Published in final edited form as:
J Biol Chem. 2007 March 9; 282(10): 7632–7640. doi:10.1074/jbc.M607909200.

IDENTIFICATION OF HISTONE H3 LYSINE 36 ACETYLATION AS A HIGHLY CONSERVED HISTONE MODIFICATION*

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Abstract

Histone lysine (K) acetylation is a major mechanism by which cells regulate the structure and function of chromatin, and new sites of acetylation continue to be discovered. Here we identify and characterize histone H3K36 acetylation (H3K36ac). By mass spectrometric analyses of H3 purified from *Tetrahymena thermophila* and *Saccharomyces cerevisiae* (yeast), we find that H3K36 can be acetylated or methylated. Using an antibody specific to H3K36ac, we show that this modification is conserved in mammals. In yeast, genome-wide ChIP-chip experiments show that H3K36ac is localized predominantly to the promoters of RNA polymerase II-transcribed genes, a pattern inversely related to that of H3K36 methylation. The pattern of H3K36ac localization is similar to that of other sites of H3 acetylation, including H3K9ac and H3K14ac. Using histone acetyltransferase complexes purified from yeast, we show that the Gcn5-containing SAGA complex that regulates transcription specifically acetylates H3K36 *in vitro*. Deletion of *GCN5* completely abolishes H3K36ac *in vivo*. These data expand our knowledge of the genomic targets of Gcn5, show H3K36ac is highly conserved, and raise the intriguing possibility that the transition between H3K36ac and H3K36me acts as an “acetyl/methyl switch” governing chromatin function along transcription units.

In eukaryotes, the regulation of chromatin structure modulates all DNA-templated processes such as DNA replication and transcription. One major mechanism that regulates the structure and function of chromatin is the covalent modification of histones. A number of different posttranslational modifications are known to occur on histones, including acetylation,

*This work was supported by grants from the NIH; GM 68088 to B.D.S., GM 072518 to J.D.L., G.M 37537 to D.F.H, GM 40922 to C.D.A., the Ford Foundation to S.A.M. and B.A.G., and The Rockefeller University “Women & Science” Fellowship to S.B.H. B.D.S. is a Pew Scholar in the Biomedical Sciences.

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methylation, phosphorylation, ubiquitylation, and sumoylation (1–5). While the majority of these modifications are restricted to the flexible N- and C-terminal 'tail' domains of these proteins, a significant number of these marks have been identified in their highly structured globular domains (6,7). The function of many of these modifications is not well understood, but several of them have been linked to transcriptional activation and repression, DNA repair, and cell cycle regulation (8–11).

Acetylation and methylation of histone lysine residues, in particular, have been shown to play key roles in the regulation of chromatin structure and function, with the majority of these modifications occurring on histone H3 (12). Acetylation is highly dynamic and has been linked to cellular processes such as transcriptional activation, DNA repair, as well as chromatin assembly (13–15). Methylation, in contrast, has been considered a stable modification that regulates transcriptional repression and activation, transcriptional elongation, heterochromatin formation, X-inactivation and polycomb-mediated gene silencing (16–23). However, recent studies have revealed that histone lysine methylation can also be enzymatically reversed (24–27).

An additional layer of functional complexity in the acetylation or methylation of lysine residues arises from the finding that lysine residues can be targeted for both acetylation and methylation, but not simultaneously. Specifically, it has been shown in mammals and fission yeast that methylation at lysine 9 on H3 (H3K9) serves as a binding site for the recruitment of the chromodomain protein HP1, initiating the formation of heterochromatin (28,29). However, acetylation on H3K9 prevents this site from being methylated, thus requiring deacetylation prior to methylation for proper heterochromatin formation (30). This interplay between acetylation, deacetylation and methylation at the same site demonstrates a dynamic relationship between gene activation and repression that has the potential to occur at other histone lysine residues. However, the possibility of "dual" modifications (here "dual" denoting the choice of being either one modification or another) occurring on lysine residues outside of H3K9 has not been widely explored (31).

Previous studies have characterized lysine 36 of histone H3 (H3K36) as a site of methylation mediated by the methyltransferase Set2 in the budding yeast *Saccharomyces cerevisiae* (32). In its methylated form, H3K36 functions in the process of transcriptional elongation and occurs predominantly in the coding regions of genes (21,22,33). Here, we show that in addition to being a site of methylation, H3K36 can also be a target for acetylation. We find that acetylation at H3K36 is conserved in mammals and, in yeast, is localized predominantly to the promoters of RNA polymerase II-transcribed genes. We also find that the Gcn5-containg SAGA complex specifically acetylates H3K36 *in vitro* and is required for H3K36ac *in vivo*. Collectively, these results identify H3K36 acetylation as a conserved modification that likely functions in transcription. Because H3K36 is also a site of methylation, these data raise the intriguing possibility that the transition between H3K36ac and H3K36me represents a novel "chromatin switch" involved in the regulation of gene transcription.

EXPERIMENTAL PROCEDURES

Yeast strains

The wild-type histone H3 and H3K36-to-alanine point mutation (K36A) strains were in the WZY42 background and have been described previously (34). The TAP-tagged *GCN5* and *SAS3* strains were in the BY4741 background and obtained from Open Biosystems. The wild-type (DY150), *gcn*5Δ (DY5925) (35), and *sas3*Δ (DY8179) strains were in the W303 background and were kindly provided by David Stillman (University of Utah).

Histone preparation

Yeast, *Tetrahymena* and mammalian histones were acid-extracted from purified nuclei as previously described (36,37). For mass spectrometric analyses, *Tetrahymena* and yeast histone H3 were purified by reverse phase (RP)-HPLC as previously described (38,39). Typically, acid-extracted core histones were separated on a C8 column (220 X 4.6 mm, Aquapore RP-300; PerkinElmer) using a linear ascending solvent B gradient of 35–60% over 75 min at 1.0 ml/min on a Beckman Coulter System Gold 126 Pump Module and 166 Detector (solvent A was 5% acetonitrile/0.1% TFA in water, and solvent B was 90% acetonitrile/0.1% TFA in water). Peak fractions corresponding to H3 were collected, dried and resuspended in water. A portion of each fraction was used to confirm the presence of purified histones by gel electrophoresis followed by Coomassie blue staining. The remainder of the fraction was used for mass spectrometric (MS) analyses.

Mass spectrometric analyses

Mass spectrometric analyses of *Tetrahymena* and yeast H3 were performed as previously described with slight modifications (36). Briefly, *Tetrahymena* H3 was digested with trypsin prior to propionylation while yeast H3 lysines were blocked by propionylation followed by digestion with either trypsin or chymotrypsin. Derivatization of H3 with propionylation reagent converts internal lysine residues (monomethylated and endogenously unmodified residues) and the amino terminus to propionyl amides causing the blockage of trypsin cleavage on the C-terminal side of lysines allowing cleavage to occur only C-terminal to arginine (40). Digestion of *Tetrahymena* H3 prior to propionylation allowed for the generation of peptides distinct from that of yeast H3 improving the chances of detecting posttranslational modifications. Following digestion and propionylation reactions, samples were gradient eluted (Agilent 1100 Series) directly into a Finnigan linear quadrupole ion trap-Fourier transform (LTQ-FT) mass spectrometer (Thermo Electron) at a flow rate of 100 nl/min operated in the MS/MS data-dependent mode. All MS/MS data were manually validated by inspection of band y-type ions.

Electrophoresis and immunoblot analyses

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblot analyses were performed using procedures and reagents from GE Healthcare. The anti-H3K36me3 (α-H3K36me3) rabbit polyclonal antibody was obtained from Abcam (catalog # ab9050) and used at a dilution of 1:2,000–1:5,000. All other rabbit histone modification-specific antibodies were obtained from Upstate Biotechnology and used at the following dilutions: 1:10,000 for H3K18ac (α-H3K18ac, catalog # 07–354), 1:5,000 for H3K14ac (α-H3K14ac, catalog #07–353), and 1:15,000–1:30,000 for the C-terminus of H3 (α-H3, catalog # 07–690). The H3K36ac antiserum (α-H3K36ac, 1:1,000–1:10,000 dilution) was developed by immunizing a rabbit with a synthetic KLH-conjugated peptide specific for acetylation at H3K36 (C-APATGGVKacKPH). Typically, histones or synthetic histone H3 peptides were resolved on SDS-PAGE gels (15% for histones and 10% for peptides), followed by transfer onto polyvinylidene difluoride (PVDF) membranes (Millipore). In some cases, membranes were stained with Ponceau S (Sigma-Aldrich) to ensure proper protein transfer. After incubation with primary antibody and addition of a horseradish peroxidase-conjugated secondary antibody (GE Healthcare), membranes were incubated with ECL-Plus substrate (GE Healthcare), and proteins were detected by exposure to X-ray films.

For peptide competition experiments, the H3K36ac antibody was incubated overnight at 4°C with 5 μg/ml of the following peptides: unmodified histone H3 (amino acids 1–21), K9/14ac histone H3 (amino acids 1–21), K14ac histone H3 (amino acids 1–21), unmodified histone H3 (amino acids 27–46), and K36ac histone H3 (amino acids 27–46). Peptides were either obtained

from Upstate Biotechnology or synthesized and verified by mass spectrometry at the University of North Carolina Microprotein Sequencing and Peptide Synthesis Facility.

Chromatin immunoprecipitation (ChIP) and DNA microarray (ChIP-chip) analyses

ChIP assays were performed as previously described (33). Antibodies and amounts used in the immunoprecipitations (IPs) are as follows: α-H3K36ac (2 μl/IP, available from Upstate Biotechnology as immunoaffinity purified antibody), α-H3K36me2 (3 μl/IP, Upstate Biotechnology, catalog # 07–274), and α- H3K9/14ac (3 μl/IP, Upstate Biotechnology, catalog # 06–599). Following DNA recovery, the ChIP-enriched DNA were amplified as described (41). Briefly, two initial rounds of DNA synthesis with T7 DNA polymerase using primer 1 (5'-GTTTCCCAGTCACGATCNNNNNNNNN-3') was followed by 25 cycles of PCR with primer 2 (5'-GTTTCCCAGTCACGATC-3'). Cy3-dUTP or Cy5-dUTP were then incorporated directly with an additional 25 cycles of PCR using primer 2. Direct microarray hybridizations of H3K36ac ChIP vs. H3K36me2 ChIP or H3K9/K14ac ChIP vs. H3K36me2 ChIP were performed using standard procedures (42). This method allowed for the direct comparison between the histone modification patterns, which showed that H3K36ac enrichment was preferentially in the promoter regions of genes while H3K36me2 enrichment was preferentially in the transcribed regions. Additional control experiments in which the reference DNA were from H3 ChIPs (for standard nucleosome occupancy normalization), H3K36ac ChIPs from a H3K36 point mutation strain (K36A), or genomic DNA also demonstrated the H3K36ac enrichment to be promoter specific (data not shown). Following hybridizations, the arrays were scanned with a GenePix 4000B scanner and data was extracted with Genepix 5.0 software. Data were normalized such that the median $\log_2$ ratio value for all quality elements on each array equaled zero, and the median of pixel ratio values was retrieved for each spot. Only spots of high quality by visual inspection, with at least 50 pixels of quality data (regression $R^2 > 0.5$) were used for analysis. All data was log-transformed before further analysis. For ChIP-chip data analyses, the $\log_2$ ratio of each spot was transformed to a z-score using the formula $z_x = (X-\mu)/\sigma$, where X is a retrieved spot value, μ is the mean of all retrieved spots from one array, and σ is the standard deviation of all retrieved spots from that same array. Z-scores from three biological replicates were averaged. Raw data can be obtained from the University of North Carolina Microarray Database at https://genome.unc.edu. Data are also available through GEO (accession number GSE5544).

Purification of native yeast histone acetyltransferase (HAT) complexes

For purification of SAGA and NuA3, 4 L of yeast cells containing Gcn5-TAP or Sas3-TAP were grown to an OD_{600} between 1.0 and 2.0. Cells were disrupted by glass bead-beating in a breaking buffer consisting of 50 mM Tris-HCl (pH 8.0), 350 mM NaCl, 10% glycerol, 0.1% triton-X-100 and protease inhibitors (PMSF, aprotinin, leupeptin, and pepstatin) followed by clarification of the extract by ultracentrifugation at 25,000 rpm for 1 h at 4°C as described (43). TAP-tagged proteins were purified in a one-step procedure from the extracts by directly binding the calmodulin binding peptide (CBP) component of the TAP tag to calmodulin resin (Stratagene; 200 μl beads) in the presence of $CaCl_2$ (1 mM final concentration) at 4°C for 4 hours. All wash and elution steps were performed in 0.8 × 4 cm Polyprep chromatography columns (BioRad). Protein-bound resin was washed two times with breaking buffer containing $CaCl_2$ at a final concentration of 1 mM. Following washes, bound proteins were eluted in twelve 250 μl fractions with elution buffer (50 mM Tris-HCl (pH 8.0), 350 mM NaCl, 10% glycerol, 2 mM EGTA, and protease inhibitors). Generally, the peak of complex elution was found in fractions 2 and 3. Purified complexes were analyzed by Coomassie blue staining, immunoblot analysis with an anti-Protein A antibody (Sigma-Aldrich) and *in vitro* HAT assays (see below). Additionally, an untagged wild-type strain was included in the purification procedure as a control to confirm that there were no contaminating activities due to nonspecific protein interactions with the calmodulin resin.

HAT assays

Acetyltransferase assays were performed as described previously with modification (43,44). Briefly, 3 μl of TAP-purified SAGA or NuA3 complex (fraction 2) was incubated with either 2 μg chicken core histones, 2 μg recombinant mononucleosomes, or 2 μg synthetic H3 peptides along with 30 μM unlabeled acetyl coenzyme A (acetyl-CoA, Sigma-Aldrich) in acetyltransferase buffer (50 mM Tris (pH 7.4), 10% glycerol, and protease inhibitors). Reactions were allowed to proceed for 30 min at 30°C in a total volume of 12 μl and analyzed by SDS-PAGE followed by Coomassie blue staining or immunoblotting. For radiolabeled HAT assays, 3 μl of TAP-purified SAGA or NuA3 complex was incubated with 2 μg synthetic H3 peptides along with 0.125 μCi [^{3}H] acetyl coenzyme A (2–10 Ci/mmol, GE Healthcare) in acetyltransferase buffer. Reactions were allowed to proceed as described above followed by spotting of reaction products onto p81 Whatman paper (Fisher Scientific) and monitoring of ^{3}H incorporation by scintillation counting (filter-binding assay). Recombinant mononucleosomes were a gift from Song Tan (Pennslyvania State University) and consisted of *Xenopus* core histones that were bacterially expressed, purified, and reconstituted with the NucB region of the MMTV promoter.

RESULTS

Histone H3 is acetylated at lysine 36 in Tetrahymena and yeast

Methylation at H3K36 is a highly conserved modification found in eukaryotes ranging from *Tetrahymena* to humans, but whether other types of modifications occur at this residue was unknown. To determine if novel modifications occur at H3K36, we analyzed H3 from *Tetrahymena* by tandem mass spectrometry. This organism, a ciliated protozoan, has proven useful in the discovery of other “ON” histone covalent modifications, notably H3K4me (45). *Tetrahymena* H3 was purified by RP-HPLC from acid-extracted bulk histones and digested with both trypsin and chymotrypsin followed by chemical derivatization using propionic anhydride reagent (46). Digests were analyzed by on-line nanoflowLC-MS/MS on a linear quadrupole ion trap-Fourier transform (LTQ-FT) mass spectrometer. Using this platform, we determined H3K36 is acetylated.

Shown in Fig. 1A is the MS/MS spectrum of a peptide produced from the propionylated tryptic digest of *Tetrahymena* histone H3. The $[M+2H]^{2+}$ of the parent ion is shown at m/z 542.3062. The high mass accuracy of the LTQ-FT mass spectrometer can easily distinguish between trimethylation and acetylation on peptides ($\Delta m = 0.0364$ Da). The accurate mass of the parent ion recorded was found to be consistent with acetylation on this peptide (+0.18 ppm) and not trimethylation. Importantly, we were able to also detect H3K36 mono-, di-, and trimethylation on other H3 peptides (data not shown), confirming that this residue can be methylated or acetylated.

We additionally surveyed histone H3 purified from budding yeast to determine if H3K36 acetylation might be present in this distinct unicellular organism. Using the approach mentioned above for *Tetrahymena* H3, we identified that yeast H3K36 was also acetylated (Fig. 1B). H3K36 acetylation was observed on a number of different peptides. Fig. 1B displays the tandem mass spectrum of the $[M+2H]^{2+}$ ion at m/z 823.4552 from a propionylated digest of one such identified H3 peptide. This peptide was identified to be the 27–40 amino acid H3 fragment and it was found to contain acetylation at both H3K27 and H3K36. These results identify a novel acetylation event at H3K36 that is conserved between *Tetrahymena* and yeast.

To further investigate the occurrence of this modification, we raised an antibody against a synthetic peptide acetylated at H3K36 and analyzed *Tetrahymena* and yeast histones by immunoblot analysis. As shown in Fig. 2A, the α- H3K36ac antibody efficiently recognized

the RP-HPLC purified *Tetrahymena* H3, originally analyzed by mass spectrometry. We determined the antibody to be specific for H3K36ac, as only a synthetic H3 peptide acetylated at H3K36 could selectively compete away the signal detected by the α-H3K36ac antibody (Fig. 2A). Other unmodified or acetylated H3 peptides did not compete for this antibody's detection of H3.

Next, we determined if this antibody could specifically recognize acetylation at H3K36 in yeast. Yeast histones isolated by acid-extraction from a wild-type or H3K36 point mutant (K36A) strain were analyzed by immunoblot analysis using the H3K36ac-specific antibody. Similar to *Tetrahymena*, the α-H3K36ac antibody detected H3 in a wild-type yeast strain but much less so in a strain where H3K36 was mutated to alanine (Fig. 2B). However, we note that high concentrations of histones loaded in these assays results in the ability of the α-H3K36 antibody to weakly recognize the backbone of H3 (Fig. 2B, K36A lane). Nonetheless, these results confirm our mass spectrometry findings that H3K36ac exists in both *Tetrahymena* and yeast.

H3K36 acetylation is preferentially enriched in the promoters of RNA polymerase II-transcribed genes genome-wide

Next, we used a ChIP-chip approach to determine the genomic distribution of H3K36ac and how it compared to the distribution of methylation found at H3K36 (47,48). The α-H3K36ac-specific antibody was used in ChIP reactions from yeast whole cell extracts. Enriched genomic DNA fragments were treated with RNase, amplified, and labeled fluorescently. DNA enriched from H3K36me2 ChIPs were prepared in a similar manner, and both samples were hybridized on the same array. Three independent sets of ChIPs were performed. Using this method, we directly compared the patterns of H3K36ac and H3K36me2 using arrays that tiled continuously over the entire genome at a resolution of ~1 kb. We observed a preferential enrichment of H3K36ac at 5′ regulatory regions (bidirectional and unidirectional promoters) relative to coding regions (ORFs) and 3′ UTRs (non-coding region downstream of two convergently transcribed genes) (Fig. 3A). Significantly, the H3K36ac pattern was found to be inversely related to that of H3K36me2, which occurs preferentially in the coding region and 3′ UTR of genes (33,48,49).

We then compared the location of the H3K36ac modification to that of other well-characterized acetylation sites on H3; namely H3K9 and H3K14 acetylation (H3K9/14ac). Using the same extracts, ChIPs were performed with an antibody that recognizes diacetylated H3K9/14, and the enriched DNA was amplified, labeled, and hybridized to DNA microarrays as described above. These experiments revealed that H3K9/K14ac was localized to promoters in a pattern strikingly similar to the pattern we observed for H3K36ac genome-wide (data not shown and see Fig. 3B). These data were also consistent with the H3K9/14 acetylation results obtained by others (47,50). We further examined the distribution of H3K36ac, H3K9/K14ac, and H3K36me2 using a high-resolution microarray containing probes that covered all of chromosome III at a resolution of 200 bp (and at 100 bp resolution for 1/3 of the chromosome). For both H3K36ac and H3K9/K14ac, the level of acetylation enrichment drops sharply as a function of distance from translational initiation site while H3K36me2 increases (Fig. 3B). These data are fully consistent with our analysis using lower-resolution arrays and confirm that these acetyl marks occur preferentially upstream of coding regions while H3K36me2 occurs primarily in the coding regions of genes.

We next asked whether H3K36ac associates with genomic regions other than those characteristic of RNA polymerase II (RNAPII) promoters. We found that genomic regions which were transcriptionally silent under our growth conditions, or regions not transcribed by RNAPII including centromeres, telomeres, and silent mating type loci, contained low levels of H3K36ac and H3K9/K14ac (Fig. 3C). These results indicate that H3K36ac is associated

strictly with active RNAPII regulatory sequences, and suggest that like H3K36me, H3K36ac may function in RNAPII-mediated gene transcription.

The Gcn5-containing SAGA histone acetyltransferase complex mediates H3K36 acetylation

We next sought to identify the responsible histone acetyltransferase(s) (HATs) that mediate this mark. Given H3K36 acetylation is enriched in the promoter regions of RNAPII-transcribed genes, and has a pattern of acetylation similar to that of H3K9/14 acetylation, we initially focused on the Gcn5-containing SAGA histone acetyltransferase (HAT) complex that mediates acetylation to the N-terminus of H3 (43). Furthermore, we noticed that the amino acid sequence surrounding H3K36 is very homologous to a preferred Gcn5 consensus site of acetylation found at H3K14 (51) (compare STGG**K14**AP vs. STGGV**K36**KP; underlined residues indicate homology and bold "K" indicates the residue targeted for acetylation).

To test if SAGA would mediate H3K36ac, we TAP-purified SAGA from yeast whole extracts using a tagged form of Gcn5 and then incubated the complex with either unmodified or modified H3 synthetic peptides along with unlabeled acetyl- CoA (acetyl donor) in a HAT assay. Following the HAT reactions, the products were electrophoresed on 10% SDS-PAGE gels and analyzed by immunoblot with the α-H3K36ac antibody. After incubation with purified SAGA, a previously unmodified H3 27–46 amino acid peptide was recognized by the H3K36ac-specific antibody (Fig. 4A). Importantly, when these same assays were performed using a radiolabeled form of acetyl-CoA, SAGA was readily able to acetylate an N-terminal H3 peptide (residues 1–21 containing H3K9 and H3K14) as well as the unmodified 27–46 peptide, but showed no activity toward a matched 27–46 peptide acetylated at H3K36 (Fig. 4B).

To learn more about the physiological relevance of SAGA-mediated H3K36 acetylation, we next asked if SAGA could acetylate H3K36 in the context of other histone proteins and DNA. Again HAT assays were performed using TAP-purified SAGA in which the complex was incubated with either free chicken core histones or recombinant mononucleosomes. As shown in Fig. 4C, SAGA was capable of acetylating both substrates at H3K36.

We also tested the NuA3 HAT complex for an activity that specifically acetylates H3K36. Like SAGA, NuA3 has been identified as a HAT complex that specifically targets H3 for acetylation (52). Previous work shows that the catalytic subunit of NuA3, Sas3, has overlapping activities with Gcn5 and can target H3K14 for acetylation *in vivo* (53). Unlike SAGA, however, we found that TAP-purified NuA3 was unable to acetylate H3K36 in the context of H3 synthetic peptides. However, NuA3 could acetylate H3K36 in the context of core histones and nucleosomes (data not shown).

Given the ability of SAGA and NuA3 to catalyze H3K36ac, we next asked whether either one of these HATs was required for H3K36ac *in vivo*. We therefore purified bulk core histones from *GCN5* and *SAS3* deletion strains and analyzed them for H3K36ac by immunoblot analysis. As shown in Fig. 4D, acetylation at H3K36 was abolished in the *GCN5* deletion, but not in the *SAS3* deletion. These results reveal that Gcn5 is the major HAT responsible for H3K36ac *in vivo*.

H3K36 acetylation is conserved in mammalian cells

Although our studies showed that several unicellular organisms contained H3K36ac, we wanted to determine how conserved this modification would be among several diverse multicellular organisms. We therefore isolated histones by acid-extraction from *Tetrahymena*, yeast, mouse, and human cells and probed them for the presence of H3K36ac. As shown in Fig. 5A, we found H3K36ac in all of the organisms analyzed, although the relative

abundance varied between species. H3K36ac appeared to be most abundant in *Tetrahymena* and yeast, followed by human and mouse. The mouse embryonic fibroblasts used for this study apparently harbor a very low level of H3K36ac, which is consistent with our mass spectrometry experiments that did not detect H3K36ac in histones isolated from these cells (see 54). In agreement with our human results, a recent mass spectrometry proteomics survey reported the existence of H3K36ac in human HeLa cells (55). Although differences exist in the relative abundance of H3K36ac found in these organisms, and perhaps within different cell types, these results reveal that H3K36ac occurs in organisms as diverse as yeast and humans. Our results also underscore differences in the employment of histone marks in different organism and in the importance of applying independent assays to assess them.

DISCUSSION

In this report, we identify and characterize a novel site of acetylation on histone H3 at lysine 36. This site was previously determined to be mono-, di-, and trimethylated in a broad range of eukaryotic organisms, and we find that acetylation at this residue is also highly conserved. Furthermore, we have determined that H3K36ac is mediated by the Gcn5-containing SAGA complex in yeast, and is preferentially enriched in the promoter regions of RNAPII-transcribed genes genome-wide. Although the exact function of this modification remains to be elucidated, our data suggests that it is involved in the transcription process.

We and others have found several clues as to how Gcn5 can target H3K36 to acetylate this site. First, previous studies have shown that Gcn5 in isolation can only target H3K14 (43,51). However, in its native SAGA complex, this enzyme has an expanded substrate range on H3 that includes H3K9, K18 and K23 (43). H3K36ac was not detected in this previous study as only H3 synthetic peptides containing amino acid residues from 5 to 28 were investigated. Our data, therefore, reveal an expanded site utilization pattern by SAGA. Second, the amino acid sequence immediately surrounding H3K36 is very similar to that of H3K14, which is a preferred site of Gcn5 acetylation (compare STGG**K^{14}**AP vs. STGGV**K^{36}**KP; underlined sequences show identity while bold lysines are the acetyl accepting residues). Structural studies of Gcn5 in complex with an H3 peptide containing H3K14 have identified several critical residues immediately surrounding H3K14 (glycine 13 and proline 16) that are important for substrate recognition and high affinity binding (56–58). Importantly, these key residues (G-**K^{14}**-X-P) are conserved in the sequence surrounding H3K36. Thus, the high similarity between residues surrounding H3K14 and H3K36 may explain how Gcn5 is capable of acetylating H3K36 *in vitro* and *in vivo*. Given H3K9/14 acetylation patterns overlap with H3K36ac, it is likely that SAGA mediates a broad H3 acetylation pattern when recruited to gene promoters.

Interestingly, while we found that H3K36ac was present in *Tetrahymena*, yeast, and mammalian cells, the general abundance of this modification varied greatly among the organisms we analyzed (Fig. 5A). While many possible reasons could account for these differences, one plausible explanation may be due to the fact that budding yeast contains only a single H3 isoform (H3.3), whereas *Tetrahymena* and mammalian cells contain multiple H3 variants (H3.1, H3.2 and H3.3; see Fig. 5B). Since H3.1 and H3.2 in flies and mammals are generally associated with silenced chromatin (which make up a large proportion of their genomes), these histone variants are not likely to be H3K36 acetylated and/or associated with active transcription. Thus, much of the bulk-isolated histones from these more complex organisms contain H3 isoforms that harbor “OFF” marks. This idea, along with our H3K36ac observations, is consistent with earlier studies that show that these same multicellular organisms have much lower levels of H3K4 methylation (a modification associated with transcriptionally competent chromatin) compared to those observed in yeast (59). Given the sequence surrounding H3K36 is highly conserved between these organisms (Fig. 5B), the

differences observed between species is not likely due to the inability of our antibody to efficiently recognize the site of H3K36.

That H3K36ac is mediated by Gcn5 and is found in the promoters of RNAPII-regulated genes suggests that acetylation at H3K36 may play a role in gene transcription. Previous studies of SAGA and Gcn5 indicate that this enzyme complex is activator recruited and targets acetylation at specific promoters during transcriptional activation (14,60–62). While the exact function of H3K36ac is unknown, we speculate that H3K36ac acts in concert with other Gcn5-mediated sites of acetylation to properly regulate transcriptional induction. Such cooperativity would be in agreement with prior studies that have shown the importance of Gcn5-mediated acetylation of multiple sites on H3 for normal cell growth and transcriptional activation (63). Additionally, the acetylation of H3K36 by SAGA, as part of this complex's expanded targeting, is consistent with studies on H4 that show a cumulative effect of acetylation is associated with the promoters of active genes (64).

In contrast to H3K36ac, methylation at H3K36 is found in the coding region of genes and is involved in transcriptional elongation. Recent reports have identified a function for this modification in maintaining an environment within coding regions that is repressive to the activation of intergenic transcription by recruiting the deacetylase complex Rpd3(S) (65–67). An important question, therefore, is whether acetylation and methylation activities compete for the same target sites, such as H3K36. Well documented is the finding that H3K9 is subject to either methylation or acetylation (see below), and a report co-submitted with this one reveals that a significant number of lysines targeted for acetylation are also targeted for methylation, and vise versa (54). These results raise the intriguing possibility that functional interplay between two or more posttranslational modifications at a single lysine residue is a general phenomenon that drives distinct biological effects within chromatin. To date, the best example of functional interplay between methylation and acetylation is with H3K9, at which acetylation is removed prior to methylation by *Su(var)3–9* in the promoters of genes (68). This activity is required for the recruitment of HP1/Swi6 that leads to the formation of heterochromatin in both fission yeast and multicellular eukaryotes. Although our analyses do not reveal if functional interplay occurs at this site, future investigations will aim to determine whether SAGA and Set2 regulate transcription initiation events through competition for H3K36.

Acknowledgments

We would like to thank Nicholas Laribee, Kelby Kizer, and Yoichiro Shibata for helpful discussions and suggestions on the manuscript. We also thank Song Tan and Brian Hnatkovich for the generous gift of recombinant mononucleosomes, David Stillman for the kind gift of HAT deletion yeast strains, Ming-Ming Zhou for the communication of unpublished results and Upstate Biotechnology for help with the H3K36ac antibody production.

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The abbreviations used are

K	lysine
H3	histone H3
ac	acetylation
me	methylation (69)
ChIP	chromatin immunoprecipitation
ChIP-chip	chromatin immunoprecipitation and DNA microarray detection
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TAP	tandem affinity purification
CBP	calmodulin binding peptide
HAT	histone acetyltransferase
MS	mass spectrometry
RNase	ribonuclease
ORFs	open reading frames
UTRs	untranslated regions
RNAPII	RNA polymerase II.

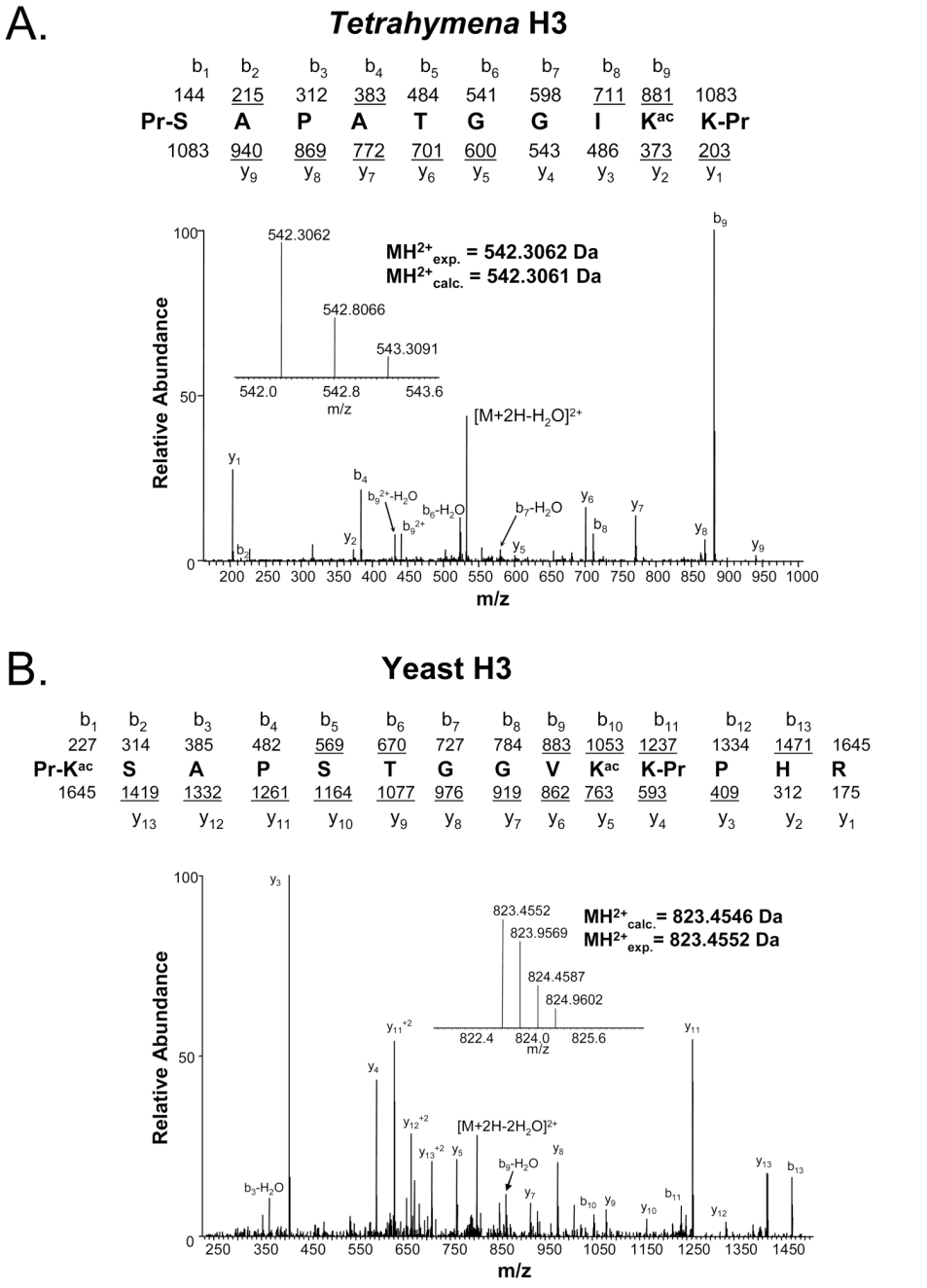


Figure 1. Identification of H3K36 acetylation in *Tetrahymena* and yeast by mass spectrometry

(A) Displayed is the MS/MS fragmentation spectrum of the $[M+2H]^{2+}$ parent ion from an H3 peptide (histone H3, amino acids 29–37) derived from a propionylated digest of *Tetrahymena* H3. Inset shows full MS of parent ion (whole peptide), from which the fragmentation spectrum was taken, at 542.3062 m/z. Scale for the y-axis of inset represents relative abundance of the parent ion and is identical to the fragmentation spectrum y-axis. Accurate mass (inset) indicates acetylation and not trimethylation on this peptide (+0.18 ppm error), while fragment ions (full spectrum) show H3K36 as the acetylation site. Experimentally observed ($MH^{2+}_{exp.}$) and calculated masses ($MH^{2+}_{calc.}$) for this acetylated peptide are indicated. Above the spectrum is the peptide sequence in which predicted b-type ions, which

contain the amino terminus of the peptide, are immediately above the sequence. Predicted y-type ions, which contain the carboxyl terminus, are immediately below the sequence. Ions observed in the spectrum are underlined and represent masses associated with the fragmented peptides from the MS/MS analyses. Note the addition of propionyl groups (Pr, adds 56 Da) on amino terminus residues and unmodified lysine residues due to chemical derivatization with propionic anyhydride reagent (see experimental procedures for explanation). *(B)* Same as in *(A)* except peptides were derived from digested RP-HPLC purified yeast H3. Displayed is the MS/MS fragmentation spectrum of the $[M+2H]^{2+}$ parent ion at m/z 823.4552. This peptide was identified as the 27–40 fragment from yeast histone H3. Inset shows full MS of parent ion at m/z 823.4552. Accurate mass indicates the addition of two acetylation modifications and not trimethylation on this peptide (+0.73 ppm error), while fragment ions show H3K27 and H3K36 as the acetylation sites. Experimentally observed ($MH^{2+}_{exp.}$) and calculated masses ($MH^{2+}_{calc.}$) for this acetylated peptide are indicated. As in *(A)*, b- and y-type ions observed in the spectrum are underlined and the peptide contains the addition of propionyl groups (Pr) on unmodified lysine and amino terminus residues.

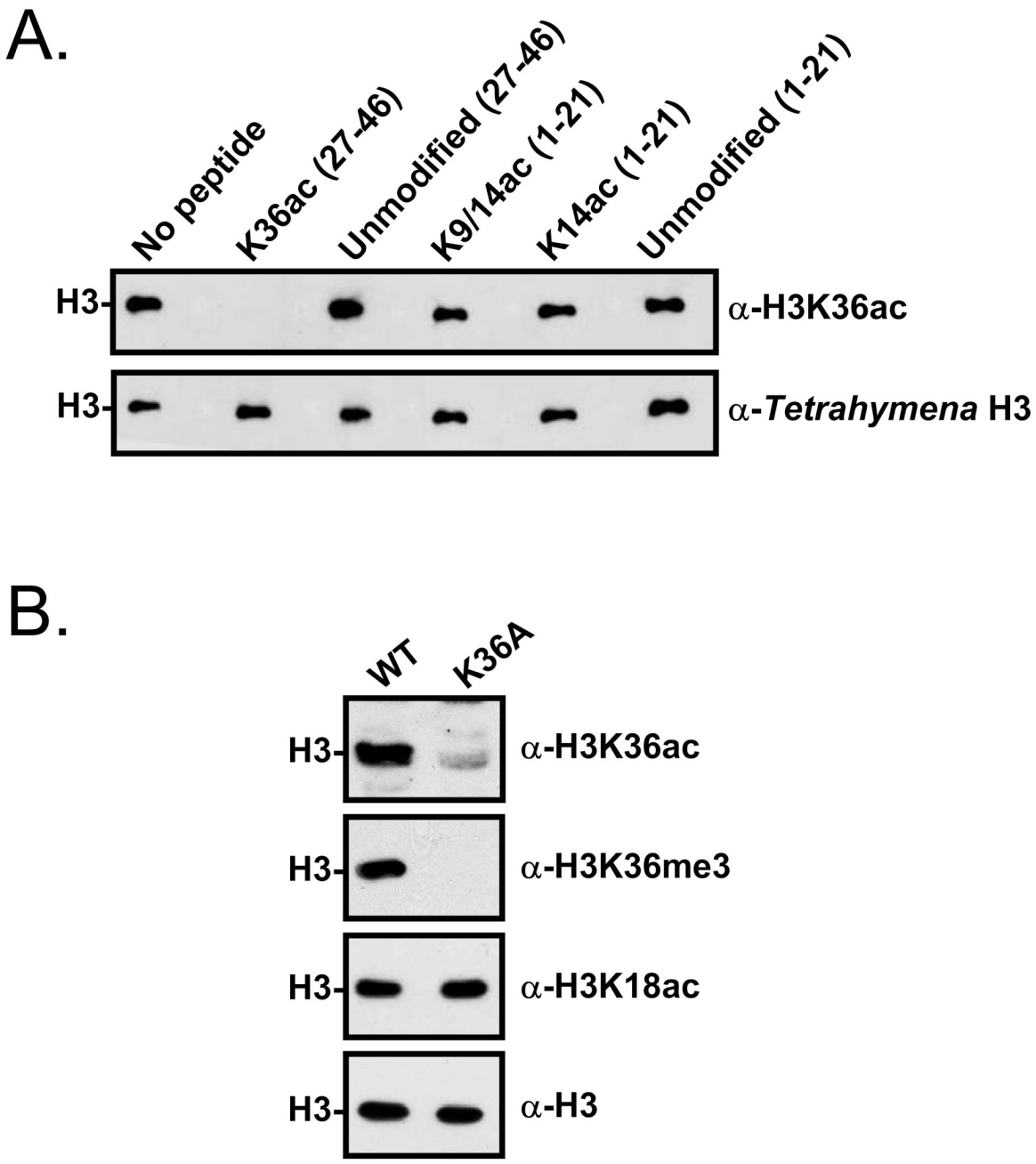


Figure 2. Detection of H3K36 acetylation in *Tetrahymena* and yeast using a specific antiserum

(A) An antibody specific to H3K36 acetylation recognizes *Tetrahymena* H3. RP-HPLC *Tetrahymena* H3 (same as used in Fig. 1A) was loaded onto adjacent lanes and resolved on a 15% SDS-PAGE gel. Following transfer to a polyvinylidene difluoride (PVDF) membrane, each lane was separated and probed with an α-H3K36 acetyl antiserum (α-H3K36ac) that was preincubated with different unmodified or modified H3 synthetic peptides as indicated. The same blots were stripped and reprobed with an antibody specific for *Tetrahymena* H3 (α-*Tetrahymena* H3) as a loading control. *(B)* The α-H3K36ac antibody specifically recognizes H3K36 acetylation in yeast. Acid-extracted histones prepared from a wild-type or H3K36 point mutant yeast strain (K36A) were resolved on a 15% SDS-PAGE gel, transferred to a PVDF

membrane, and probed for H3K36ac. An antibody specific for the C-terminus of H3 (α-H3) was used as a loading control. Antibodies specific for H3K18 acetylation (α-H3K18ac) and H3K36 trimethylation (α-H3K36me3) were used as additional controls.

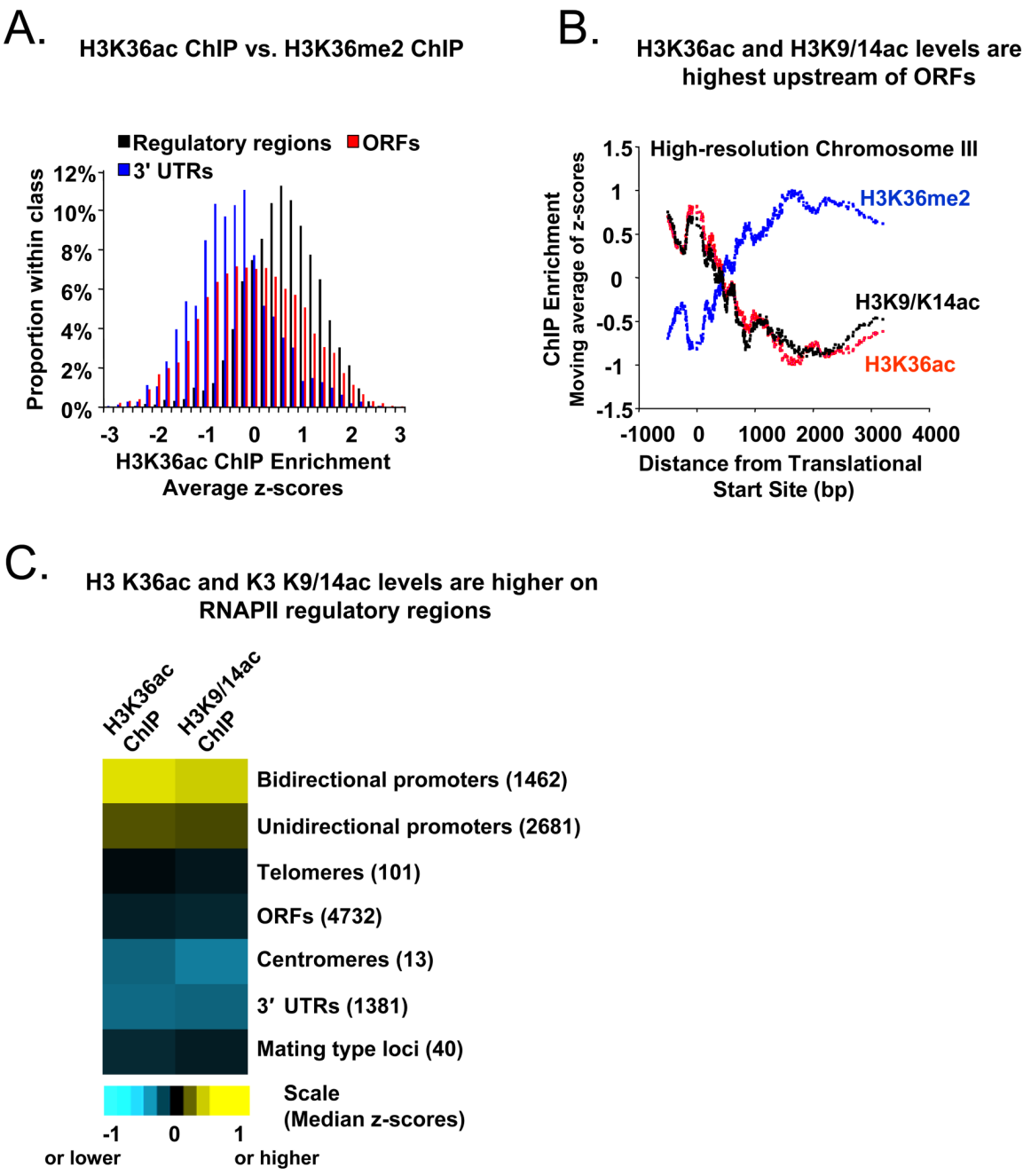


Figure 3. H3K36 acetylation is localized predominantly to the promoters of RNA polymerase II-transcribed genes genome-wide

(A) The distribution of average z-scores (units are standard deviation from the mean) for 5′ regulatory regions (black), ORFs (red), and 3' UTRs (blue) derived from ChIP-chip experiments in which H3K36ac ChIPs were compared directly to H3K36me2 ChIPs. Thus, the H3K36ac and H3K36me2 ratios shown here are inversely related. Similar promoter enrichment results for H3K36ac were obtained when the H3K36ac ChIPs were compared to a genomic DNA reference, or references composed of histone H3 ChIPs (experimental procedures). *(B)* A moving-average plot (window size=40, step size=1) of average z-scores from three independent experiments comparing H3K36ac ChIPs (red), H3K9/14ac ChIPs

(black) and H3K36me2 ChIPs (blue) on a high resolution DNA microarray covering all of chromosome III. ChIP enrichment is plotted as a function of the distance from the translational start site among genes greater than 1 kb in length. *(C)* H3K36ac distribution genome-wide. Colors (scale at bottom) represent the median of all z-scores recorded from all arrayed elements in the indicated functional class (labeled on right, number of elements indicated in parentheses). Data were derived from three independent replicates.

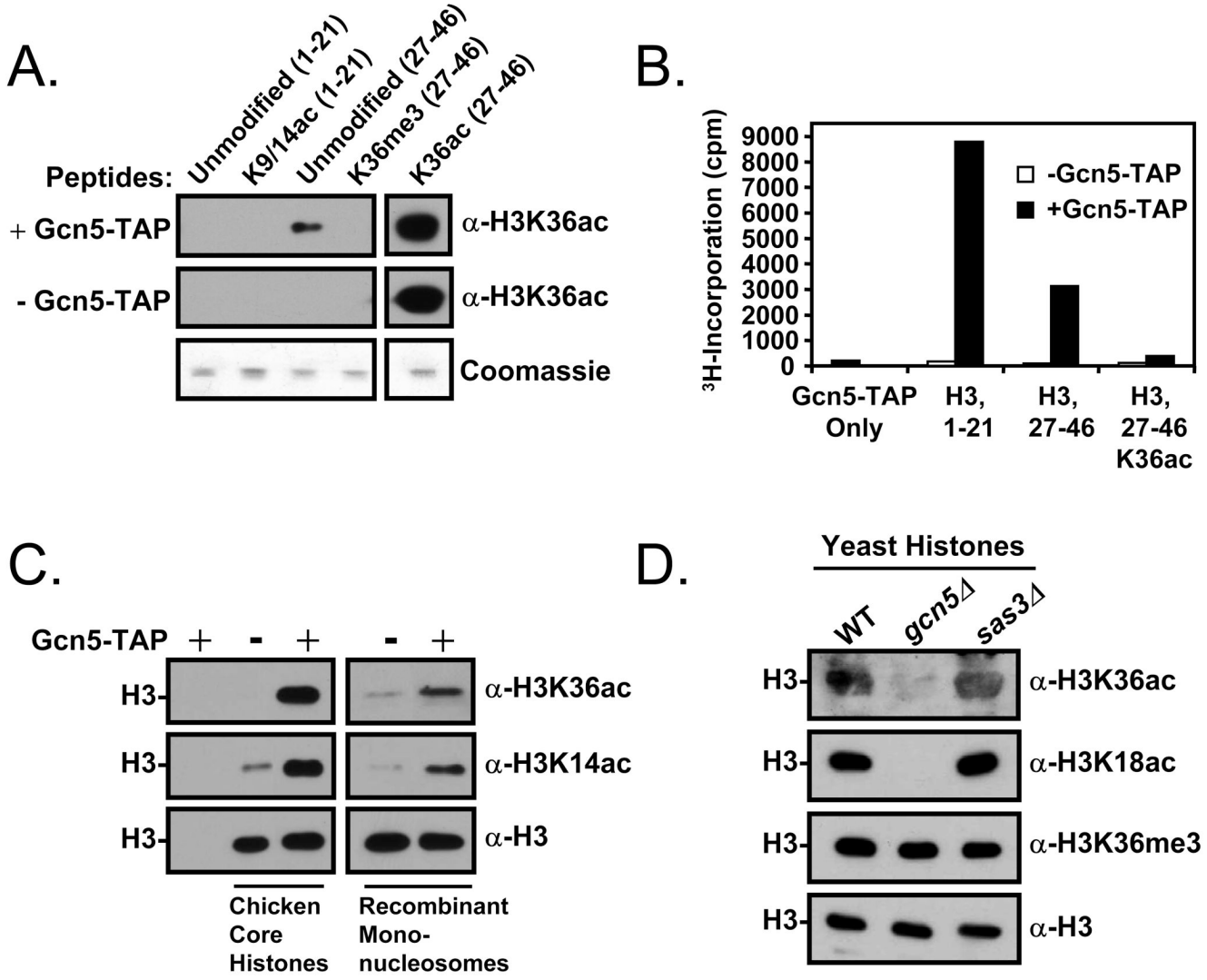
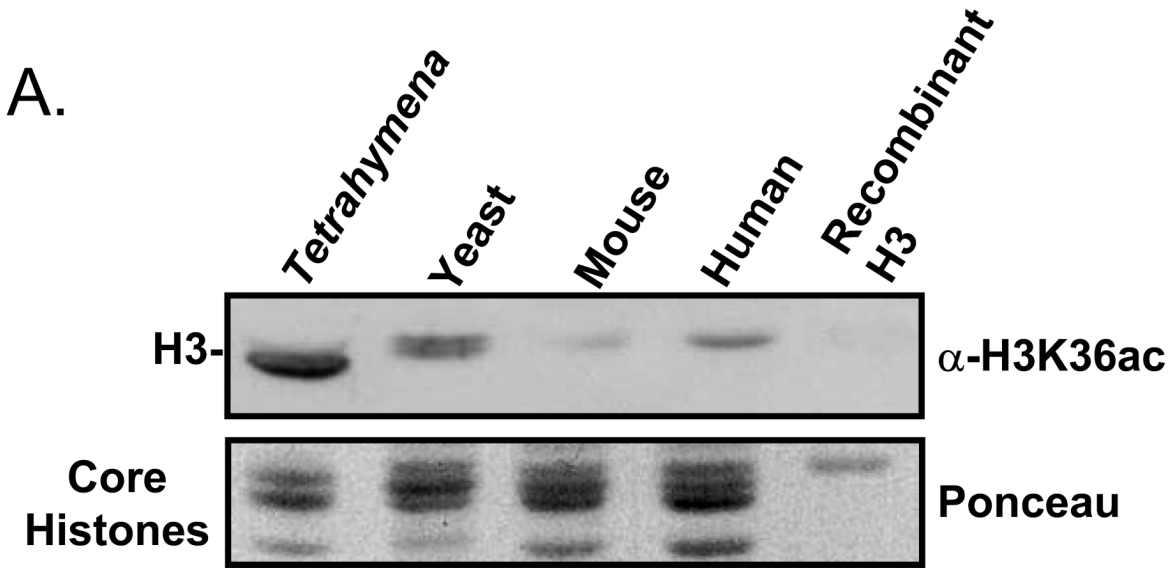


Figure 4. The Gcn5-containing SAGA complex acetylates H3K36

(A) Shown are the results of a HAT assay in which TAP-purified SAGA complex was incubated with either unmodified or modified H3 synthetic peptides along with unlabeled acetyl coenzyme A (acetyl-CoA). Reaction products were resolved on a 10% SDS-PAGE gel, transferred to a PVDF membrane, and analyzed by immunoblot for H3K36ac. An H3 synthetic peptide acetylated at H3K36 was used as a control for antibody detection. Parallel reactions were performed and examined by Coomassie staining to monitor loading (lower panel). *(B)* Displayed is a graph representing the results of a HAT assay in which TAP-purified SAGA complex was incubated with either unmodified or modified H3 synthetic peptides along with [^{3}H] labeled acetyl-CoA. ^{3}H incorporation was analyzed by filter-binding assay and monitored by scintillation counting. *(C)* Shown are the results of a HAT assay in which TAP-purified SAGA complex was incubated with either chicken core histones or recombinant mononucleosomes along with unlabeled acetyl-CoA as in *(A)*. Reaction products were resolved on a 15% SDS-PAGE gel, transferred to PVDF membrane, and analyzed by immunoblot for H3K36ac. Parallel reactions were performed and analyzed by immunoblot for H3K14ac as a control. The same blot was stripped and reprobed with an antibody specific for the C-terminus of H3 (α-H3) to monitor loading. Note slight antibody detection of histone H3 backbone in the

absence of TAP-purified SAGA in the recombinant (unmodified) mononucleosomes reactions. *(D)* Gcn5 is responsible for mediating H3K36ac in yeast. Acid-extracted histones prepared from wild-type, *gcn5* Δ or *sas3*Δ strains were resolved on a 15% SDS-PAGE gel, followed by transfer to a PVDF membrane, and analyzed by immunoblot for the presence of H3K36ac. An antibody specific for the C-terminus of H3 (α-H3) was used as a loading control. Antibodies specific for H3K18ac and H3K36me3 were used as additional controls.



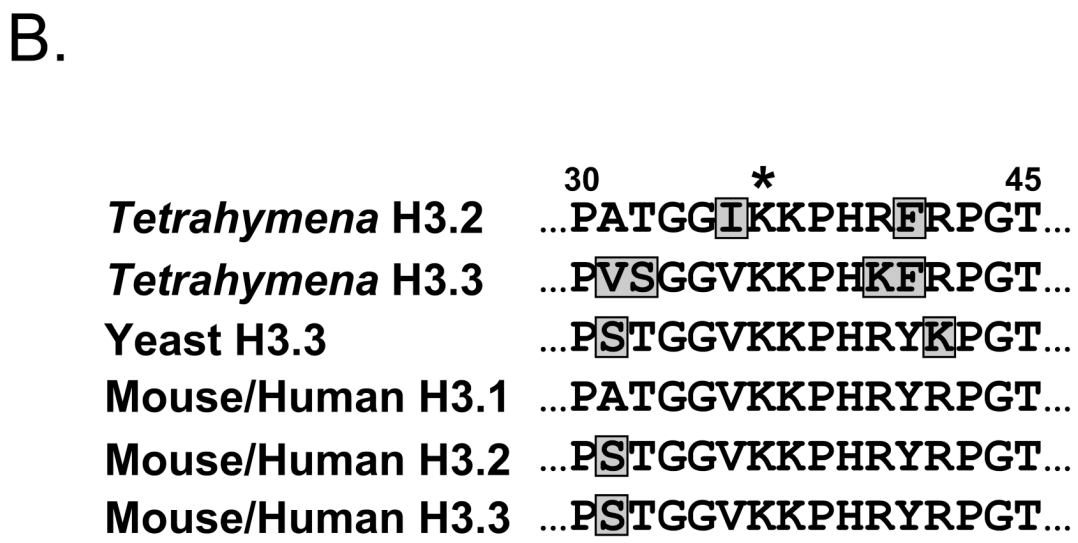


Figure 5. Histone H3K36 acetylation is conserved in mammals

(A) Acid-extracted histones from *Tetrahymena*, yeast (*S. cerevisiae*), mouse (mouse embryonic fibroblasts) and human (HEK293) cells, along with recombinant H3 from *Xenopus*, were resolved on a 15% SDS-PAGE gel, transferred to a PVDF membrane and probed for H3K36ac (upper panel). Prior to immunoblot analysis, the membrane was Ponceau S stained to confirm equal loading of protein (lower panel). *(B)* Alignment of histone H3 protein sequences (amino acids 30–45) from different eukaryotic species. Divergent residues are highlighted in gray boxes. Asterisk indicates the position of lysine 36 in the H3 sequence.