Modifications of Human Histone H3 Variants during Mitosis†

Benjamin A. Garcia,[‡,§] Cynthia M. Barber,[§,||] Sandra B. Hake,[⊥] Celeste Ptak,[‡] Fiona B. Turner,[||] Scott A. Busby,[‡] Jeffrey Shabanowitz,[‡] Richard G. Moran,[#] C. David Allis,[⊥] and Donald F. Hunt*[,‡,▽]

Department of Chemistry, University of Virginia, Charlottesville, Virginia 22904, Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, Virginia 22908, Laboratory of Chromatin Biology, The Rockefeller University, New York, New York 10021, Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, Virginia 23298, and Department of Pathology, University of Virginia, Charlottesville, Virginia 22904

Received May 16, 2005; Revised Manuscript Received July 29, 2005

ABSTRACT: Phosphorylation of histone H3 is a hallmark event in mitosis and is associated with chromosome condensation. Here, we use a combination of immobilized metal affinity chromatography and tandem mass spectrometry to characterize post-translational modifications associated with phosphorylation on the N-terminal tails of histone H3 variants purified from mitotically arrested HeLa cells. Modifications observed in vivo on lysine residues adjacent to phosphorylated Ser and Thr provide support for the existence of the "methyl/phos", binary-switch hypothesis [Fischle, W., Wang, Y., and Allis, C. D. (2003) *Nature 425*, 475−479]. ELISA with antibodies selective for H3 at Ser10, Ser28, and Thr3 show reduced activity when adjacent Lys residues are modified. When used together, mass spectrometry and immunoassay methods provide a powerful approach for elucidation of the histone code and identification of histone post-translational modifications that occur during mitosis and other specific cellular events.

Packaging and organization of enormous amounts of DNA in eukaryotic chromosomes is accomplished by forming a dynamic higher-order nucleosome complex containing histone proteins, the monomeric repeating units of chromatin. About 146 bp of DNA are wrapped around a histone octamer consisting of two H2A−H2B dimers and an H3−H4 tetramer (*1*, *2*). Although histone proteins themselves are highly conserved, variation to the nucleosomes is provided by the incorporation of specialized histone variants or extensive covalent modifications. Histone post-translational modifications have been observed mainly on the flexible highly charged amino terminal tails that extend beyond the surface of the nucleosome (*3*, *4*). Lysine (Lys) residues on histone proteins can be modified through acetylation, methylation, and ubiquitination, arginine (Arg) residues through methylation, while phosphorylation can occur at serine (Ser) and threonine (Thr) residues. More importantly, emerging evidence suggests that histone modifications may occur in deliberate patterns associated with specific nuclear events. This idea has been referred to as the "histone code" hypothesis and states that specific single modifications or combinations of modifications on histone residues increase binding affinity for certain chromatin-associated proteins and may direct distinct downstream events (*5*). For example, methylation (which can occur as mono-, di-, or trimethylation) of Lys9 or Lys27 on histone H3 is linked to gene silencing by the recruitment of heterochromatin protein 1 (HP1) and polycomb effector molecules, respectively (*6*−*7*). Conversely, histone acetylation of any H3 Lys residue or methylation of Lys4 or Lys79 has been associated with the transcriptional activation of genes (*8*−*10*). Although the importance of histone H3 methylation and acetylation has been the focus of countless studies and these reactions are reviewed in several reports (*6*−*11*), the significance of histone H3 phosphorylation remains unclear.

Phosphorylation of histone H3 at Ser10 has been demonstrated to be closely associated with chromosome condensation during mitosis and meiosis in various eukaryotic organisms (*12*−*16*). Genetic work in *Tetrahymena* micronuclei also showed that a mutation of Ser10 to an alanine residue led to abnormal chromosome condensation and segregation with a large extent of chromosome loss during mitosis (*14*). However, this point mutation did not have the same effect in *Saccharomyces cerevisiae* (*15*); thus it was concluded that other similar phosphorylation marks on histone H3 or other histones may exist and play important roles during mitotic chromosome condensation. In fact, another phosphorylation site of H3 at Ser28 has also been characterized and linked to chromosome condensation, albeit to a much lesser degree than Ser10 phosphorylation (*17*). Additionally, inhibition of H3 phosphorylation prevents the initiation of chromosome condensation and entry into mitosis, while inhibition of H3 dephosphorylation causes irregularities during mitotic exit (*18*−*20*). Histone H3 phosphorylation has also been occasionally observed outside of mitosis. Some studies have demonstrated that histone H3 phosphorylation at Ser10 rapidly increases during the expression of early

† This work was supported by grants from the National Institutes of Health to D.F.H. (GM37537) and C.D.A. (GM40922), The Rockefeller University's Women & Science Fellowship Program to S.B.H., and the Ford Foundation to B.A.G.

* To whom correspondence should be addressed. Tel: 434-924-3610. Fax: 434-982-2781. E-mail: dfh@virginia.edu.

‡ Department of Chemistry, University of Virginia.

§ B.A.G. and C.M.B. contributed equally to this work.

|| Department of Biochemistry and Molecular Genetics, University of Virginia.

⊥ The Rockefeller University.

Virginia Commonwealth University.

▽ Department of Pathology, University of Virginia.

10.1021/bi050906n CCC: $30.25
Published on Web 09/03/2005

response proto-oncogenes such as *c-fos* and *c-jun* upon growth factor treatment or other types of stimulation (*21*). H3 phosphorylation has also been observed to be triggered by trichostatin A or ultraviolet B treatment (*22*, *23*). Therefore, while H3 (Ser10 and Ser28) phosphorylation is well documented and likely an important step for normal chromosome condensation, the exact functions of these modifications in this and other cellular processes remain elusive.

To better understand histone H3 phosphorylation, it is necessary to identify and characterize the complete profile of histone H3 modifications occurring during mitosis. The focus of traditional methods for the investigation of histone post-translational modifications has been based primarily on metabolic labeling and/or immunoassay techniques. Antibody-dependent assays such as Western blot, immunoprecipitation, and immunofluorescence are highly sensitive and do not require a well-purified sample. Nevertheless, complications may arise when multiple modifications occur on the same histone, as simultaneous presence of other modifications may impact the specificity of the site-specific antibody (*24*, *25*). In addition, the generation of site-specific antibodies is a labor-intensive process.

Recently mass spectrometry (MS)[1] has become an important tool for the analysis of histone post-translational modifications, but these experiments have primarily focused on the characterization of modifications on Lys and Arg residues, and mention little if any phosphopeptide information (*26*−*29*). Therefore, a prime objective of this work is to use phosphoproteomic MS methods in combination with immunoassay techniques to globally characterize phosphorylated H3 proteins for a comprehensive look at the modification patterns present on all three human H3 variants (H3.1, H3.2, and H3.3) from HeLa cells arrested in mitosis. This report shows that an exceedingly complex pattern of histone H3 modifications exist *in vivo*, which remain poorly understood, and may prove difficult to analyze by immunoassay methods alone. Less known H3 phosphorylation sites at Thr3 and Ser31 (histone H3.3 variant) were also identified by MS using our approach. ELISA experiments demonstrated that the sole use of antibodies for detection of histone post-translational modifications might be problematic and difficult to fully interpret. In conclusion, this work will provide better methods to produce more complete information for evaluating the function of histone H3 phosphorylation in the "histone code" during mitosis and other cellular processes.

MATERIALS AND METHODS

Cell Culture and Preparation of Histone Protein Samples. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GibcoBRL, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (*30*). The cells were maintained in a humidified 37 °C incubator with 5% CO_2 atmosphere. To arrest cells in mitosis, nocodazole (1.5 μg/mL) was added for 18 h followed by agitation to release loosely associated mitotic cells from the tissue culture plates. Adherent cells were released by trypsinization and washed in phosphate buffered saline solution, pH 7.5, and then all cells were pelleted by centrifugation at 2500*g* for 10 min at 4 °C. Cell pellets were resuspended in 500 μL of nuclear isolation buffer (NIB250, 5 mM Tris-HCl, pH = 7.5, 60 mM KCl, 15 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM DTT, 2mM sodium vanadate, 1 μg/mL microcystein, 250 mM sucrose, 2.5 μg/mL leupeptin; 2.5 μg/mL pepstatin; 2.5 μg/mL aprotinin; 2.5 μg/mL antipain; 2.5 μg/mL chymostatin and 1 mM PMSF + 0.6% NP-40 detergent), and the released nuclei were pelleted by centrifugation at 15000*g* for 5 min at 4 °C. For extraction of histone proteins, pelleted nuclei were resuspended in approximately 500 μL of NIB250 buffer and 2 mL of 0.4 N sulfuric acid was added while incubating the solution on ice for 30 min. Insoluble proteins were pelleted by centrifugation at 14000*g* for 10 min. The acid soluble histone proteins were then precipitated with 20% trichloroacetic acid. The resulting protein pellet was washed with 1 mL of 1% HCl in acetone, followed by two washes (1 mL each) with 100% acetone and dissolved in 200 μL of deionized H_2O.

Fluorescence-Activated Cell Sorting (FACS) Analysis. 1 × 10^7 cells from asynchronous and nocodazole-arrested HeLa cell cultures were fixed with 75% ethanol, stained with 2 μL of propidium iodide (500 μg/L), and analyzed by FACS at the University of Virginia FACS core facility.

Reverse Phase HPLC (RP-HPLC) Purification of Histones. Bulk histones obtained by acid extraction of nocodozole-arrested HeLa cells were fractionated on a C8 column (220 by 4.6 mm Aquapore RP-300, Perkin-Elmer) using a linear ascending gradient of 35−60% solvent B (solvent A, 5% acetonitrile, 0.1% TFA; solvent B, 90% acetonitrile, 0.1% TFA) over 75 min at 1.0 mL/min on a Beckman Coulter System Gold 126 pump module as previously described (*31*). The elution profile was monitored at 214 nm, and 0.8 mL fractions were collected. Fractions from each peak were pooled, dried under vacuum, and stored at −80 °C. Prior to MS analysis, an aliquot from each fraction was resolved on a 15% SDS−PAGE gel (*32*) to access purity of fractionated histones.

Propionylation and Enzymatic Digestion of Histone H3. Histone H3 (150 pmol) in 15 μL of ammonium bicarbonate (100 mM, pH 8) was treated with 15 μL of a 3:1 mixture of methanol (75 μL) and propionic anhydride (25 μL) (Aldrich, Milwaukee, WI) (*33*). The pH of the resulting solution was adjusted to 8 by dropwise addition of ammonium hydroxide (15 N). After standing at 37 °C for 15 min the reaction mixture was taken to dryness on a Speed Vac, and the derivatization and solvent removal steps were then repeated. Propionylated H3 was dissolved in 100 mM ammonium bicarbonate (50 μL, pH 8) and digested with either chymotrypsin (Roche, Indianapolis, IN) or trypsin (Promega, Madison, WI) (substrate:enzyme ratio of 20:1) for 6 h at 37 °C. Digestion was terminated by adding concentrated acetic acid to the mixture and by freezing the solution.

Mass Spectrometry (MS). Peptides generated from an H3 enzymatic digestion were loaded onto capillary precolumns (360 μm o.d. × 75 μm i.d., Polymicro Technologies, Phoenix, AZ) packed with irregular C18 resin (5−20 μm, YMC Inc., Wilmington, NC) and washed with 0.1% acetic

[1] Abbreviations: Pr, propionyl; pS, phosphoserine; pT, phosphothreonine; Ac, acetyl; Me, methyl; IMAC, immobilized metal affinity chromatography; MS, mass spectrometry; RP-HPLC, reverse-phase high-performance liquid chromatography; LTQ-FT, linear quadrupole ion trap Fourier transform; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; MS/MS, tandem mass spectrometry.

acid at a flow rate of 5 μL/min for 10 min. Precolumns were connected with Teflon tubing to capillary analytical columns (360 μm o.d. x 50 μm i.d., Polymicro Technologies, Phoenix, AZ) packed with C18 resin (5 μm, YMC Inc., Wilmington, NC) constructed with an integrated electrospray emitter as previously described (*34*). All samples were analyzed by nanoflow HPLC-microelectrospray ionization on either a Finnigan LCQ Deca XP ion trap (Thermo Electron, San Jose, CA) or a Finnigan linear quadrupole ion trap Fourier transform ion cyclotron resonance (LTQ-FT) mass spectrometer (Thermo Electron, San Jose, CA) equipped with a 7-T magnet. The gradient used on a model 1100 series HPLC solvent delivery system (Agilent, Palo Alto, CA) consisted of 0−60% B in 60 min or 0−60% B in 120 min (solvent A = 0.1% acetic acid, solvent B = 70% acetonitrile in 0.1% acetic acid) or other similar gradients. The LCQ and LTQ-FT mass spectrometers were both operated in the data-dependent mode throughout the HPLC gradient. For LCQ data-dependent experiments, the mass spectrometer acquired 1 full MS spectrum and 5 subsequent MS/MS spectra of the 5 most abundant parent ions. LTQ-FT experiments were performed by acquiring MS spectra using the FT-ICR (resolution = 100,000 at *m*/*z* 400) with the 10 most abundant ions being isolated and fragmented in the linear ion trap. The LTQ-FT-MS was externally calibrated using a standard peptide mixture over a broad *m*/*z* range. All MS/MS spectra were searched against sequences of human histone H3 isoforms downloaded from the NCBI database using the Mascot program (Matrix Science, London, U.K.) (*35*). The mass tolerances used were 2.0 Da for parent ions and 0.5 Da for fragment ions. Propionylation (Pr, +56 Da), propionylation-Me (PrMe, +70 Da) and acetylation/trimethylation (+42 Da) were searched on lysine residues as variable modifications. Phosphorylation (serine and threonine residues, +80 Da) was also searched as a variable modification. A static modification (Me, +14 Da) was used as modifications on all Asp and Glu residues, as well as C-termini when peptides were converted to their corresponding methyl esters. All peptide assignments of the corresponding MS/MS spectra were validated by manual inspection. All intact H3 protein mass spectra were deconvoluted using the ProMass 2.3 software (Novatia LCC, Princeton, NJ).

Immobilized Metal Affinity Chromatography (IMAC). To prepare the samples for IMAC, peptides were first converted to their corresponding methyl esters by addition of 100 μL of methanolic HCl and incubated at room temperature for 1 h (*36*). Methanolic HCl consists of mixing 160 μL of acetyl chloride in 1 mL of MeOH producing a 2 M methanolic HCl solution. IMAC columns were constructed by packing capillary columns (360 μm o.d. × 150 μm i.d.) with 10 cm of POROS 20 MC (Applied Biosystems, Framingham, MA). The IMAC columns were activated with 100 mM $FeCl_3$ solution (Aldrich, Milwaukee, WI) at a flow rate of 1 μL/min for 20 min. Histone peptides generated from the enzymatic digest were redissolved in 20 μL of a solution containing equal parts of methanol, 0.1% acetic acid, and acetonitrile and then loaded onto the IMAC column. After sample loading, the column was washed with 0.01% acetic acid (15 μL) and phosphopeptides were eluted with 20 μL of a 250 mM Na_2HPO_4 (pH = 6, Aldrich, Milwaukee, WI) or a 250 mM ascorbic acid (Aldrich, Milwaukee, WI) solution onto C18 packed capillary columns.

Enzyme-Linked Immunosorbent Assay (ELISA). A synthetic peptide of the H3 1−20 residues containing Ser10 phosphorylation was prepared at the Baylor University peptide facility and was used to produce an H3 Ser10 phos polyclonal rabbit antibody (Covance Inc., Princeton, NJ). The monoclonal H3 Ser28 phos antibody was given as a gift (*37*). The H3 Ser10 phos monoclonal antibody (clone 3H10) and Thr3 phos polyclonal antibody were purchased from Upstate Biotechnology Inc. (Lake Placid, NY). ELISA procedures were performed as described previously (*38*). Briefly, ELISA employed a bound horseradish peroxidase enzyme conjugate that detected the hydrolysis of *o*-phenylenediamine dihydrochloride (Sigma Co., Milwaukee, WI), at a wavelength of 492 nm. H3 Ser28-phos ELISA utilized the following synthetic peptides: H3 residues 21−33, ATKAARKSAPATG; H3 Ser28-phos residues 21−33, ATKAARK**pS**APATG; H3 Ser10-phos residues 1−15, ARTKQTARK**pS**TGGKA; H3 K27-Ac/Ser28-phos residues 21−33, ATKAAR$\mathbf{K}_{Ac}$**pS**APATG; H3 K27-methyl/Ser28-phos residues 21−33, ATKAAR$\mathbf{K}_{Me}$**pS**APATG; and CENP-A Ser7-phos residues 4−17, RRR**pS**RKPEAPRRRS. H3 Ser10-phos (both poly and monoclonal antibodies) ELISA used the following synthetic H3 peptides: H3 Ser10-phos 1−15, ARTKQTARK**pS**TGGKA; H3 K9-Ac/Ser10-phos 1−15, ARTKQTAR$\mathbf{K}_{Ac}$**pS**TGGKA; H3 K9-methyl/Ser10-phos 1−15, ARTKQTAR$\mathbf{K}_{Me}$**pS**TGGKA; H3 Ser28-phos residues 21−33, ATKAARK**pS**APATG; H3 residues 1−15, ARTKQTARKSTGGKA; and CENP-A Ser7-phos residues 4−17, RRR**pS**RKPEAPRRRS. Synthetic H3 peptides used in the H3 Thr3-phos ELISA experiments included the following: unmodified H3 residues 1−15, ARTKQTARKSTGGKA; H3 Ser10-phos residues 1−15, ARTKQTARK**pS**TGGKA; H3 Ser28-phos residues 21−33, ATKAARK**pS**APATG; H3 Thr11-phos residues 7−20, ARKS**pT**GGKAPRKQL; H3 Thr3-phos residues 1−11, AR**pT**KQTARKST; and H3 Thr3-phos/K4-methyl residues 1−11, AR**pT**K_{Me}QTARKST. Synthetic peptides were immobilized to the microtiter dishes by overnight incubation of peptide solutions.

RESULTS

Mass Spectrometry (MS) Characterization of Phosphorylated H3 Variants. To separate human H3 variants, an approach previously used to separate alfalfa and *Drosophila* H3 variants (*39*, *40*) and which we successfully optimized for mammalian cells in a previous study (*41*) was utilized. Mammalian cells contain three different histone H3 proteins: H3.1, H3.2, and H3.3. H3.1 and H3.2 differ by only one amino acid at position 96 (Cys → Ser), while H3.1 and H3.3 differ by five amino acids at positions 31 (Ala → Ser), 87 (Ser → Ala), 89 (Val → Ile), 90 (Met → Gly), and 96 (Cys → Ser). Acid-extracted bulk histones from mitotically arrested HeLa cells were fractionated by RP-HPLC using a C8 column as shown in Supplemental Figure 1A (Supporting Information). H2B is the first of the core histones to elute followed by a fraction containing a mixture of both H4 and H2A and then by a fraction containing solely H2A isoforms. H3 variants are the last of the core histones to elute and are separated into two peaks. RP-HPLC fractionation of histone H3 allowed for 5 fractions to be obtained from each peak in the chromatographic separation (peak 1 HPLC fractions 66−70 and peak 2 HPLC fractions 72−76). In peak 1, protein eluting in the first three HPLC fractions and protein eluting

in the last two HPLC fractions were pooled separately, and all fractions of peak 2 were pooled. The purity of separated histone H3 proteins from the fractions spanning the two peaks was assessed by SDS−PAGE analysis (Supplemental Figure 1B). As can be seen from the SDS−PAGE analysis, all H3 proteins in both peaks were present at roughly the same MW, indicating that all H3 molecules were at full length. ESI-MS spectra of H3 proteins from the first three fractions of peak 1, the last two fractions of peak 1, and the pooled fractions of peak 2 are shown in Supplemental Figure 2 (Supporting Information). Reconstructed mass spectra created using the deconvolution software ProMass 2.3 display the molecular weight of each H3 protein in the peak fractions and are shown as insert mass spectra. The most abundant deconvoluted molecular weights of the pooled early peak 1 fractions, the late peak 1 fractions, and the peak 2 fractions were 15407, 15351, and 15423 Da, respectively. All molecular weights of the pooled HPLC fractions were higher than the calculated monoisotopic molecular weights of the H3 isoforms (H3.1 = 15 263 Da, H3.2 = 15 247 Da and H3.3 = 15 187 Da), suggesting that the H3 proteins in all the HPLC fractions are full intact H3 proteins modified by post-translational modifications.

Compositions of the two HPLC histone H3 peaks were then confirmed by tandem mass spectrometry of the HPLC fractions. As mentioned earlier, careful collection of HPLC fractions allowed for 5 fractions to be collected across each peak. In peak 1, protein eluting in the first three and protein eluting in the last two HPLC fractions were pooled separately and each fraction was propionylated and digested separately with trypsin and chymotrypsin. Digests were then analyzed by LC−MS/MS. In the early three fractions of peak 1 (fractions 66−68), peptides corresponding to histone H3.2 were mostly detected (Supplemental Table 1, Supporting Information). Digests of the last two fractions in peak 1 (fractions 69−70) generated peptides that were unique to histone H3.3 (Supplemental Table 2, Supporting Information), such as the peptide RFQSAAIGALQEASEAY (MS/MS spectrum of the $[M + 2H]^{2+}$ ion at 934.4626 *m*/*z* of this peptide is shown in Supplemental Figure 3, Supporting Information). This MS result is in agreement with our previous study that indicated that histone H3.3 only eluted in later HPLC peak 1 fractions as detected by a histone H3.3 specific antibody (*41*). Sequence coverage for the H3.2 and H3.3 variants based on peptides observed in the two digests were both over 90% and spanned from the N-terminus to the C-terminus. Purity of the early and late eluting fractions of peak 1 was estimated from MS ion abundances observed for all peptides corresponding to residues 27−40 that contain either alanine (H3.2) or serine (H3.3) at position 31. Histone H3.2 fractions were found to be 90% pure, while histone H3.3 fractions were found to be 80% pure. No unique peptides from histone H3.1 were detected in the first HPLC peak. For peak 2, all five fractions were pooled and analyzed as above. MS/MS fragmentation of a $[M + 2H]^{2+}$ ion at *m*/*z* 1347.1352 (Supplemental Figure 4, Supporting Information) determined the sequence of the peptide to be ALQEACEAYLVGLFEDTNLCAIH, solely found on histone H3.1. A list of peptides from the tryptic and chymotryptic digest of HPLC peak 2 is shown in Supplemental Table 3 (Supporting Information). From this analysis, it was determined that histone H3.1 was near exclusively present in the second H3 HPLC peak. Sequence coverage for H3.1 based on peptides observed in the two digests was also over 90%. No unique peptides corresponding to histone H3.2 were detected, but some trace amounts of histone H3.3 were observed. Ion abundances observed for sequences containing residues 27−40 that contain either alanine (H3.1) or serine (H3.3) at position 31 indicated that the composition of peak 2 was 99% H3.1 and 1% H3.3. Therefore, we have demonstrated that the fractionation and subsequent enrichment of mammalian H3 variants by RP-HPLC is possible.

Previous mass spectrometry studies have focused on mapping the post-translational modifications on histones isolated from asynchronously grown cells where phosphorylation is at a minimum (*26*−*29*). Here we analyze histone H3 variants isolated from HeLa cells arrested in mitosis where phosphorylation is at a maximum and characterize the modifications that exist in combination with phosphoserine and phosphothreonine. All H3 variants were first treated with propionic anhydride reagent before digestion with trypsin or chymotrypsin. Previously we determined that reacting highly basic histone peptides with propionylation reagent results in the removal of charge from the side chains of monomethylated and endogenously unmodified lysine residues and the N-termini of peptides by generating a propionyl amide chemical bond (+56 Da), thus also increasing retention on a C18 column (*33*). Tandem mass spectra of highly charged peptides are also difficult to interpret, and removal of charge through propionylation assists in this manner as well. The digestion of propionylated H3 isoforms also blocks trypsin from cleaving on the C-terminal side of unmodified lysines (endogenously modified lysines are not proteolyzed by trypsin). Consequently, a trypsin digest of propionylated histone H3 results in proteolytic cleavage only C-terminal to Arg residues generating a known set of peptides that can be readily analyzed by MS. Shown in Figure 1A is the MS/MS spectrum recorded on the $[M + 2H]^{2+}$ ion at *m*/*z* 554.2823 for a peptide observed in the trypsin digest of propionylated histone H3.1. This mass corresponds to residues 9−17 containing two methyl marks and 1 acetyl mark plus either pSer or pThr. Acetyl and trimethyl marks differ in mass by 0.036 Da and are easily distinguished on the LTQ-FT mass spectrometer. Observed fragment ions (above and below the figure inset) confirm the sequence to be K_{Me2}**pS**TGGK_{Ac}APR. Ser10 phosphorylation is known to accompany chromosome condensation and segregation during mitosis and meiosis in ciliate, yeast, worm, and vertebrate organisms (*13*−*16*). Additionally, several reports have also associated this modification site with transcriptional activation (*21*, *42*). Although H3 Ser10 phosphorylation is well documented and likely to be an important step in chromosome condensation, the exact role of this modification remains to be elucidated. Histone H3 containing a Ser10 to Ala mutation fails to exhibit a clear mitotic phenotype in yeast (*15*). This suggests that other potentially redundant mitotic phosphorylation marks exist on either H3 or other histones. Several additional sites are reported in the present work. Shown in Figure 1B is the tandem mass spectrum recorded on the $[M + 2H]^{2+}$ ion at *m*/*z* 862.4678. This mass corresponds to residues 27−40 of histone H3.2 containing three methyl marks and either pSer or pThr. Observed fragment ions (above and below the figure inset) place the phosphate and trimethyl groups on Ser28 and Lys27,

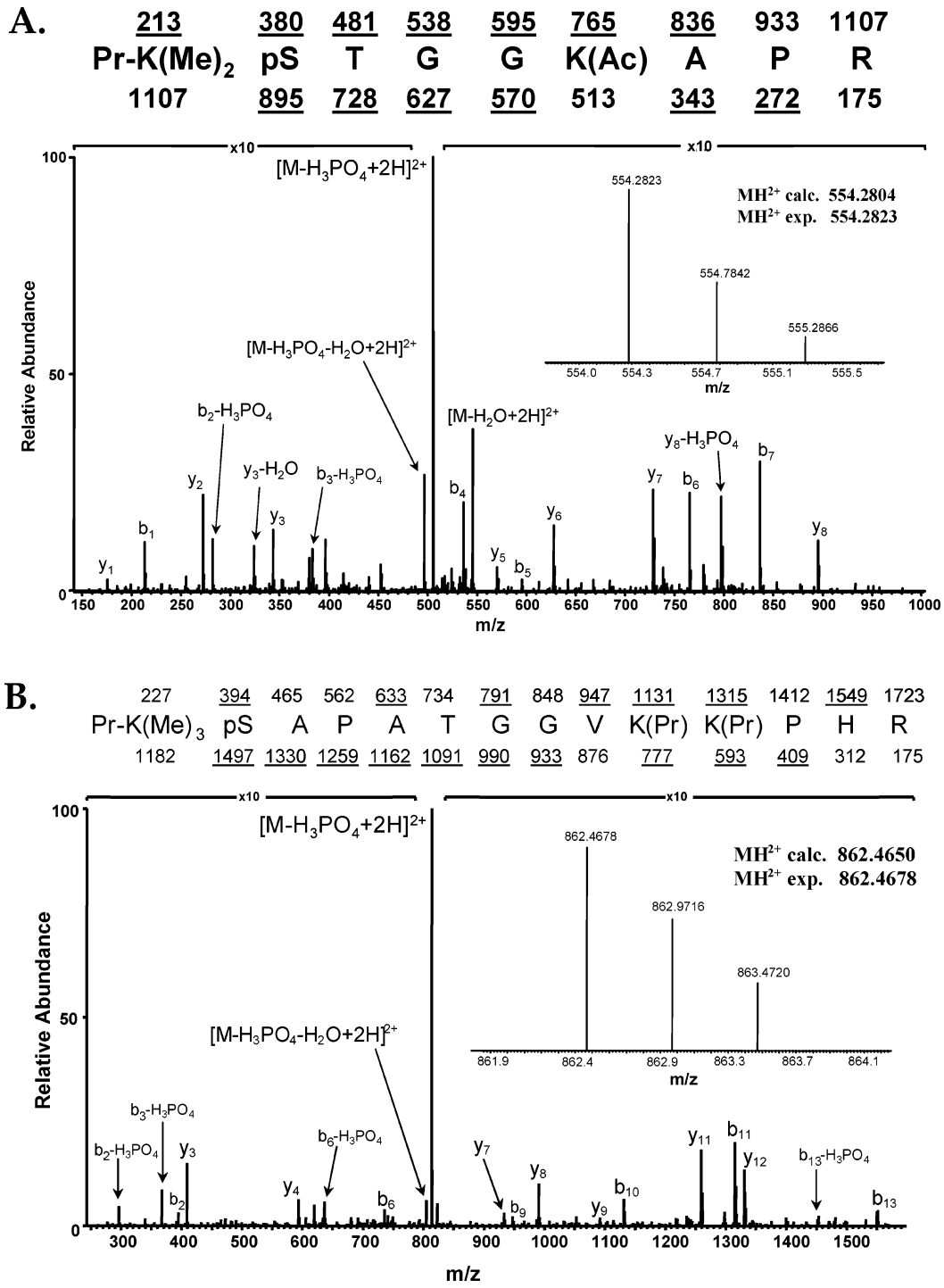

FIGURE 1: Tandem mass spectra of propionylated, post-translationally modified histone H3 peptides. Measured and theoretical masses for the parent $[M + 2H]^{2+}$ ions are shown in the figure insets. Propionylation of the N-terminus and the epsilon side chains of Lys residues are indicated by Pr (+56 Da). (A) MS/MS spectrum recorded on the $[M + 2H]^{2+}$ ion (*m*/*z* 554.2823) corresponding to the H3.1 residues 9−17 containing dimethyl, acetyl, and phospho marks on Lys9, Lys14, and Ser10, respectively. (B) MS/MS spectrum recorded on the $[M + 2H]^{2+}$ ion (*m*/*z* 862.4678) corresponding to the H3.2 residues 27−40 containing trimethyl and phospho marks on Lys27 and Ser28, respectively.

respectively, and confirm the sequence as K_{Me3}**pS**APATGGVKKHR.

The histone H3.3 variant is found at transcriptionally active loci (*43*) and is enriched in post-translational modifications associated with active chromatin (acetylated Lys14, Lys18, and Lys23; methylated Lys4, Lys36, and Lys79) (*40*). Nevertheless, phosphopeptides were not detected in that earlier study (*40*). In the present work, we were able to resolve the H3.2 and H3.3 variants by RP-HPLC and to characterize the post-translational modifications on each phosphorylated isoform. Note that the H3.3 isoform contains a Ser at position 31, while the same residue in the other two isoforms is Ala. Thus, the tryptic peptide containing residues 27−40 of H3.3 is 16 Da heavier than the corresponding peptides from H3.2 and H3.1. Shown in Figure 2 is the MS/MS spectrum recorded on the $[M + 2H]^{2+}$ ion at *m*/*z* 863.4601. This mass corresponds to residues 27−40 of histone H3.3 containing two methyl marks and either pSer

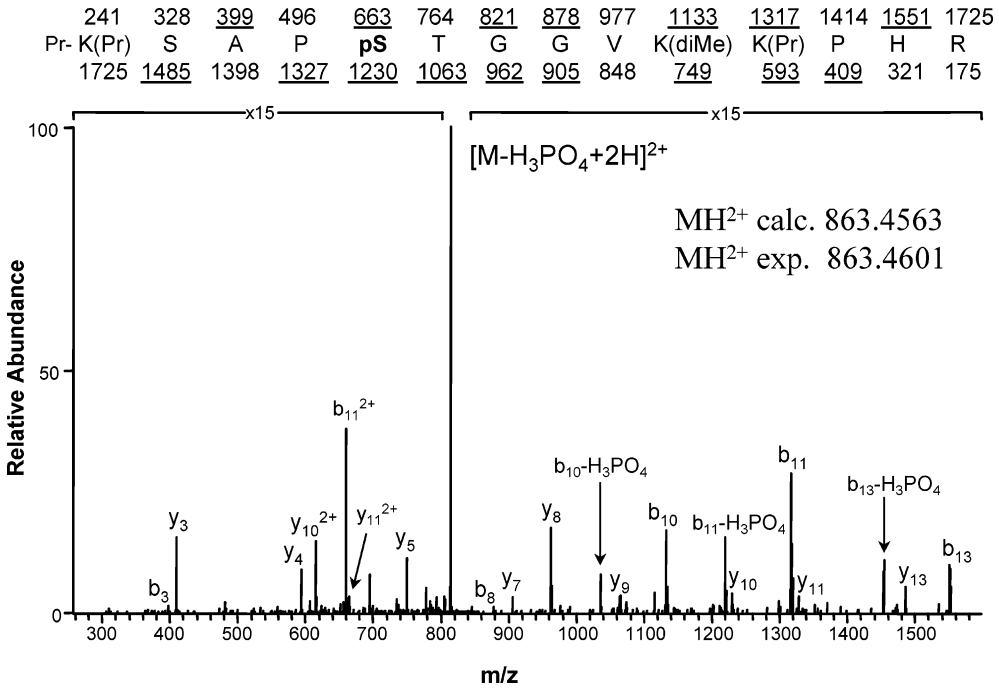

FIGURE 2: Identification of Ser31 as a novel phosphorylation site on the histone H3 variant H3.3. MS/MS spectrum recorded on the $[M + 2H]^{2+}$ ion (*m/z* 863.4601) corresponding to residues 27−40 containing phospho and dimethyl marks on Ser31 and Lys36, respectively.

or pThr. The observed fragment ions place the two methyl groups on Lys36 and the phosphate on Ser31, respectively. Phosphorylation of Ser31 is the first variant specific modification to be identified on any of the three H3 isoforms. We recently determined this Ser31 phosphorylation mark to be localized to regions surrounding centromeres during mitosis (*41*), and current work is underway to determine its biological significance. This study expands on that initial analysis and comprehensively lists all of the phosphorylated peptides (all phosphorylation sites and lysine modifications found in combination with those phosphorylation sites) observed on the H3.3 variant.

Shown in Table 1 is a list of all phosphopeptides sequenced by tandem mass spectrometry from each of the histone H3 variants. We observe that Ser10 and Ser28 are both phosphorylated on all three variants and are easily detected by using conventional C18, nanoflow-HPLC interfaced to the LTQ-FTMS instrument. To detect phosphopeptides at lower levels, we converted propionylated H3 peptides to methyl esters and then enriched the sample for phosphopeptides by using immobilized metal affinity chromatography (IMAC). Phosphopeptides identified by this approach are labeled (*b*) in Table 1. We also note that phosphopeptides that contain methyl marks on Lys were found in greater abundance than those with acetyl marks on Lys residues, as acetylated phosphopeptides were only detected after IMAC enrichment. All phosphopeptides detected in the conventional C18 analyses were seen in the IMAC−LC−MS/MS experiments. Tandem mass spectra of two phosphopeptides only observed after IMAC enrichment are shown in Figure 3. Displayed in Figure 3A is the MS/MS spectrum recorded on the $[M + H]^+$ ion at *m/z* 809.3916 for a peptide generated in a chymotrypsin digest of H3.1. The observed mass corresponds to residues 1−5 containing a single phosphorylation mark. The observed fragment ions define the sequence as AR**pT**KQ. Note that the spectrum recorded on the corresponding synthetic peptide is identical (Figure 3 inset). In support of this phosphorylation site, immunocytochemical evidence for phosphorylation of histone H3 at Thr3 has also been recently reported (*44*, *45*). We also employed IMAC to detect a propionylated chymotryptic peptide containing residues 1−19 of H3.1. The MS/MS spectrum recorded on the $[M + 3H]^{3+}$ ion (*m/z* 842.1017) for this peptide is shown in Figure 3B. The observed mass corresponds to residues 1−19 plus two sites of phosphorylation. Fragment ions in the MS/MS spectrum define the locations for the two phosphorylation marks as Thr3 and Ser10 (AR**pT**KQARK**pS**TGGKAPRKQ). All lysine residues and the N-terminus are fully propionylated, and the C-terminus is modified by conversion to its methyl ester. Inspection of the y type ion series (C-terminal end of the peptide) clearly showed that one of the phosphorylation sites is found on Ser10 and not Thr11. The ion at 1068 Da (y_9) corresponds to an unmodified Thr11 residue, and the ion at 1235 Da (y_{10}) corresponds to the Ser10 residue with an addition of phosphate. Additionally, the y_{10} ion also loses phosphoric acid (H_3PO_4, −98 Da) further pointing to Ser10 as one of the phosphorylation sites on this peptide. Looking at the b type ions from the N-terminus we found that a fragment ion at 465 Da (b_3) is present and also loses 98 Da (H_3PO_4), indicating that Thr3 is also phosphorylated on this particular peptide. Several other doubly phosphorylated peptides containing both Thr3 and Ser10 phosphorylation were observed with various internal lysine modifications as well (data not shown). Although histone H3 has been reported to be phosphorylated at Thr11 (*46*), no evidence of this residue being phosphorylated was observed using our methods. However, it is possible that this result may be dependent on the cell cycle stage investigated or that this modification may be present below our current MS detection limits. In addition, no other phosphorylated peptides from the core domain of the histone H3 proteins were observed with our methods.

Table 1: Modifications Associated with Phosphorylated Variants of Histone H3 during Mitosis as Determined by Tandem Mass Spectrometry[a]

modification(s)	H3.1	H3.2	H3.3
3−8 Residue Peptides			
T3-P	455.7234[b] (455.7236)	455.7233[b] (455.7236)	455.7236[b] (455.7236)
T3-P, K4-Me	nd[c]	nd	462.7313[b] (462.7314)
9−17 Residue Peptides			
S10-P	575.2881 (575.2871)	575.2889 (575.2871)	575.2879 (575.2871)
S10-P, K9-Me	582.2951 (582.2949)	582.2954 (582.2949)	582.2949 (582.2949)
S10-P, K9-Me_2	561.2892 (561.2896)	561.2898 (561.2896)	561.2893 (561.2896)
S10-P, K9-Me_3	568.2973 (568.2972)	568.2975 (568.2972)	568.2972 (568.2972)
S10-P, K9-Ac	575.2874[b] (575.2868)	575.2867[b] (575.2868)	575.2872[b] (575.2868)
S10-P, K14-Ac	575.2868[b] (575.2868)	575.2871[b] (575.2868)	575.2862[b] (575.2868)
S10-P, K9-Me, K14-Ac	575.2890 (575.2868)	575.2875 (575.2868)	575.2886 (575.2868)
S10-P, K9-Me_2, K14-Ac	554.2823 (554.2815)	554.2817 (554.2815)	561.2895[b] (561.2893)
S10-P, K9-Me_3, K14-Ac	561.2892 (561.2891)	561.2897 (561.2891)	561.2890 (561.2891)
S10-P, K9-Ac, K14-Ac	nd	nd	568.2787[b] (568.2787)
27−40 Residue Peptides			
S28-P	869.4589 (869.4563)	876.4649[b] (876.4641)	884.4656[b] (884.4615)
S28-P, K27-Me	876.4680 (876.4641)	876.4666 (876.4641)	891.4703[b] (891.4694)
S28-P, K27-Me_2	855.4598 (855.4588)	855.4595 (855.4588)	870.4621[b] (870.4641)
S28-P, K27-Me_3	862.4672 (862.4663)	862.4678 (862.4663)	nd
S28-P, K36-Me	876.4651 (876.4641)	876.4656 (876.4641)	891.4718[b] (891.4694)
S28-P, K36-Me_2	855.4600 (855.4588)	855.4601 (855.4588)	863.4579 (863.4563)
S28-P, K36-Me_3	nd	869.4760[b] (869.4741)	nd
S28-P, K27-Me, K36-Me	883.4758 (883.4719)	883.4756 (883.4719)	898.4777[b] (898.4772)
S28-P, K27-Me, K36-Me_2	862.4682 (862.4666)	862.4680 (862.4666)	877.4713[b] (877.4719)
S28-P, K27-Me, K36-Me_3	nd	869.4770 (869.4742)	nd
S28-P, K27-Me_2, K36-Me	862.4676 (862.4666)	862.4679 (862.4666)	877.4711[b] (877.4719)
S28-P, K27-Me_2, K36-Me_2	841.4619 (841.4614)	841.4617 (841.4614)	856.4664[b] (856.4666)
S28-P, K27-Me_2, K36-Me_3	nd	nd	nd
S28-P, K27-Me_3, K36-Me	869.4774 (869.4742)	869.4768 (869.4742)	877.4738 (877.4716)
S28-P, K27-Me_3, K36-Me_2	848.4698 (848.4689)	nd	nd
S28-P, K27-Me_3, K36-Me_3	nd	nd	nd
S31-P, K36-Me_2	n/a[d]	n/a	863.4601 (863.4563)
S31-P, K27-Me_3, K36-Me	n/a	n/a	877.4734 (877.4716)
S28-P, S31-P	n/a	n/a	nd

[a] MS/MS spectra were acquired on a LTQ-FT mass spectrometer. Measured and theoretical (shown in parentheses) monoisotopic *m*/*z* values for $[M + 2H]^{2+}$ ions were within 5 ppm of each other for all modified peptides. [b] Peptides detected only after enrichment by IMAC. [c] Not detected. [d] Not applicable.

ELISA Analysis Demonstrates Epitope Occlusion. Most biochemical characterizations of histone modifications are based on the generation and use of site-specific histone antibodies. Although some dual-modification antibodies have been produced recently (*24*), the majority of histone antibodies have been directed against single modifications on histone proteins. Our MS experiments on mitotically arrested H3 variants showed that the extent of multiple modifications on these proteins is quite extensive and thus may be problematic for antibody analysis. An example of this potential problem in using site-specific antibodies to monitor single modification sites is demonstrated in the next experiments. ELISA experiments using a collection of synthetic H3 peptides modified in various combinations and immobilized to microtiter dishes are shown in Figure 4. As shown in Figure 4A, an ELISA experiment using a monoclonal H3 Ser10 phos antibody demonstrated that the recognition of this antibody toward a synthetic peptide containing only Ser10 phosphorylation (ARTKQTARKpSTGGKA) was very high. However, the recognition of the Ser10 phosphorylation site on synthetic dual-modified peptides containing Lys9 Acetyl/Ser10 phos and Lys9 monomethyl/Ser10 phos by the Ser10 phos antibody was significantly disrupted (Figure 4A). This result is in agreement with a previous report that showed that a Ser10 phospho antibody lost recognition of the epitope when the nearby residues at Lys9 or Lys14 became acetylated upon trichostatin A treatment, an effect known as epitope occlusion (*25*). Similar results were also obtained with experiments using a monoclonal antibody to H3 Ser28 phos (Figure 4B), showing that methylation or acetylation of Lys27 adjacent to Ser28 phosphorylation severely hindered the binding of the H3 Ser28 phos antibody to the synthetic phosphopeptide. This trend was also observed in studies using H3 polyclonal antibodies to Ser10 and Thr3 phosphorylation (Supplemental Figure 5A and 5B, respectively, Supporting Information). Although all antibodies used in this study (both monoclonal and polyclonal) did easily recognize the correct synthetic phosphorylated peptides, the presence of modifications on adjacent lysine residues severely hindered the antibody binding to dual-modified peptides. The generation of antibodies against dual-modified peptides should aid in the detection of highly modified histone H3 proteins, although similar problematic issues could still occur if other endogenous modifications were present in the epitope recognized by the antibody. At the very least, this potential problem shows the need for careful interpretation of data from the sole use of site-specific antibodies and demonstrates the advantage of using both MS and immunoassay methods for histone modification studies.

DISCUSSION

In this work, mass spectrometry and immunoassay analyses of phosphorylated histone H3 purified from mitotically arrested human cells are provided. These complementary

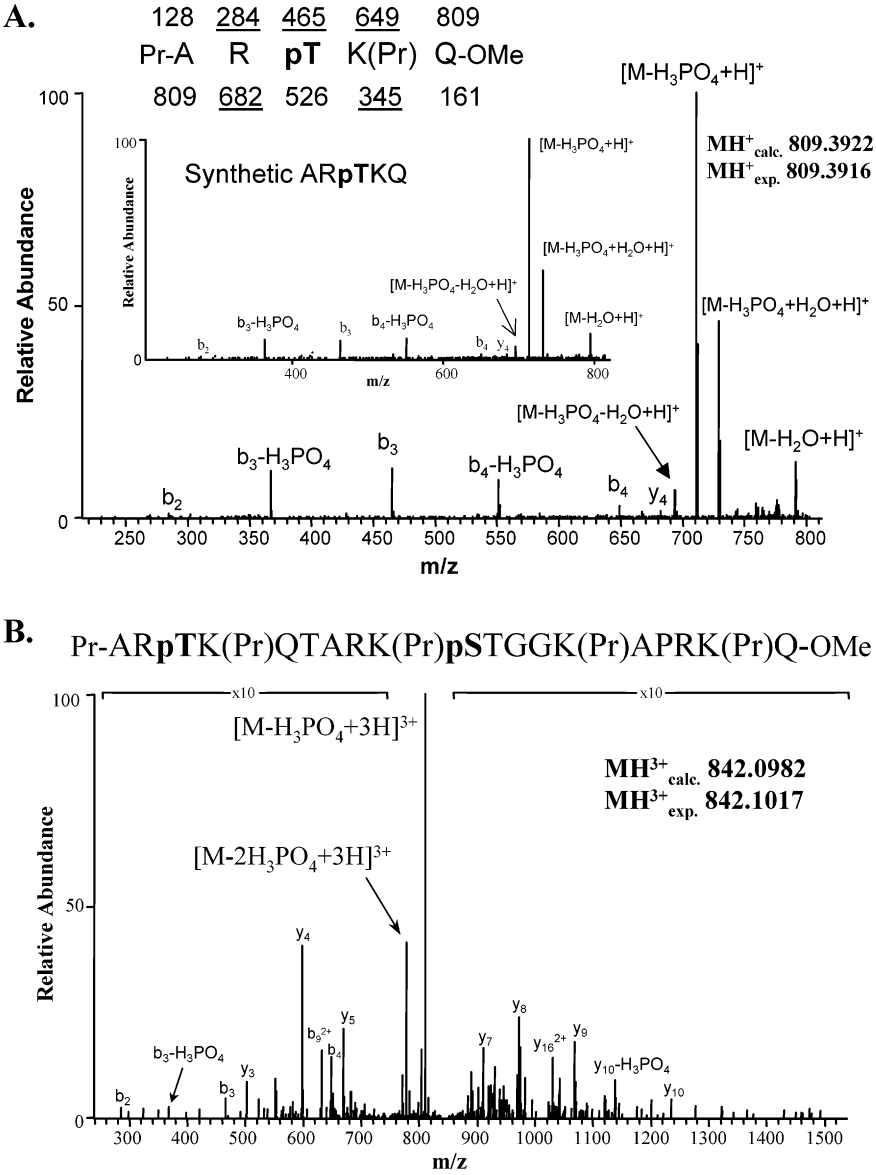

FIGURE 3: Identification of Thr3 as a site of phosphorylation in histone H3. Histone H3.1 was digested with chymotrypsin. The resulting peptides were propionylated, converted to methyl esters, enriched for phosphopeptides by IMAC, and analyzed by on-line nanoflow HPLC−MS/MS. (A) MS/MS spectrum recorded on the $[M + H]^+$ ion (*m*/*z* 809.3916) corresponding to residues 1−5 containing a phosphorylation mark on Thr3. The inset shows the MS/MS spectrum recorded on the corresponding synthetic peptide. (B) MS/MS spectrum recorded on the $[M + 3H]^{3+}$ ion (*m*/*z* 842.1017) corresponding to residues 1−19 containing 2 sites of phosphorylation. Observed fragment ions of types b and y define the sites of phosphorylation as Thr3 and Ser10.

approaches document an extremely diverse combination of histone H3 modifications existing in vivo whose function(s) remain to be fully investigated. It is now clear that many histone modifications coexist on the same molecule and this information needs to be considered in any future work focusing on deciphering modification patterns or a "histone code" throughout the cell cycle. LC−MS/MS experiments showed that phosphorylated H3 proteins are decorated with abundant methylation modifications on nearby and adjacent lysine residues during mitosis. This finding is in contrast to an earlier report suggesting that the coexistence of H3 Lys9 methylation and Ser10 phosphorylation was unlikely. For example, an H3 Ser10-phos peptide inhibited SUV39H1-dependent methylation of Lys9. Reciprocally, a dimethylated H3 Lys9 peptide reduced the ability of a mitotic kinase, Ipl1/aurora B, to phosphorylate the adjacent Ser10 site on the peptide in vitro (*47*). These experiments using purified enzymes and peptide substrates implied that only a limited number of potential combinations of modifications on a single histone molecule might exist. This report also described increased levels of histone H3 Ser10 phosphorylation in mouse embryonic fibroblasts with mutations in the methyltransferase *suv39h1* as well. Nevertheless, our MS results indicate that phosphorylation and methylation are endogenously found on the same H3 protein on adjacent residues such as Lys9/Ser10, Lys27/Ser28, and Thr3/Lys4 (Table 1). In fact, phosphopeptides containing lysine methylation were qualitatively found in much higher abundances than phosphopeptides containing lysine acetylation. This was evident from the fact that phosphopeptides with Lys9 or Lys27 methylation could be observed from normal LC−MS/MS analyses, while phosphopeptides with single or double acetylation (such as Lys9 and Lys14) were only observed after IMAC enrichment (Table 1). This observation also contrasts work showing that Ser10 phosphorylation enhances Lys14 acetylation by Gcn5 both in vitro and in vivo (*48*,

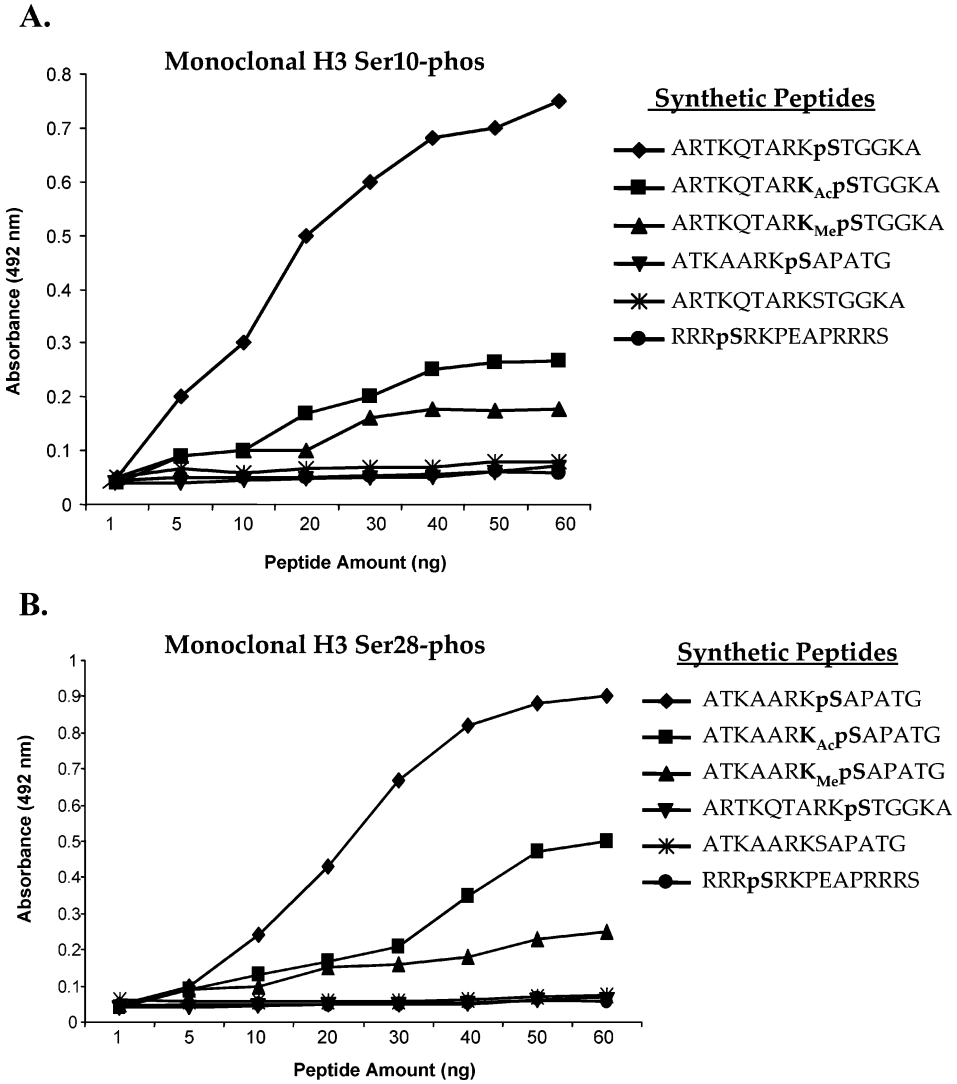

FIGURE 4: ELISA analysis using monoclonal H3 phospho antibodies. (A) ELISA analysis using a monoclonal H3 Ser10 phos antibody. Experiments were performed using the following synthetic peptides immobilized on microtiter dishes: H3 Ser10 phos residues 1−15, H3 K9-Ac/Ser10 phos residues 1−15, H3 K9-methyl/Ser10 phos residues 1−15, H3 Ser28 phos residues 21−33, H3 residues 1−15, and CENP-A Ser7 phos residues 4−17. (B) ELISA analysis using a monoclonal H3 Ser28 phos antibody. Experiments were performed as described in panel A using the following synthetic peptides: H3 residues 21−33, H3 Ser28 phos residues 21−33, H3 Ser10 phos residues 1−15, H3 K27-Ac/Ser28 phos residues 21−33, H3 K27-methyl/Ser28 phos residues 21−33, and CENP-A Ser7 phos residues 4−17. Peptide amounts ranged from 1 to 60 ng (*x*-axis), and absorbance was measured at 492 nm (*y*-axis).

49) and precedes Lys14 acetylation at specific promoters (*50*, *51*). It is good to keep in mind that our work represents a global identification of phosphorylation from bulk histone H3 extracted from mitotically arrested HeLa cells, while the previous report describes the partial linking of Lys14 acetylation to Ser10 phosphorylation at distinct signaling pathways in *Saccharomyces cerevisiae* (*49*). Nevertheless, a previous MS report has documented that Lys14 can be acetylated in the absence of Ser10 phosphorylation, which also suggests that Ser10 phosphorylation is not a prerequisite for acetylation at Lys 14 (*26*).

Additionally, the observation of endogenous phosphorylation on Ser and Thr residues and abundant methylation modifications on adjacent Lys residues (Table 1) also provide some in vivo support for the proposed histone "methyl/phos" binary switch hypothesis (*52*). The binary switch hypothesis states that post-translational modifications to adjacent amino acid residues on histone proteins, such as methylation of K27 and phosphorylation of S28 on histone H3, could lead to loss of binding to an effector module (such as the methyl HP1 or Polycomb) and could act as regulatory switches during distinct cellular processes (*52*). Therefore, the potential exists for the phosphorylation of Ser10 to regulate binding of effector molecules to methylated Lys9 and vice versa (also possibly Lys27/Ser28 and Thr3/Lys4 switches) as methylation is thought to be a more stable mark that may be maintained over several cell generations (*53*). Additionally, methylated Lys9 is bound by the chromodomain of HP1 and other Lys9 binding effectors (*54*), and this interaction is critical for heterochromatin formation and associated gene silencing (*6*, *7*, *55*, *56*). The dynamic rearrangements of chromatin higher-order structure during mitosis may require the release of tightly associated chromatin-binding proteins, such as HP1. Therefore, as articulated by Fischle et al. (*52*), the reversible cycle of histone phosphorylation (and dephosphorylation) may govern the ability of such effectors both to bind methylated lysine marks and to release these binding factors at the appropriate stage of the cell cycle or development.

Fractionation of histone H3 by RP-HPLC also allowed for the assignment of modifications to specific phosphorylated H3 variants. All previous histone H3 investigations have not specifically looked at individual H3 isoform phosphorylation but rather only probed bulk histone H3 phosphorylation by biochemical means. Henikoff and co-workers have recently shown that, in *Drosphophila* cells, the H3.3 variant marks active chromatin and contains more modifications associated with transcriptionally active chromatin such as Lys4 methylation and lysine acetylation than H3 (human H3.2) (*40*, *43*). Although no quantification experiments involving stable isotope labeling were employed in these studies, we note that our IMAC−MS results (Table 1) seem to be in agreement with that study as Thr3 phosphorylation was found together with Lys4 methylation only on the H3.3 variant. In addition, a 9−17 residue peptide with Ser10 phosphorylation, Lys9 acetylation, and Lys14 acetylation was also only detected on the H3.3 isoform. Finally, methylation at Lys27 in combination with Ser28 phosphorylation was also found in higher abundances on H3.1 and H3.2 when compared to H3.3 (Table 1), as this combination of modifications was only observed on histone H3.3 after IMAC enrichment. Therefore, future work on histone H3 phosphorylation cannot simply ignore that modification differences in H3 variants may exist and should be taken into account.

ELISA experiments resulted in two major observations. First, ELISA with H3 phospho-specific antibodies showed that, at the minimum, caution is required in the interpretation of experiments that are based entirely on the use of site-specific antibodies, as epitope occlusion can complicate experiments. As this problem of epitope occlusion can vary from antibody to antibody and from one modification site to another, considerable care must be taken in order to determine exactly what epitope(s) the antibody recognizes, and if modification of neighboring residues impacts the reactivity of the antibody. Epitope occlusion has previously been shown for Ser10 phosphorylation (*25*), and our studies have observed this phenomenon to be true for Ser28 and Thr3 phosphorylation as well. These studies illustrate the strength in using MS in combination with immunological assays for the analysis of histone post-translational modifications, as MS can circumvent the problems associated with the use of site-specific antibodies such as specificity, cross-reactivity, and epitope occlusion through interference by neighboring modifications. Second, the ELISA experiments also lend support to the "methyl/phos" binary switch hypothesis (*52*). This hypothesis was formulated in part by the observation of the weak interaction of chromatin associated proteins with their respective histone modification ($K_d = 10^{-4}-10^{-6}$ M for both chromodomain/methyl-lysine and bromodomain/acetyl-lysine interactions) (*57*, *58*), that could lead to a rapid "on−off" binding and could be possibly even further modulated by adjacent modifications. Our ELISA results show that even molecules with higher affinities for histone modifications (site-specific antibodies) can be affected by the introduction of neighboring modifications within the recognized epitope, causing loss of binding to the dual-modified histone sequence. Currently, the generation of dual modification detecting antibodies is underway for more detailed studies of adjacent post-translationally modified residues on histone H3.

MS also has advantages over single modification antibody analysis as it permits for the simultaneous detection of multiple modifications on the same peptide and identification of unexpected modifications (as shown in Figures 2 and 3 for Ser31 and Thr3 phosphorylation) as well. IMAC enrichment in combination with MS also allowed for the detection of lower abundant phosphopeptides not observed in normal MS analyses such as peptides containing phosphorylation at Thr3 or acetyl containing phosphopeptides. Recent results from the analysis of Thr3 phosphorylation suggest that the spatial-temporal pattern of Thr3 phosphorylation is distinct from Ser10 phosphorylation (*44*). In addition, it has been suggested that differently phosphorylated chromosomes are segregated to different regions of mitotic chromatin (*44*). However, our MS observation of peptides containing simultaneous Thr3 and Ser10 phosphorylation (Figure 3B) shows that these marks can coexist on the same H3 protein in vivo. Through the use of an H3 Thr3-phospho-specific antibody our results (data not shown) are consistent with the timing and localization of H3 Thr3 and Ser10 phosphorylation overlapping, similar to a different report (*45*). Therefore, these phosphorylated residues may be simultaneously present on the same H3 molecule within mitotic chromatin and may not be mutually exclusive events as previously suggested (*44*). However, in contrast as we have shown previously, we did not detect simultaneous phosphorylation of Ser28 and Ser31 on the same H3.3 peptide, suggesting that these modifications are mutually exclusive (*41*). As with all antibody-dependent approaches, we recognize that the interpretation of immunostaining and blotting results may be affected by epitope recognition and masking issues. Thus, we favor the use of alternative methods such as the MS approach described here, in combination with immunoassay techniques for a more complete analysis of histone post-translational modifications. This is the first comprehensive phosphoproteomic analysis of histone H3 variants from any cellular phase, and the results will definitely impact future work on deciphering the role of phosphorylation in the "histone code".

SUPPORTING INFORMATION AVAILABLE

Separation of histone proteins by RP-HPLC and identification of H3 variants in the HPLC peaks fractions (Supplemental Figures 1−4 and Supplemental Tables 1−3) and the ELISA analysis of polyclonal antibodies to Ser10 and Thr3 (Supplemental Figure 5). This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Kornberg, R. D., and Lorch, Y. (1999) Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome, *Cell 98*, 285−294.
2. Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997) Crystal structure of the nucleosome core particle at 2.8 Å resolution, *Nature 389*, 251−260.
3. Jenuwein, T., and Allis, C. D. (2001) Translating the histone code, *Science 293*, 1074−1080.
4. Turner, B. M. (2002) Cellular memory and the histone code, *Cell 111*, 285−291.
5. Strahl, B. D., and Allis, C. D. (2000) The language of covalent histone modifications, *Nature 403*, 41−45.
6. Lachner, M., O'Carroll, D., Rea, S., Mechtler, K., and Jenuwein, T. (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins, *Nature 410*, 116−120.

7. Jones, D. O., Cowell, I. G., and Singh, P. B. (2000) Mammalian chromodomain proteins: their role in genome organisation and expression, *Bioessays 22*, 124−137.
8. Annunziato, A. T., and Hansen, J. C. (2000) Role of histone acetylation in the assembly and modulation of chromatin structures, *Gene Expression 9*, 37−61.
9. Santos-Rosa, H., Schneider, R., Bannister, A. J., Sherriff, J., Bernstein, B. E., Emre, N. C., Schreiber, S. L., Mellor, J., and Kouzarides, T. (2002) Active genes are tri-methylated at K4 of histone H3, *Nature 419*, 407−411.
10. van Leeuwen, F., Gafken, P. R., and Gottschling, D. E. (2002) Dot1p modulates silencing in yeast by methylation of the nucleosome core, *Cell 109*, 745−756.
11. Zhang, Y., and Reinberg, D. (2001) Transcription regulation by histone methylation: interplay between different covalent modifications of the core histone tails, *Genes Dev. 15*, 2343−2360.
12. Hendzel, M. J., Wei, Y., Mancini, M. A., Van Hooser, A., Ranalli, T., Brinkley, B. R., Bazett- Jones, D. P., and Allis, C. D. (1997) Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation, *Chromosoma 106*, 348−360.
13. Wei, Y., Mizzen, C. A., Cook, R. G., Gorovsky, M. A., and Allis, C. D. (1998) Phosphorylation of histone H3 at serine 10 is correlated with chromosome condensation during mitosis and meiosis in Tetrahymena, *Proc. Natl. Acad. Sci. U.S.A. 95*, 7480−7484.
14. Wei, Y., Yu, L., Bowen, J., Gorovsky, M. A., and Allis, C. D. (1999) Phosphorylation of histone H3 is required for proper chromosome condensation and segregation, *Cell 97*, 99−109.
15. Hsu, J. Y., Sun, Z. W., Li, X., Reuben, M., Tatchell, K., Bishop, D. K., Grushcow, J. M., Brame, C. J., Caldwell, J. A., Hunt, D. F., Lin, R., Smith, M. M., and Allis, C. D. (2000) Mitotic phosphorylation of histone H3 is governed by Ipl1/aurora kinase and Glc7/PP1 phosphatase in budding yeast and nematodes, *Cell 102*, 279−291.
16. Kaszas, E., and Cande, W. Z. (2000) Phosphorylation of histone H3 is correlated with changes in the maintenance of sister chromatid cohesion during meiosis in maize, rather than the condensation of the chromatin, *J. Cell Sci. 113*, 3217−3226.
17. Goto, H., Tomono, Y., Ajiro, K., Kosako, H., Fujita, M., Sakurai, M., Okawa, K., Iwamatsu, A., Okigaki, T., Takahashi, T., and Inagaki, M. (1999) Identification of a novel phosphorylation site on histone H3 coupled with mitotic chromosome condensation, *J. Biol. Chem. 274*, 25543−25549.
18. Van Hooser, A., Goodrich, D. W., Allis, C. D., Brinkley, B. R., and Mancini, M. A. (1998) Histone H3 phosphorylation is required for the initiation, but not maintenance, of mammalian chromosome condensation, *J. Cell Sci. 111*, 3497−3506.
19. de la Barre, A. E., Gerson, V., Gout, S., Creaven, M., Allis, C. D., and Dimitrov, S. (2000) Core histone N-termini play an essential role in mitotic chromosome condensation, *EMBO J. 19*, 379−391.
20. Guo, X. W., Th'ng, J. P., Swank, R. A., Anderson, H. J., Tudan, C., Bradbury, E. M., and Roberge, M. (1995) Chromosome condensation induced by fostriecin does not require p34cdc2 kinase activity and histone H1 hyperphosphorylation, but is associated with enhanced histone H2A and H3 phosphorylation, *EMBO J. 14*, 976−985.
21. Nowak, S. J., and Corces, V. G. (2004) Phosphorylation of histone H3: a balancing act between chromosome condensation and transcriptional activation, *Trends Genet. 20*, 214−220.
22. Zhong, S., Goto, H., Inagaki, M., and Dong, Z. (2003) Phosphorylation at serine 28 and acetylation at lysine 9 of histone H3 induced by trichostatin A, *Oncogene 22*, 5291−5297.
23. Zhong, S. P., Ma, W. Y., and Dong, Z. (2000) ERKs and p38 kinases mediate ultraviolet B-induced phosphorylation of histone H3 at serine 10, *J. Biol. Chem. 275*, 20980−20984.
24. Cheung, P. (2004) Generation and characterization of antibodies directed against di- modified histones, and comments on antibody and epitope recognition, *Methods Enzymol. 376*, 221−234.
25. Clayton, A. L., Rose, S., Barratt, M. J., and Mahadevan, L. C. (2000) Phosphoacetylation of histone H3 on c-fos- and c-jun-associated nucleosomes upon gene activation, *EMBO J. 19*, 3714−3726.
26. Zhang, K., Tang, H., Huang, L., Blankenship, J. W., Jones, P. R., Xiang, F., Yau, P. M., and Burlingame, A. L. (2002) Identification of acetylation and methylation sites of histone H3 from chicken erythrocytes by high-accuracy matrix-assisted laser desorption ionization-time-of-flight, matrix-assisted laser desorption ionization-postsource decay, and nanoelectrospray ionization tandem mass spectrometry, *Anal. Biochem. 306*, 259−269.
27. Zhang, K., Williams, K. E., Huang, L., Yau, P., Siino, J. S., Bradbury, E. M., Jones, P. R., Minch, M. J., and Burlingame, A. L. (2002) Histone acetylation and deacetylation: identification of acetylation and methylation sites of HeLa histone H4 by mass spectrometry, *Mol. Cell. Proteomics 1*, 500−508.
28. Zhang, L., Eugeni, E. E., Parthun, M. R., and Freitas, M. A. (2003) Identification of novel histone post-translational modifications by peptide mass fingerprinting, *Chromosoma 112*, 77−86.
29. Pesavento, J. J., Kim, Y. B., Taylor, G. K., and Kelleher, N. L. (2004) Shotgun annotation of histone modifications: a new approach for streamlined characterization of proteins by top down mass spectrometry, *J. Am. Chem. Soc. 126*, 3386−3387.
30. Rice, J. C., Nishioka, K., Sarma, K., Steward, R., Reinberg, D., and Allis, C. D. (2002) Mitotic specific methylation of histone H4 Lys 20 follows increased PR-Set7 expression and its localization to mitotic chromosomes, *Genes Dev. 16*, 2225−2230.
31. Sobel, R. E., Cook, R. G., and Allis, C. D. (1994) Non-random acetylation of histone H4 by a cytoplasmic histone acetyltransferase as determined by novel methodology, *J. Biol. Chem. 269*, 18576−18582.
32. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680−685.
33. Syka, J. E., Marto, J. A., Bai, D. L., Horning, S., Senko, M. W., Schwartz, J. C., Ueberheide, B., Garcia, B., Busby, S., Muratore, T., Shabanowitz, J., and Hunt, D. F. (2004) Novel linear quadrupole ion trap/FT mass spectrometer: performance characterization and use in the comparative analysis of histone H3 post-translational modifications, *J. Proteome Res. 3*, 621−626.
34. Martin, S. E., Shabanowitz, J., Hunt, D. F., and Marto, J. A. (2000) Subfemtomole MS and MS/MS peptide sequence analysis using nano-HPLC micro-ESI Fourier transform ion cyclotron resonance mass spectrometry, *Anal. Chem. 72*, 4266−4274.
35. Pappin, D. J. C., Hojrup, P., and Bleasby, A. J. (1993) Rapid identification of proteins by peptide-mass fingerprinting, *Curr. Biol. 3*, 327−332.
36. Ficarro, S. B., McCleland, M. L., Stukenberg, P. T., Burke, D. J., Ross, M. M., Shabanowitz, J., Hunt, D. F., and White, F. M. (2002) Phosphoproteome analysis by mass spectrometry and its application to Saccharomyces cerevisiae, *Nat. Biotechnol. 20*, 301−305.
37. Goto, H., Yasui, Y., Nigg, E. A., and Inagaki, M. (2002) Phosphorylation and reorganization of vimentin by p21-activated kinase (PAK), *Genes Cells 7*, 91−97.
38. Muller, S., Isabey, A., Couppez, M., Plaue, S., Sommermeyer, G., and Van Regenmortel, M. H. (1987) Specificity of antibodies raised against triacetylated histone H4, *Mol. Immunol. 24*, 779−789.
39. Waterborg, J. H. (1990) Sequence analysis of acetylation and methylation in two histone H3 variants of alfalfa, *J. Biol. Chem. 265*, 17157−17161.
40. McKittrick, E., Gafken, P. R., Ahmad, K., and Henikoff, S. (2004) Histone H3.3 is enriched in covalent modifications associated with active chromatin. *Proc. Natl. Acad. Sci. U.S.A. 101*, 1525−1530.
41. Hake, S. B., Garcia, B. A., Kauer, M., Baker, S. P., Shabanowitz, J., Hunt, D. F., and Allis, C. D. (2005) Serine 31 phosphorylation of histone variant H3.3 is specific to regions bordering centromeres in metaphase chromosomes, *Proc. Natl. Acad. Sci. U.S.A. 102*, 6344−6349.
42. Strelkov, I. S., and Davie, J. R. (2002) Ser-10 phosphorylation of histone H3 and immediate early gene expression in oncogene-transformed mouse fibroblasts, *Cancer Res. 62*, 75−78.
43. Ahmad, K., and Henikoff, S. (2002) The histone variant H3.3 marks active chromatin by replication-independent nucleosome assembly, *Mol. Cell 9*, 1191−1200.
44. Polioudaki, H., Markaki, Y., Kourmouli, N., Dialynas, G., Theodoropoulos, P. A., Singh, P. B., and Georgatos, S. D. (2004) Mitotic phosphorylation of histone H3 at threonine 3, *FEBS Lett. 560*, 39−44.
45. Dai, J., Sultan, S., Taylor, S. S., and Higgins, J. M. (2005) The kinase haspin is required for mitotic histone H3 Thr 3 phosphorylation and normal metaphase chromosome alignment, *Genes Dev. 19*, 472−488.
46. Preuss, U., Landsberg, G., and Scheidtmann, K. H. (2003)Novel mitosis-specific phosphorylation of histone H3 at Thr11 mediated by Dlk/ZIP kinase, *Nucleic Acids Res. 31*, 878−885.

47. Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B. D., Sun, Z. W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C. P., Allis, C. D., and Jenuwein, T. (2000) Regulation of chromatin structure by site-specific histone H3 methyltransferases, *Nature 406*, 593−599.
48. Edmondson, D. G., Davie, J. K., Zhou, J., Mirnikjoo, B., Tatchell, K., and Dent, S. Y. (2002) Site-specific loss of acetylation upon phosphorylation of histone H3, *J. Biol. Chem. 277*, 29496−29502.
49. Lo, W. S., Gamache, E. R., Henry, K. W., Yang, D., Pillus, L., and Berger, S. L. (2005) Histone H3 phosphorylation can promote TBP recruitment through distinct promoter- specific mechanisms, *EMBO J. 24*, 997−1008.
50. Cheung, P., Tanner, K. G., Cheung, W. L., Sassone-Corsi, P., Denu, J. M., and Allis, C. D. (2000) Synergistic coupling of histone H3 phosphorylation and acetylation in response to epidermal growth factor stimulation, *Mol. Cell 5*, 905−915.
51. Lo, W. S., Trievel, R. C., Rojas, J. R., Duggan, L., Hsu, J. Y., Allis, C. D., Marmorstein, R., and Berger, S. L. (2000) Phosphorylation of serine 10 in histone H3 is functionally linked in vitro and in vivo to Gcn5-mediated acetylation at lysine 14, *Mol. Cell 5*, 917−926.
52. Fischle, W., Wang, Y., and Allis, C. D. (2003) Binary switches and modification cassettes in histone biology and beyond, *Nature 425*, 475−479.
53. Bannister, A. J., Zegerman, P., Partridge, J. F., Miska, E. A., Thomas, J. O., Allshire, R. C., and Kouzarides, T. (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain, *Nature 410*, 120−124.
54. Taverna, S. D., Coyne, R. S., and Allis, C. D. (2002) Methylation of histone h3 at lysine 9 targets programmed DNA elimination in tetrahymena, *Cell 110*, 701−711.
55. Ayyanathan, K., Lechner, M. S., Bell, P., Maul, G. G., Schultz, D. C., Yamada, Y., Tanaka, K., Torigoe, K., and Rauscher, F. J. (2003) Regulated recruitment of HP1 to a euchromatic gene induces mitotically heritable, epigenetic gene silencing: a mammalian cell culture model of gene variegation, *Genes Dev. 17*, 1855−1869.
56. Wallrath, L. L. (1998) Unfolding the mysteries of heterochromatin, *Curr. Opin. Genet. Dev. 8*, 147−153.
57. Festenstein, R., Pagakis, S. N., Hiragami, K., Lyon, D., Verreault, A., Sekkali, B., and Kioussis, D. (2003) Modulation of heterochromatin protein 1 dynamics in primary mammalian cells, *Science 299*, 719−721.
58. Dhalluin, C., Carlson, J. E., Zeng, L., He, C., Aggarwal, A. K., and Zhou, M. M. (1999) Structure and ligand of a histone acetyltransferase bromodomain, *Nature 399*, 491−496.

BI050906N