 S1 phospho-specific antibody [α-phos (S1) H4]. MMS-induced damage resulted in a dramatic increase of H4 S1 phosphorylation within 1 hr and peaked at 3 hr after treatment (Figure 1A). Equal sample loading was ensured by acetylation-specific antibody (α-acetyl H4) and Coomassie stain (Figures 1A and S3A). Parallel samples analyzed by flow cytometry showed no S-phase arrest at the time of H4 S1 phosphorylation induction (Figure S3B). In these experiments, yeast had arrested in the S phase within 27-hr after MMS treatment [13], but this arrest occurred long after the increase of H4 S1 phosphorylation. Hence, our data suggest that H4 phosphorylation is most likely not associated with DNA replication or synthesis of nascent histones during S phase.

To further confirm that H4 S1 phosphorylation is not dependent on cell-cycle stage, we arrested *MATa* yeast cells at G1 with α factor and then released them to proceed through the cell cycle in synchrony (Figure S4A) or released them into hydroxyurea (HU) to achieve an S-phase population of cells (Figure S5A). Whole-cell extracts were probed in Western analyses with H3 S10 phospho-specific antibody [α-phos (S10) H3] and α-phos (S1) H4. As expected, H3 S10 phosphorylation was enhanced during G2/M [14], but only baseline levels of H4 S1 phosphorylation were evident at any cell-cycle stage (Figures S4B and S5B), suggesting that MMS-induced H4 S1 phosphorylation is most likely due to a DNA-damage response independent of cell-cycle effects.

To further establish that the enhanced signal detected by α-phos (S1) H4 is specific to S1 in H4, H4 S1 was mutated to alanine (S1A), a nonphosphorylatable residue. As expected, this mutation abolishes the MMS-induced signal, indicating that H4 S1 is the site of phosphorylation during DNA damage (Figure 1B). Next, we sought to determine whether the enhancement of H4 S1 phosphorylation occurs during different types of DNA damage. Yeast was treated with phleomycin and MMS, which produce double-strand breaks (DSBs), or with UV radiation, which primarily induces several dipyrimidine photoproducts; only treatment with DSB inducers resulted in H4 S1 phosphorylation (Figure 1C).

We next sought to investigate the localization of H4 S1 phosphorylation in relation to sites of DSBs. A single DSB was induced at the mating-type (*MAT*) locus after expression of the homothallic switching (HO) endonuclease was induced [15, 16]. In chromatin-immunoprecipitation (ChIP) experiments, H4 S1 phosphorylation is specifically enhanced 20- to 25-fold at nucleosomes

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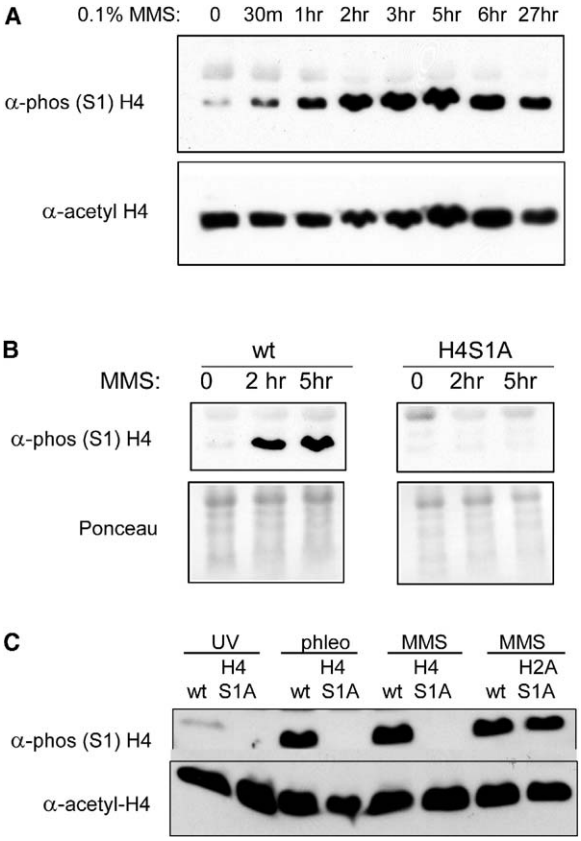

Figure 1. Phosphorylation of H4 S1 by Inducers of DNA Double-Strand Breaks

(A) Yeast was treated with 0.1% MMS and harvested at successive time points after induction. Whole-cell yeast extracts were probed with α-phos (S1) H4 by Western blot. α-acetyl H4 was used to estimate equal loading.

(B) H4 S1A mutant yeast was treated with 0.1% MMS and analyzed with α-phos (S1) H4. Ponceau S stain of the membrane indicates the levels of transferred protein.

(C) Wild-type (wt), H4 S1A, or H2A S1A yeast were treated with 100 J/m^2 UV radiation, 500 mg/ml phleomycin, or 0.1% MMS and analyzed by Western blot.

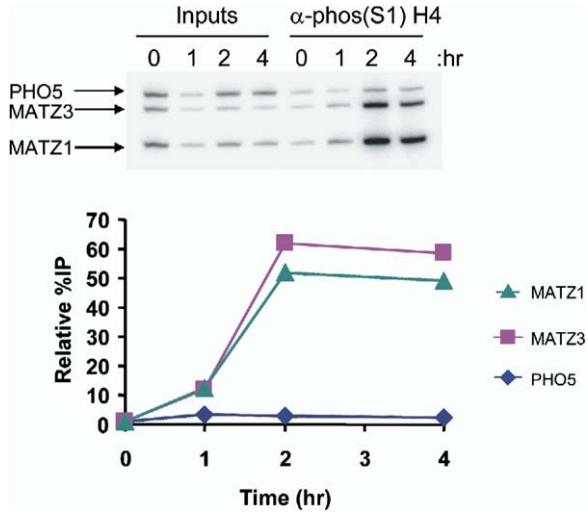

Figure 2. Localization of H4 S1 Phosphorylation at Double-Strand Breaks

ChIP analyses with MATα yeast that harbors deletions of both silent-mating-type loci and contains an integrated Gal10-HO gene. HO expression and cleavage at the MAT locus were induced by galactose and were followed by formaldehyde crosslinking and immunoprecipitation of chromatin with α-phos (S1) H4. Primers were used to detect regions adjacent to (MATZ1) or distal to (MATZ3) the HO DNA double-strand break [28]. Primers specific to the PHO5-promoter region were used as a control.

within 1 kb of the DSB (primers MATZ1 and Z3) as compared to unlinked loci on a different chromosome, such as *PHO5* (Figure 2). This increase occurred within 1 hr of DSB induction and lasted for at least 4 hr. Preliminary experiments indicated that levels of H4 S1 phosphorylation at HO-induced breaks in *rad51* and *rad54* mutants, which are deficient in homologous recombination (HR), were similar to levels at breaks in the wild-type (B.W. and C.L.P., unpublished data). These data suggest that ongoing recombination is not required for this phosphorylation event to occur. Taken together, our results demonstrate that H4 S1 phosphorylation is specifically localized to DSB in vivo and may be involved in aspects other than recombination during DSB repair.

To gain further understanding of the roles that H4 S1 phosphorylation plays in DNA damage, we sought to identify the responsible kinase(s). MEC1 and TEL1 kinases phosphorylate H2A S129 and are the main regulators of the DNA-damage pathway in yeast [8]. Therefore, we tested the effects of *mec1-21* and *mec1-21/tel1* mutations on the levels of MMS-induced H4 S1 phosphorylation and found little, if any, difference between these kinase mutants and the wild-type (Figure S6). Hence, we performed an in-gel kinase assay with nuclear extracts from MMS-treated yeast and histone H4 as the substrate (data not shown) and also performed a proteomic screen for H4 S1 kinases (T.K., S.L.B., J. Patacek, and M. Snyder, unpublished data). Unexpectedly, results from both approaches converged on one candidate kinase, suggesting that the catalytic subunits CKA1 and/or CKA2 of casein kinase II (CK2) were involved in the DNA-damage-induced phosphorylation of H4 at S1. In support of this possibility, we show that recombinant human CK2 and purified yeast CK2 complexes are able to phosphorylate H4 in vitro with either purified H4 or histone octamers as a substrate (Figure 3A, data not shown). We further explored this link by treating CK2 mutant strains [17] with MMS to determine the effect on H4 S1 phosphorylation in vivo. The MMS-induced H4 S1 phosphorylation is greatly diminished in a *cka1* null yeast strain and is largely undetectable in the double *cka1* null/*cka2* temperature-sensitive (ts) strain at the nonpermissive temperature (Figure 3B). These results, together with the in vitro kinase data, suggest that CK2 is directly involved in regulating this DNA-damage-induced phosphorylation event.

To further investigate a potential link among CK2, H4 S1 phosphorylation, and DNA damage, we sought to determine the sensitivity of CK2 mutants to the DNA-damaging agents phleomycin and MMS. We found that the CK2 mutant most deficient in phosphorylating H4

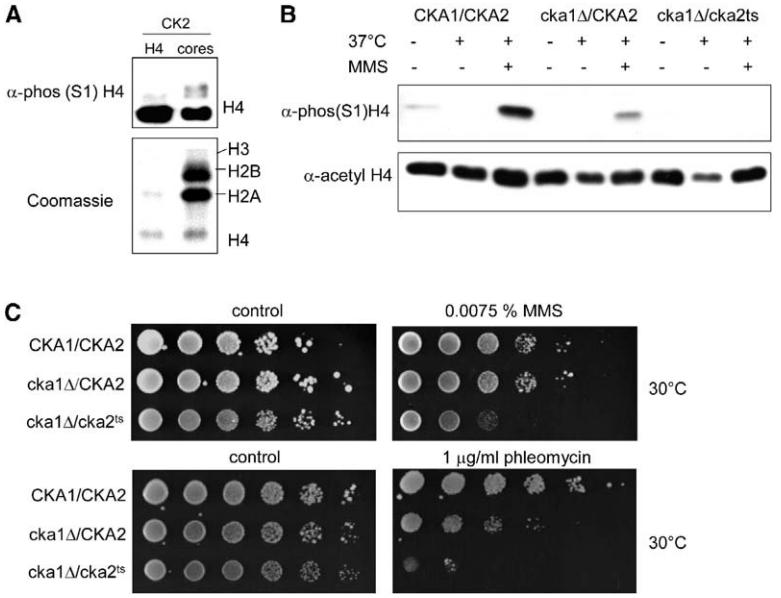

Figure 3. H4 S1 Phosphorylation Is Mediated by CK2

(A) In vitro kinase reactions with human recombinant CK2 and chicken H4 and core histones were probed with α-phos (S1) H4 by Western analyses.

(B) Wild-type and CK2 mutant yeast, containing deletion and/or temperature-sensitive mutations of the CK2 catalytic subunits, were treated with 0.1% MMS and analyzed by Western blot. Probing with α-acetyl-H4 allowed an approximation of equal loading.

(C) Serial dilutions of the indicated yeast strains and their isogenic wild-type control (W303) plated on medium containing 0.005% MMS or 1 μg/ml phleomycin.

S1 in vivo is also the most sensitive to DNA-damaging agents (Figure 3C), suggesting that H4 S1 might be one of the physiological substrates for CK2. However, H4 S1A and H4 S1E mutants do not exhibit sensitivity to DNA-damaging agents (Figure S7), implying that damage-induced H4 S1 phosphorylation is involved in a pathway that has other redundant or compensatory mechanisms.

Because the ChIP experiments demonstrated that H4 S1 phosphorylation occurs at HO-induced breaks (Figure 2), we investigated the role of H4 S1 phosphorylation in nonhomologous end joining (NHEJ). In an NHEJ plasmid-religation assay, a restriction-enzyme-digested plasmid cleaved within a selectable marker was transformed into H4 S1E, S1D, S1T, and wild-type strains. In the H4 S1E and S1D strains that may mimic constitutive S1 phosphorylation, the survival rates exceed those of the wild-type strain, (Figure 4A) suggesting that H4 S1 phosphorylation enhances the efficiency of plasmid rejoining. In contrast, the H4 S1T and S1A mutants display a phenotype similar to that of the wild-type strain (Figure 4A, data not shown), suggesting that the enhancement of DNA-religation efficiency is specific to a mutation that mimics phosphorylation. Similarly, the H2A S129E mutant, but not S129A, affects the accessibility of chromatin to nuclease digestion [8]. Our data and the data of Downs et al. [8] suggest that specific functions are conferred by the addition of a phosphate moiety to histone tails. Thus, it appears that the phosphorylation of H4 S1 is involved in promoting DSB repair by NHEJ. Consistent with this interpretation, the CK2 mutant that is most deficient in H4 S1 phosphorylation is also the least capable of rejoining enzyme-digested plasmids (Figure 4A). The NHEJ repair was accurate in each of these strains; the restriction site of the religated plasmid was maintained in the DNA isolated from resulting colonies (Figure 4B). Taken together, our results suggest that CK2 phosphorylation of H4 S1 and possibly other targets appears to contribute to NHEJ repair.

Here, we show that H4 S1 phosphorylation is induced during DNA damage and is specifically localized to the DSB. This phosphorylation is mediated by CK2 and appears to be involved in promoting DSB repair. Interestingly, CK2 has been implicated in the DNA-damage response because in yeast it regulates POLI/III-dependent gene expression [18] and controls adaptation to the

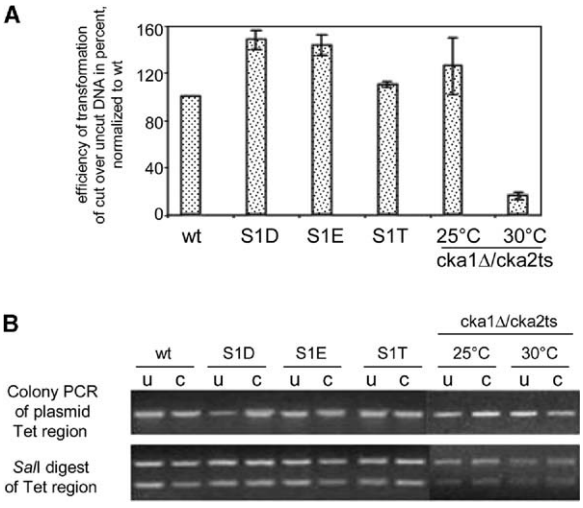

Figure 4. H4 S1 Phosphorylation Promotes NHEJ Repair

Wild-type (wt), H4 S1 mutants, and cka1Δ/cka2^{ts} mutants were transformed with uncut or SalI-digested plasmids YCp50.

(A) The percentage of transformation with cut versus uncut DNA was calculated for each strain. The efficiency was then normalized to the appropriate wild-type control, which was set to 100%. The graph values and standard errors represent the average of two separate transformation experiments.

(B) PCRs of the Tetr region at the YCp50 were performed for colonies transformed with either uncut (u) or SalI-digested (c) YCp50. These colonies consisted of either wild-type or H4 S1 mutant yeast. The PCR product was then digested with SalI to determine if the religation of cut YCp50 plasmid was accurate.

DNA-damage checkpoint [19], and because in mammalian cells it regulates p53 activity [20] and facilitates XRCC1-mediated repair of single-strand breaks [21]. Our data suggest that in yeast the phosphorylation of histone H4 serine 1 requires CK2 and that this event occurs at nucleosomes within 1 kb of DNA DSBs. In addition, CK2 and constitutively phosphorylated H4 S1 appear to promote DSB repair. The exact mechanism is unclear, but H4 S1 phosphorylation could mediate the DSB repair by recruiting the required *trans*-acting nuclear factors. Alternatively, H4 S1 phosphorylation may trigger the formation of a specialized *cis*-mediated chromatin structure that permits more-efficient religation of broken DNA ends at DSBs [22]. Regardless of the mechanism, we favor the view that H4 S1 phosphorylation is part of a poorly understood and poorly appreciated "histone code" for DNA-damage response. Investigation of these modification patterns will provide further insights into the mechanisms of genomic instability and tumorigenesis [23, 24].

Experimental Procedures

Strains and Plasmids

JHY62 (*H4 S1A*) was derived from wild-type yeast strain JHY86 [*MAT*α *ura3-52 leu2-3,112 trp1-289 his3Δ1 Δ(hht1 hhf1) Δ(hht2 hhf2)* pJH18 (*CEN ARS TRP1 HHT2 HHF2*)] [14]. Other histone H4 mutations were generated by site-directed mutagenesis of pRM204 (*HHF2-HHT2 CEN-ARS1TRP*). The following plasmids were plasmid shuffled into yeast strain FY1716 [*MAT*a *his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128*δ (*hht1-hhf1*)*Δ::LEU2* (*hht2-hhf2*)*Δ::HIS3* pDM9(*HHT1-HHF1 CEN-ARS1 URA3*)]: pTK54 (*hhf2 S1A-HHT2 CEN-ARS1 TRP*); pTK56 (*hhf2 S1D-HHT2 CEN-ARS1 TRP*); pTK57 (*hhf2 S1E-HHT2 CEN-ARS1 TRP*); and pTK58 (*hhf2 S1T-HHT2 CEN-ARS1 TRP*). Yeast strain ySL151, isogenic to FY1716 but carrying pRM204 instead of pDM9, was used as the wild-type H4 control strain.

YPH250 strain (*MAT*a *CKA1 CKA2*) [25] was transformed with pRS315 to make it isogenic to YDH6 (Δcka1 CKA2) and YDH8 (Δcka1 cka2^{ts}) [17].

Preparation of Whole-Cell Extracts, Electrophoresis, and Western Blotting

Yeast cells were grown in YPD to an OD_{600} density of 0.8 to 1.0, pelleted, washed with sterile distilled water, and frozen at –80°C. Yeast whole-cell extracts (WCE) were isolated as described previously [26]. In brief, the cell pellets were resuspended in breaking buffer (10 mM Tris [pH 7.4], 300 mM sorbitol, 600 mM NaCl, 5 mM $MgCl_2$, and 5 mM EDTA) and fresh protease and phosphatase inhibitors (1 μg/ml aprotinin, pepstatin, and leupeptin; 1 mM PMSF; and 1 μM microcystin-LR). Cells were disrupted with acid-washed glass beads in a mini-beadbeater, and the clarified supernatant was collected for electrophoresis on 15% SDS-PAGE gels as described previously [27]. Resolved proteins were transferred to PVDF membrane and probed with primary α-phos (S1) H4 or α-acetyl-H4 rabbit sera (1:5,000 and 1:10,000 dilutions, respectively, in 2% milk, Tris-buffered saline, and 1% Tween-20). HRP-conjugated donkey anti-rabbit secondary antibody (Amersham Pharmacia Biotech) was used at a 1:5,000 dilution and chemiluminescence was performed with the ECL plus kit (Amersham Pharmacia Biotech).

Exposure of Yeast to DNA-Damaging Agents

Yeast cultures at OD_{600} density of 0.8 to 1.0 were treated with 0.1% MMS (Sigma) or 500 μg/ml phleomycin (Research Products International) for 2 hr; alternatively, cultures were treated with 100 J/m^2 UV radiation, media were changed, and cultures were grown in the dark for 1 hr. For temperature-sensitive strains, yeast was grown to log phase at 25°C, MMS was added to 0.1%, and cultures were shifted to 37°C for 3 hr. For serial dilution assays on plates, yeast cells were diluted 10-fold in H_2O in a 96-well plate and spotted on SC-agar plates containing 0.0075% MMS or 1 μg/ml phleomycin. Plates were incubated at 30°C for 48 hr.

Chromatin Immunoprecipitation

Galactose induction of HO endonuclease and chromatin immunoprecipitation (ChIP) were performed as described previously [28]. In brief, yeast cells grown in YEP media containing 2% raffinose were induced to express HO upon galactose addition (2% final). At successive time points, samples were cross-linked with 1% formaldehyde for 15 min at 25°C, and the reactions were stopped by incubation in ice water. Samples were further prepared as described previously [29] and analyzed by radioactive semiquantitative PCR [30].

In Vitro Kinase Assay

Histones were enriched from chicken erythrocyte nuclear extracts with 0.4 N H_2SO_4, the soluble fraction of which was precipitated with 20% trichloroacetic acid, and resuspended in H_2O. Reverse-phase purified chicken H4 was kindly provided by C. Mizzen (University of Illinois at Urbana-Champaign). Histones were treated with shrimp alkaline phosphatase (Promega) (0.05 U/μg) for 30 min at 37°C and then for 30 min at 65°C. Human recombinant CK2 (UBI) (100 ng/reaction) was incubated with 2 μg H4 or 8 μg core histones, 0.08 mM cold ATP, and kinase buffer (20 mM MOPS, [pH 7.2], 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM Na_3VO_4, and 1 mM DTT) for 15 min at 30°C. Reactions were quenched with SDS loading buffer, resolved on 15% SDS-PAGE gel, and analyzed by Western blot with α-phos (S1) H4.

Nonhomologous End Joining Assay

Five ml of o/n YPD/SC-Leu cultures was grown at 30°C or 25°C (CK2 strains), used to inoculate fresh media at 0.125 OD_{600}, and shaken at 30°C or 25°C until an OD_{600} of 0.5 was reached. The cultures were harvested, washed once in water and once in 0.1 M LiOAc, and resuspended in 0.1 M LiOAc. The cells were adjusted to ~0.1 OD_{600}/μl in the case of histone mutants or to ~0.05 OD_{600}/μl in the case of CK2 mutants.

Competent yeast cells were transformed with equivalent amounts of uncut or SalI-digested yCP50. Ten microliters of sheared, heat-denatured salmon-sperm DNA (10 mg/ml) was added along with 400 μl of 40% polyethylene glycol in 0.1 M LiOAc into the transformation reaction and incubated for 30 min at 30°C. All transformation reactions were carried out in duplicate. Cells were heat shocked for 15 min at 42°C, harvested, resuspended in sterile water, plated on SC-Ura (wild-type and H4 mutant strains) or on SC-Ura-Leu (CK2 strains), and incubated at 30°C unless otherwise indicated for 2–3 days. Resulting colonies were counted, and the transformation efficiency was calculated as percentage normalized to wild-type. The graph values and standard errors represent the average of two separate transformation experiments.

Colony PCR, followed by SalI digestion of the Tet region of yCP50, was performed to assay for the accuracy in the repair of the digested plasmid used for transformation.

Supplemental Data

Nine supplemental figures can be found with this article online at http://www.current-biology.com/cgi/content/full/15/7/656/DC1/.

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