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Histone H3 Thr 45 phosphorylation is a replication-associated post-translational modification in *S. cerevisiae*

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Abstract

Post-translational histone modifications are crucial for the regulation of numerous DNA-templated processes, and are thought to mediate both alteration of chromatin dynamics and recruitment of effector proteins to specific regions of the genome¹. In particular, histone Ser/Thr phosphorylation regulates multiple nuclear functions in the budding yeast *Saccharomyces cerevisiae*, including transcription, DNA damage repair, mitosis, apoptosis and sporulation². Although modifications to chromatin during replication remain poorly understood, a number of recent studies have described acetylation of the histone H3 N-terminal α -helix (α N helix) at Lys 56 as a modification that is important for maintenance of genomic integrity during DNA replication and repair^{3,4}. Here, we report phosphorylation of H3 Thr 45 (H3-T45), a histone modification also located within the H3 α N helix in *S. cerevisiae*. Thr 45 phosphorylation peaks during DNA replication, and is mediated by the S phase kinase Cdc7–Dbf4 as part of a multiprotein complex identified in this study. Furthermore, loss of phosphorylated H3-T45 causes phenotypes consistent with replicative defects, and prolonged replication stress results in H3-T45 phosphorylation accumulation over time. Notably, the phenotypes described here are independent of Lys 56 acetylation status, and combinatorial mutations to both Thr 45 and Lys 56 of H3 cause synthetic growth defects.

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AUTHOR CONTRIBUTIONS S.P.B. performed purifications of TAP-tagged Cdc7 complex, recombinant Cdc7 and Dbf4 expression and purification, *in vitro* histone and peptide kinase assays, mass spectrometric identification of Thr 45 phosphorylation, antibody characterization, yeast strain generation, mutagenesis, growth assays, synchronization experiments, inductions, and associated western blotting. J.P. identified and isolated the native Cdc7 histone kinase complex, generated the Cdc7–TAP strain and performed the Cdc7 immunoprecipitation and associated kinase assays. S.A. and J.R.Y. performed the mass spectrometric identification of the kinase complex subunits and subsequent data analysis. Q.Q. and M.M.S. performed and analysed the FACS data. J.S. and D.F.H. assisted with the mass spectrometric mapping of the histone phosphorylation mark and data analysis. P.A.G. conducted experimental design and analysis. S.P.B. and P.A.G. wrote the manuscript.

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METHODS Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturecellbiology/>

Together, these data identify and characterize H3-T45 phosphorylation as a replication-associated histone modification in budding yeast.

We set out to identify histone kinases in *S. cerevisiae* through extensive chromatographic separation of candidate activities. A number of histone-modifying enzymes, including kinases, have been successfully purified from yeast extracts using Ni^{2+} -NTA agarose resin in the absence of any affinity tags^{5,6}. In this study, yeast whole-cell extracts were bound to Ni^{2+} -NTA agarose and fractionated through multiple rounds of chromatography (Supplementary Information, Fig. S1a). Resultant fractions containing distinct histone kinase activities from the final separation were silver-stained and analysed by tandem mass spectrometry (Fig. 1a, b). This study revealed a multiprotein complex containing the S phase-regulating kinase Cdc7–Dbf4. Accompanying protein bands from this silver-stained complex were identified, but await validation. As Cdc7 has not previously been reported as a core histone kinase, it was subsequently immunoprecipitated from extracts to verify this activity (Fig. 1c). Our results suggest that the native Cdc7–Dbf4 complex is capable of phosphorylating core histones.

The Ser/Thr kinase Cdc7 is a key regulator of cell-cycle progression into S phase⁷. Enzymatic activity of Cdc7 is regulated by association with the unstable regulatory protein Dbf4 (ref. 8), whose expression fluctuates throughout the cell cycle, peaking late in G1 before onset of S phase⁹. Cdc7 has been linked directly to chromatin and to numerous nuclear functions requiring access to DNA¹⁰, and thus histones represent a likely target for its enzymatic activity. Specifically, Cdc7–Dbf4 binds to chromatin at replication origins¹¹, where it has been implicated in the phosphorylation of the Mcm2–7 helicase complex^{12,13}. To further investigate the histone kinase activity of Cdc7, we generated a yeast strain bearing a TAP tag located downstream of the endogenous *CDC7* locus, allowing purification of native Cdc7 and its associated proteins by calmodulin affinity. The complex was partially purified by conventional chromatography, then bound to calmodulin–sepharose beads and eluted using EGTA (Supplementary Information, Fig. S1b). Gel filtration chromatography indicates that Cdc7 exists in a multiprotein complex with a relative molecular mass exceeding 1,000K, consistent with the number of protein bands observed in our original silver stain (Supplementary Information, Fig. S1c). The final calmodulin elution was subsequently assessed for kinase activity using histone core octamers to identify the specific histones most likely to be targets *in vivo*. Our results indicate that histone H3 is a major *in vitro* target of native Cdc7 when in the context of the histone octamer (Fig. 1d).

To identify specific sites of histone phosphorylation, kinase assays were repeated using recombinant histone H3, and the products were analysed by tandem mass spectrometry. Trypsin digestion and MS/MS analysis of H3 phosphorylated *in vitro* revealed a phosphopeptide corresponding to residues 41–49, with the sequence YRPGpTVALR (Fig. 2a). To verify this result, we subsequently performed kinase assays with peptides containing Thr 45 or several previously described H3 phosphorylation sites, either pre-phosphorylated or unmodified. Only the peptide containing unmodified Thr 45 gave counts significantly greater than the auto-phosphorylation observed in the control experiment (no peptide) when incubated with the Cdc7–TAP complex (Supplementary Information, Fig. S2a). Additionally, Cdc7 and Dbf4 co-expressed recombinantly and purified from *Escherichia coli* were able to phosphorylate H3-T45 *in vitro* (Fig. 2b; Supplementary Information, Fig. S2b). Taken together, these data indicate that H3-T45 is a specific *in vitro* substrate of Cdc7.

Thr 45 lies within the H3 α N helix, a remarkably conserved region that makes critical contacts with DNA when assembled into the nucleosome core particle (Supplementary Information, Fig. S3a, b)^{14,15}. In particular, Thr 45 is located precisely at the points of entry

and exit of DNA on the nucleosome, and the Thr side chain of this residue interacts directly with the DNA entry gyre¹⁶. Thus, phosphorylation of this residue may heavily influence DNA–histone interactions within the core particle. Interestingly, a number of recent papers have described acetylation of H3 Lys 56 (H3-K56), a residue that is also located within the α N helix¹⁷. This modification is associated with proper cell-cycle progression, recovery from replicative stress, and promotion of genomic stability^{3,4,18}.

To verify that phosphorylation of Thr 45 occurs *in vivo*, we generated an antibody specific for this modification. Peptide dot blots indicate that this antibody recognizes phosphorylated Thr 45, and does not cross-react with several known H3 phosphorylation sites (Fig. 2c). Yeast cells expressing either wild-type or *T45A*-mutated histone H3 were grown to mid-log phase, and whole-cell extracts were analysed by western blotting. As expected, H3-T45 phosphorylation signal was observed in the wild-type extracts but absent from the *T45A* mutant, indicating that Thr 45 is indeed phosphorylated *in vivo* (Fig. 2d). To investigate the dependence of this modification on Cdc7, we used *bob1* and *bob1 cdc7 Δ* yeast strains. Mutation of the *MCM5* gene, termed *bob1*, allows for deletion of the otherwise essential *CDC7* gene¹⁹. When we performed the same western blot experiment in these yeast strains, we found that H3-T45 phosphorylation signal was markedly decreased in the absence of Cdc7 (Fig. 2d), supporting the findings of our initial *in vitro* assays. Thus, Cdc7 mediates phosphorylation of H3-T45 both *in vitro* and *in vivo*.

We next sought to determine whether this modification is enriched during replication. Yeast cultures were grown to mid-log phase and treated with the ribonucleotide reductase inhibitor hydroxyurea to cause S phase arrest. After 2 h of treatment, the level of Thr 45 phosphorylation was increased over that observed in asynchronous cells (Fig. 3a; Supplementary Information, Fig. S4a). Notably, Mec1 and Tel1, the yeast homologues of the ATM/ATR checkpoint kinases, are not required for induction of Thr 45 phosphorylation (Fig. 3b). We also synchronized cells with nocodazole and collected histone samples at 15 min intervals subsequent to release (Fig. 3c). Western blot analysis showed that H3-T45 phosphorylation fluctuates throughout the cell cycle and peaks before expression of the G2/M cyclin Clb2 and in coordination with H3-K56 acetylation, which is enriched during S phase³. These findings link the timing of Thr 45 phosphorylation to replication.

As Thr 45 phosphorylation occurs during S phase, we reasoned that loss of this modification may cause replication defects. In addition to the *T45A* mutant described above, we generated a yeast strain mutated at H3-T45 to glutamic acid, to investigate the effects *in vivo* of either inhibited or constitutive phosphorylation. After 5 days, growth of *T45A* mutants was slow, compared with wild-type yeast, whereas *T45E* mutants seemed non-viable, indicating that genome-wide constitutive incorporation of a negative charge at this residue is very poorly tolerated (Fig. 4a). The slow growth of *T45A* was further investigated by growth curve analysis in culture, which shows that *T45A* mutants progress at a much slower rate than wild-type yeast, comparable to yeast lacking *CDC7* (Fig. 4b).

We also sought to determine whether loss of H3-T45 phosphorylation would lead to a heightened sensitivity to replication stress and/or DNA damage. Tenfold serial dilutions indicate that both *T45A* mutation and deletion of *CDC7* cause sensitivity to hydroxyurea and the topoisomerase I inhibitor camptothecin (CPT), indicating that loss of Thr 45 phosphorylation causes replication defects (Fig. 4c). Importantly, neither mutation of Thr 45 nor loss of Cdc7 affected Lys 56 acetylation levels (Fig. 2d); thus, the phenotypes observed in the *T45A* and *bob1 cdc7 Δ* mutants do not simply occur because of alterations in the proximal Lys 56 modification state.

We further reasoned that if phosphorylation of H3-T45 is important for resistance to hydroxurea and CPT, then treatment of yeast cultures with these agents may lead to accumulation of this modification. We found that replication stress does indeed increase Thr 45 phosphorylation levels after 1–2 h of treatment in culture (Fig. 4d). Importantly, treatment of yeast with the DNA alkylating agent methyl methanesulphonate (MMS) was not found to induce Thr 45 phosphorylation. This result agrees with our finding that *T45A* yeast are highly sensitive to replication stress, but has no apparent sensitivity to MMS by serial dilution (Fig. 4c).

Finally, we compared *T45A* with both *K56R* and *T45A-K56R* yeast to further verify that the two modifications function independently. The slow growth phenotype observed in *T45A* seemed much more severe than that of *K56R*, and was heightened when the two mutations were present in combination, as assessed by streaking on medium plates, or by growth in culture (Fig. 5a, b). Furthermore, we found that neither mutation alone had a significant effect on the post-translational modification status of the other residue (Fig. 5c). Finally, we analysed asynchronous cultures of the mutants by flow cytometry to assess their DNA content profiles (Fig. 5d). Compared with yeast expressing wild-type H3, *H3-K56R* yeast showed a higher proportion of cells in the G2-M stage of the cell cycle, consistent with the previously described profile of yeast lacking the H3-K56 acetyltransferase Rtt109 (ref. 18). By contrast, *T45A* yeast showed a delay at all cell-cycle stages, with a significantly greater amount of time spent in G1/S, compared with wild-type yeast. Our results show that the Thr 45 and Lys 56 mutants behave distinctly, and thus the post-translational modifications of the two residues function independently.

Taken together, our findings describe H3-T45 phosphorylation, a post-translational modification in a crucial region of the nucleosome core particle. Additionally, we have identified a histone kinase responsible for generating this modification, the S phase regulatory kinase Cdc7–Dbf4, which exists partially in its native state as a component of a much larger multiprotein complex. Phosphorylated Thr 45 is present in greatest abundance during S phase, and loss of this modification results in slow growth and drug sensitivities, which indicates replication defects, consistent with the phenotypes caused by a loss of Cdc7.

Given that Thr 45 phosphorylation is not essential for cell survival, we find it likely that this modification is not required for entry into S phase. Although Cdc7 has been implicated in the firing of replication origins, it is also thought to function in preserving the integrity of actively progressing replication forks in the event of replication stress and fork stalling²⁰. A recent study showed that *Xenopus laevis* Cdc7 regulates DNA replication reinitiation during the S phase checkpoint recovery in extracts that had been treated with etoposide²¹. Furthermore, constitutive genomewide mimicking of Thr 45 phosphorylation via *T45E* mutation is very poorly tolerated. This is not surprising given the close proximity of the negatively charged DNA-phosphate backbone to this residue, and may indicate that localized placement of a phosphate molecule on this Thr serves to disrupt DNA–histone contacts at the onset of replication, or adjacent to sites of stalled replication forks. Indeed, mutation of Thr 45 has recently been shown to affect nucleosome dynamics *in vitro* with respect to the wrapping of DNA around the nucleosome core particle¹⁶. Given the direct contact between the Thr 45 side chain and the DNA entry gyre, it seems likely that transient phosphorylation of this residue could have a dramatic effect on nucleosome stability and DNA accessibility.

Furthermore, recent studies have also identified Thr 45 phosphorylation in human neutrophils, where it functions in apoptosis²². Notably, it is not uncommon for phosphorylation of a single histone residue to function in multiple pathways, as has been well documented for phosphorylation of histone H3 Ser 10 (ref. 23). To investigate whether

H3-T45 phosphorylation similarly functions in yeast apoptosis, we monitored the modification upon treatment of yeast cultures with H₂O₂ (1 mM). We found no discernable increase in Thr 45 phosphorylation levels after 2 h of treatment (Supplementary Information, Fig. S4b), indicating that H3-T45 probably does not function in this apoptotic pathway in yeast. Nevertheless, the findings that this residue may function in different pathways across multiple organisms provide further evidence that its position in the histone octamer is critical to nucleosome function.

Interestingly, the phenotypes of the *T45A* mutant in response to replication stress seemed more severe than those observed in the *bob1 cdc7Δ* strain. It is possible therefore that Thr 45 is phosphorylated by another enzyme(s) that contributes to genomic stability. Furthermore, our studies showed that *T45A* does not result in sensitivity to MMS, and Thr 45 phosphorylation is not induced by prolonged treatment with MMS in culture. Given the extreme sensitivity of *rtt109Δ* and *H3-K56* mutants to MMS reported in previous studies^{3,4,18}, along with our findings that Lys 56 acetylation and Thr 45 phosphorylation seem to have no interdependency, we propose that the phenotypes described here are independent of H3-K56 acetylation, and thus are indicative of a distinct replicative function. Our findings reveal a mechanism of Cdc7–Dbf4 function during S phase, which ultimately may provide insight into their possible roles in the development of cancer.

METHODS

Yeast strains

Histone mutants were generated as described previously²⁴. See Supplementary Information, Table S1 for complete strain information.

Medium

For S phase arrest, mid-log phase yeast growing in YPD medium were treated with hydroxyurea (200 mM) for 2 h. For M phase arrest and release, mid-log phase yeast growing in YPD medium were treated with nocodazole to a final concentration of 15 μg ml⁻¹. After 3 h, yeast cells were spun down and washed with sterile water twice, then resuspended in fresh YPD medium. A 10 ml sample was collected every 15 min for 150 min after release, and extracted with trichloroacetic acid (TCA). For assessment of sensitivity to DNA damage and replication stress, mid-log phase yeast was serially diluted (10-fold) onto YPD plates with or without methyl methanesulphonate (MMS, 0.05%), hydroxyurea (200 mM), or camptothecin (CPT, 15 μg ml⁻¹) and incubated for 5 days at 30°C. For treatment in culture, yeast was grown to a density of 10³⁰ cells ml⁻¹ in YPD and treated with either hydroxyurea (200 mM), CPT (50 μg ml⁻¹), MMS (0.5%), or H₂O₂ (1 mM), and 10 ml samples were collected and TCA-extracted at 30 min time-points for 2 h.

Kinase assays and histone isolation

Chromatographic fractions and γ³²P-labelled ATP (Amersham) were incubated with core histones (Millipore), recombinant H3 (Millipore), H3 peptides (Lake Placid Biologicals), or core histone octamers for 30 min at 30°C. Octamers were purified as described previously²⁵. Reaction products were electrophoresed and exposed to autoradiography film, or spotted onto Whatman filters and counted in a scintillation counter (Beckman Coulter) as described previously²⁶.

Western blotting

Immunoblot analysis of chromatographic fractions was performed using anti-protein A antibody (Sigma). Whole-cell extracts for histone western blots were prepared by TCA extraction as described previously²⁷, and immunoblot analyses were performed using anti-

histone H3 (Active Motif), anti-histone H3-T45 Phos (Active Motif), anti-histone H3-K56 Acetyl (Abcam), or anti-His (Santa Cruz). For dot blots, the indicated amounts of histone peptides were spotted onto nitrocellulose and probed with anti-histone H3-T45 Phos.

Flow cytometry

Yeast cells isolated in triplicate from log phase cultures were fixed with 70% ethanol, treated with RNase A ($10 \mu\text{g ml}^{-1}$) for 2 h at 37°C , stained with SYTOX Green ($2 \mu\text{M}$; Invitrogen), and analysed with a BD FACSCalibur flow cytometer as described previously²⁸. The fractions of the populations in G1, S, and G2/M phases were estimated by nonlinear least squares curve fitting as described previously²⁹. Apparent generation times were calculated from the doubling times of exponentially growing cultures. Using the generation times and the percentage of cells in each phase, the temporal lengths of the G1, S, and G2/M were estimated as described previously³⁰. The G1 lengths reported are averages calculated by setting mother and daughter cell cycle times to be equal³⁰. The means and standard deviations for each strain were determined from three independent cultures established from different single colonies.

Purification of kinase complexes

The purification scheme used to isolate native histone kinase complexes in this study is shown in Supplementary Information, Fig. S1a, and is adapted from the isolation of histone acetyltransferase (HAT) protein complexes from yeast described previously³¹. Briefly, whole-cell extracts from 12 litres of yeast grown to mid-log phase in YPD were bound to Ni^{2+} -NTA agarose resin (Qiagen), and poured over a Poly-Prep chromatography column (Bio-Rad). Resin was washed with imidazole (20 mM), followed by elution with imidazole (300 mM). Ni^{2+} eluate was loaded directly onto a 1 ml Mono Q HR 5/5 column (GE Healthcare), and bound proteins were eluted with a 25-ml linear gradient from 100 – 500 mM NaCl. Peak fractions, as assessed by histone kinase activity, were pooled and concentrated to 0.7 ml with a Centrprep YM-30 concentrator (Millipore). Samples were then loaded on a Superose 6 HR 10/30 column (GE Healthcare) in 350 mM NaCl. Peak Superose 6 fractions were diluted to 100 mM NaCl, and loaded onto a 1 ml Mono S HR 5/5 column (GE Healthcare). Bound proteins were eluted with a 25-ml linear gradient from 0.1 – 1 M NaCl. Peak Mono S fractions were pooled and diluted to 100 mM NaCl, and loaded onto a 1 ml Mono Q HR 5/5 column (GE Healthcare). Bound proteins were eluted with a 10-ml linear gradient from 0.1 – 1 M NaCl. Peak Mono Q fractions were diluted to 100 mM NaCl and loaded onto a 1 ml heparin Sepharose column (GE Healthcare). Bound proteins were eluted with a 10-ml linear gradient from 0.1 – 1 M NaCl. Peak heparin Sepharose fractions were diluted to 100 mM and loaded onto a 1-ml histone agarose column (Sigma). Bound proteins were eluted with a 10-ml linear gradient from 0.1 – 1 M NaCl. Peak histone agarose fractions were diluted to 100 mM NaCl and loaded onto a Mini Q PC 3.2/3 column (GE Healthcare). Bound proteins were eluted with a 4.8-ml gradient from 100 – 500 mM NaCl. The calibration of the Superose 6 column was as follows: dextran blue (M_r 2,000K), fraction 15; apoferritin (M_r 443K), fraction 20/21; alcohol dehydrogenase (M_r 150K), fraction 23/24; carbonic acid (M_r 29K), fraction 28/29.

Partial purification of Cdc7–TAP was performed as previously described for the SAGA and SLIK HAT complexes³², with modifications. Briefly, whole-cell extracts from 4 l of *CDC7–TAP* yeast grown to mid-log phase in YPD were bound to and eluted from Ni^{2+} -NTA agarose resin as described above. Ni^{2+} eluate was loaded directly onto a Tricorn Mono Q 5/50 GL column (GE Healthcare), and eluted with a 25-ml linear gradient of 100 – 500 mM NaCl. Peak fractions of Cdc7 (by anti-protein A western blot) correlating with histone kinase activity were pooled and concentrated to 0.7 ml using a Centrprep YM-30 concentrator (Millipore). Samples were then loaded onto a Superose 12 HR 10/30 column

(GE Healthcare) in 350 mM NaCl. The calibration of the Superose 12 column was as follows: dextran blue (M_r 2,000K), fraction 11/12; apoferritin (M_r 443K), Fraction 18/19; alcohol dehydrogenase (M_r 150K), fraction 21/22. Peak Superose 12 fractions were diluted to 150 mM NaCl and bound to and eluted from 100 μ l calmodulin sepharose resin (Stratagene) as described previously³³.

Immunoprecipitation

α Cdc7 antibody (8 μ l; gift from R. Sclafani, University of Colorado, Denver, USA) or pre-immune serum was bound to 20 μ l protein A sepharose beads (GE Healthcare) for 1 h, and washed with binding buffer (150 mM NaCl, 50 mM Hepes (pH 7.5), 10% glycerol, 0.1% Tween-20, 1 mM PMSF). Ni^{2+} eluate (75 μ l) was then bound to beads for 2 h at 4°C in binding buffer. Beads were washed three times, and resuspended in 25 μ l of binding buffer. A 5 μ l aliquot of the slurry was incubated with 2 μ g of core histones and assessed for histone kinase activity as described above.

Mass spectrometry

For identification of native kinase complex components (Supplementary Information, Fig. S5), silver-stained protein bands from peak fractions were excised and in-gel trypsin-digested, and peptides were identified by microcolumn high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry and database searching as described previously³⁴.

For identification of phosphorylated histone H3 residues, kinase reactions were performed as described above, except that γ -³²P-labelled ATP was replaced with unlabelled ATP. After 30 min incubation, histones were acid extracted from reactions using 0.4 N H_2SO_4 , precipitated with TCA and lyophilized, as described previously³⁵. Samples were derivatized by treatment with propionylation reagent (25 μ l propionic anhydride, 75 μ l methanol) before and after digestion with trypsin (Promega) as described previously³⁶ to convert ϵ amino acid groups of Lys residues and peptide N termini to propionyl amides. Derivatized peptides were enriched for phosphorylation by immobilized metal affinity chromatography (IMAC) as described previously³⁷, with slight modifications. Briefly, samples were first treated with esterification reagent (160 μ l acetyl chloride, 1 ml methanol) and incubated at room temperature for 1 h to convert peptides to corresponding methyl esters. Samples were then lyophilized and reconstituted in a 1:1:1 mixture of methanol, MeCN, and 0.1% acetic acid, and loaded onto IMAC columns constructed by packing fused-silica capillary columns (360 μ m o.d. $\times$ 100 μ m i.d.) with POROS MC (PerSeptive Biosystems). IMAC columns were activated with 100 mM FeCl_3 before loading samples. Samples were then washed with 0.01% acetic acid and eluted with 250 mM ascorbic acid onto capillary precolumns (360 μ m o.d. $\times$ 75 μ m i.d.) packed with irregular (5–20 μ m) C18 resin. Samples bound to pre-column were washed with 0.1% acetic acid and connected by Teflon tubing to an analytical capillary column (360 μ m o.d. $\times$ 50 μ m i.d.) packed with regular (5 μ m) C18 resin and equipped with a laser-pulled emitter tip. Samples were analysed by nanoflow HPLC-microelectrospray ionization on a linear quadrupole ion trap-Fourier Transform Ion Cyclotron Resonance (LTQ-FT-ICR) mass spectrometer (Thermo Electron)³⁸, and MS/MS spectra were interpreted manually.

Expression of recombinant proteins

Cdc7 and Dbf4 were expressed individually or together in DH5 α competent cells (Invitrogen) from plasmid BG1805 (Open Biosystems). Proteins were partially purified using Ni^{2+} -NTA agarose resin (Qiagen), and incubated in kinase assays with histone H3 and analyzed by immunoblotting as described above.

Image construction

Nucleosome images were created using PyMol software at <http://people.virginia.edu/~dta4n/biochem503/nucleosome.html>.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Identification of the Cdc7–Dbf4 histone kinase complex. **(a)** Histone kinase assays using purified chromatographic fractions from yeast whole-cell extracts. Fractions from the final Mini Q column are shown. **(b)** Silver stain of Mini Q fraction 20, the peak of histone kinase activity from **a**. Proteins identified by mass spectrometry and/or confirmed by western blot are labelled; unlabelled bands await confirmation. **(c)** Histone kinase assays using proteins immunoprecipitated by either anti-Cdc7 antibody or pre-immune sera from extracts that were bound to and eluted from Ni²⁺-NTA agarose resin. **(d)** Kinase assays using histone octamers and the purified native Cdc7–TAP complex as indicated.

Figure 2.

Mapping of Cdc7-dependent histone phosphorylation. **(a)** Cdc7–TAP histone kinase assay reaction products were analysed by mass spectrometry. Tandem MS spectrum of the precursor $[M + 2H]^{2+}$ ion at 591.8054 m/z is shown. Propionyl and methyl ester derivatives are indicated by (Pr) and (OMe), respectively. Underlined mass values indicate b and y ions identified. **(b)** Western blots of H3 kinase assays using recombinant Cdc7 and Dbf4 either co-expressed or expressed independently. **(c)** Dot blot showing specificity of anti-phospho H3-T45 (H3-T45 phosph) antibody. **(d)** T45A mutation or CDC7 deletion reduces H3-T45 phosphorylation signal in western blots.

Figure 3.

Histone H3 T45 phosphorylation is linked to replication in yeast. **(a)** Yeast expressing His-tagged histone H3 were arrested with hydroxyurea (HU, 200 mM) and assessed for H3-T45 phosphorylation or total H3 (His). **(b)** Yeast strains lacking Sml1 (WT), Mec1, and/or Tel1 were either left untreated or treated with HU (200 mM) for 3 h, extracted with trichloroacetic acid, and assessed by western blotting for histone H3 T45 phosphorylation and total H3. **(c)** Western blots of wild-type yeast released from M phase arrest were probed with the indicated antibodies.

Figure 4.

Mutation of T45 causes sensitivity to replication stress. **(a)** Viabilities of yeast expressing wild-type (WT), *T45A*, or *T45E* histone H3 plated on complete supplement mixture (CSM)-Leu for selection of the mutant plasmid, and 5'-FOA for counter-selection of the wild-type (WT) plasmid. **(b)** Growth curve analysis of *H3-T45A* mutation and *CDC7* deletion relative to parental strains. **(c)** Serial dilution to assess hypersensitivities of *H3-T45A* and *cdc7Δ* deleted yeast to methyl methanesulphonate (MMS), hydroxyurea (HU), or camptothecin (CPT). **(d)** Treatment with HU or CPT increases H3-T45 phosphorylation, as detected by western blotting. Results in panels **b** are shown as the mean $\pm$ s.e.m. ($n = 3$). Uncropped images of blots are shown in Supplementary Information, Fig. S6.

Figure 5.

H3-T45 phosphorylation is distinct from H3-K56 acetylation. **(a)** Viability of yeast expressing wild-type (WT) H3, or mutations of H3-T45 and H3-K56, alone or in combination. **(b)** Growth curve analysis of *H3-K56R*, *H3-T45A*, and *H3-T45A-K56R* mutations relative to parental strain. OD; optical density. **(c)** Western blots showing Thr 45 phosphorylation and Lys 56 acetylation are unaffected by mutation of the opposite residue. **(d)** Flow cytometry profiles of asynchronous yeast cultures expressing WT, *K56R*, or *T45A* histone H3. Fractions of cell populations in G1, S, and G2/M phases and estimated temporal lengths of each phase are indicated. Results in panels **b** are shown as the mean $\pm$ s.e.m. ($n = 3$). Uncropped images of blots are shown in Supplementary Information, Fig. S6.