Global Assessment of Combinatorial Post-translational Modification of Core Histones in Yeast Using Contemporary Mass Spectrometry

LYS4 TRIMETHYLATION CORRELATES WITH DEGREE OF ACETYLATION ON THE SAME H3 TAIL

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A global view of all core histones in yeast is provided by tandem mass spectrometry of intact histones H2A, H2B, H4, and H3. This allowed detailed characterization of >50 distinct histone forms and their semi-quantitative assessment in the deletion mutants gen5Δ, spt7Δ, ahc1Δ, and rig2Δ, affecting the chromatin remodeling complexes SAGA, SLIK, and ADA. The “top down” mass spectrometry approach detected dramatic decreases in acetylation on H3 and H2B in gen5Δ cells versus wild type. For H3 in wild type cells, tandem mass spectrometry revealed a direct correlation between increases of Lys4 trimethylation and the 0, 1, 2, and 3 acetylation states of histone H3. The results show a wide swing from 10 to 80% Lys4 trimethylation levels on those H3 tails harboring 0 or 3 acetylations, respectively. Reciprocity between these chromatin marks was apparent, since gen5Δ cells showed a 50% decrease in trimethylation levels on Lys4 in addition to a decrease of acetylation levels on H3 in bulk chromatin. Deletion of Set1, the Lys4 methyltransferase, was associated with the linked disappearance of both Lys4 methylation and Lys14 or Lys18, 23 acetylation on H3. In sum, we have defined the “basis set” of histone forms present in yeast chromatin using a current mass spectrometric approach that both quickly profiles global changes and directly probes the connectivity of modifications on the same histone.

In eukaryotic cells, genetic information is complexed with histone proteins in a highly organized structure known as chromatin. The nucleosome, the fundamental structural unit of chromatin, contains an octamer formed by two copies of each of the core histones H2A, H2B, H3, and H4, about which is wrapped 146 bp of DNA (1). The dynamics of chromatin structure directly influence the accessibility of DNA, which in turn affects gene transcription, replication, and recombination (2). Core histone proteins are evolutionarily conserved and have flexible N-terminal tails protruding from the nucleosome. These tails are subject to a variety of post-translational modifications (PTMs) such as phosphorylation, ubiquitination, methylation, and acetylation (3). Such PTMs change the interactions of histone proteins with DNA and chromatin remodeling complexes, which are responsible for diverse functions within the cell (4). Different combinations of covalent modifications form the essence of what has been termed the histone code (5).

Acetylation is perhaps the best characterized PTM on histones and generally is associated with increased DNA accessibility not only by the neutralization of the positive charges on lysine residues but also as interaction sites capable of recruiting chromatin remodeling complexes (6). Previous studies have shown that particular histones and particular sites within histones become acetylated under distinct physiological conditions (7). The selection of acetylation sites could be accomplished either by a set of different histone acetyltransferases (HATs) or by the fine tuning of HAT specificity by additional factors. So far, a variety of proteins with HAT activity have been discovered in Saccharomyces cerevisiae (6, 8), but their in vivo substrate specificities have only been partially investigated by antibodies against some of the known acetylation sites (9). It is still a major challenge to define the global impact on each histone’s PTM pattern upon cellular perturbation (e.g. a HAT knock-out). A study of HAT deletion effects using a method with less bias (i.e. one that can visualize all abundant PTMs simultaneously in an unbiased fashion) and still provide site-specific PTM information will help our understanding of how HATs work together in vivo and illuminate the interplay between acetylation and other PTMs.

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The Abbreviations used are: PTM, post-translational modification; HAT, histone acetyltransferase; ECD, electron capture dissociation; RPLC, reverse phase liquid chromatography; ESI, electrospray ionization; FTMS, Fourier transform mass spectrometry; WT, wild type; HPLC, high pressure liquid chromatography; aa, amino acids; ChIP, chromatin immunoprecipitation; SAGA, Spt-Ada-Gcn5-acetyltransferase.  

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Gcn5 was first shown to be required for transcriptional activation and later recognized to have HAT activity (10). To date, it is one of the best characterized HATs both structurally and functionally. Recombinant Gcn5 acylates free H3 at Lys14 preferentially and H4 at Lys9 and Lys16 weakly in vitro, confirming the selectivity of Gcn5 for certain lysine residues within specific histones (11). For histone H3, this specificity is extended to other sites (Lys9, Lys14, Lys18, and Lys23) when Gcn5-containing complexes, such as SAGA and ADA, are used with nucleosome substrates in vitro (12), indicating that its HAT activity is regulated by interacting proteins. Later, the Grunstein laboratory used highly specific antibodies to reexamine the substrate specificity of Gcn5 in vivo (9). Their study demonstrated that Gcn5 is required for acetylation at Lys11 and Lys16 of H2B and Lys9, Lys18, Lys23, and Lys27 of H3 with the exception of Lys14. In the course of these studies, almost no effect on H4 (9, 13) was found, whereas others support that Gcn5 deletion can affect acetylation of H4 in addition to H3 (14). These results are informative but do not reveal if the acetylation sites affected by Gcn5 are on the same histone molecule or whether they are affected equivalently, because antibodies can only detect one PTM at a time and different antibodies have different affinities. Even when antibodies showed no change of modification at one site, there could be change of combinations of modifications. Due to the limitations of antibodies, a more informative and precise method is necessary to fully understand the substrate specificity of Gcn5.

Previous studies have shown a direct correlation between Gcn5 HAT activity and activation of specific model genes, including HIS3, PHOS, and HO (15–17). A more comprehensive understanding of Gcn5 HAT activity utilized microarray analysis to show expression differences for over 60% of genes in yeast (18, 19). A combination of serial analysis of gene expression and chromatin immunoprecipitation methods has shown that Gcn5 plays a global role in regulating histone H3 and H4 acetylation (20). However, direct measurements of the effect of Gcn5 on the global levels of PTMs on all core histone proteins have not been described.

Here, we used top down mass spectrometry (21, 22) to examine the global effects of Gcn5 and eight other mutants on the expression and modification of all four core histone proteins. We first established the “basis set” of all abundant PTM combinations on intact histones in wild type cells, including site-specific PTM localization, using electron capture dissociation (ECD) for tandem mass spectrometry (23, 24). Recently, this general approach has been demonstrated on diverse forms of human histones (25–27), with quantitative determination of specific combinations of modifications and PTM occupancy at specific sites (including the ability to resolve “positional isomers” of acetylation for H4) (28). Besides characterizing all core histones, ECD analysis of the H3 tail from wild-type chromatin also revealed a correlation between methylation and acetylation; there is a wide swing from 10 to 80% Lys4 trimethylation levels on the species with 0 and 3 acetylation states, respectively.

With the top down perspective, global alteration of histone modification in gcn5Δ cells was striking and readily apparent; levels of hyperacetylated forms of H2B and H3 were greatly diminished, whereas acetylation of H4 and H2A was only slightly affected. gcn5Δ cells showed reduced acetylation at specific sites in H3 and a 30% decrease in the overall trimethylation level on Lys4, underscoring the reciprocity between these two chromatin marks. Set1 mutants also showed the loss of acetylation at Lys14 and Lys18 or Lys23 on the same molecules where Lys4 trimethylation was also lost. Results from eight other mutants are also presented and highlight the potential to profile a large number of conditions for their effects on bulk chromatin.

EXPERIMENTAL PROCEDURES

Yeast Cell Culture—S288C S. cerevisiae cells (MATa his3Δ leu2Δ met15Δ ura3Δ) with the interruption of Gcn5, Ahc1, Rtg2, Ada1, Spt7, Ard1, Nat1, or Nat4, respectively, were purchased from Open Biosystems. All of the strains were grown in YPD (1% yeast extract, 2% bactopeptone, 2% glucose, pH 7) at 30 °C to midlog phase until an A600 = 0.6 units. Growth curves of wild type and gcn5Δ cells were established by recording cell density at A600 every 1.5 h. Cells were harvested by centrifugation at 4 °C for 5 min at 10,000 × g and washed twice with sterile water before storage of the cell pellets at −80 °C.

Set1 mutant MBY1217 (MATα his3Δ ade2::hisG leu2Δ ura3Δ met15Δ trp1Δ63 ty1his3AI-236r, Ty1lade2AI515 set1Δ::TRP1) and the parental strain were obtained from the laboratory of Scott Briggs (Purdue University). Construction of plasmids expressing full length Set1-(1–1080) and the N-terminal truncated Set1-(829–1080) were described in previous studies (29, 30). Cells were grown on SC medium (0.67% (w/v) yeast nitrogen base supplemented with amino acids, 2% glucose) lacking uracil to midlog phase and an A600 of 0.6 units. Cells were harvested as described above.

Histone Extraction and Separation—Frozen cell pellets were thawed on ice and extracted three times with 2.5 volumes of yeast protein purification reagent (Yper; Pierce), 0.1 M dithiothreitol, 5 mM microcysteine (Sigma), 500 μM 4-(2-aminoethyl)benzenesulfonyl fluoride (Roche Applied Science) and 100 mM sodium butyrate. Briefly, resuspended cells were incubated on ice for about 30 min, lysates were clarified by 5 min of centrifugation at 18,000 × g at 4 °C, and the supernatant was removed. Following two additional repetitions, the cytoplasm-reduced cell pellet was then washed three times with sterile water to remove excess Yper. Histone extraction was performed twice by incubation of the cell pellet with 2.5 volumes of 8 M deionized urea and 0.4 M sulfuric acid for 30 min on ice. The sample was spun down again, and clarified supernatants were pooled and immediately loaded onto an equilibrated disposable C4 column (J. T. Baker Inc. BAKERBOND sptTM wide pore butyl (C4) disposable extraction column). After washing the column with 10 volumes of buffer A (0.1% trifluoroacetic acid in distilled H2O), histones were eluted with buffer B (60% acetonitrile, 0.1% trifluoroacetic acid). Acetonitrile was then removed from the collected fractions using a SpeedVac.

Before further separation by RPLC, the sample was oxidized by incubating at room temperature in 3% formic acid and H2O2 for 4 h (31). Oxidized protein was loaded onto a 4.6-mm × 250-mm C8 or C18 Vydac column. Proteins were fractionated in 100 min from 30 to 60% B (A, 5% CH3CN with 0.1% triflu-
oroacetic acid; B, 90% CH₃CN with 0.094% trifluoroacetic acid) and detected by absorbance at 214 nm. Histone proteins were eluted between 55 and 95 min (Fig. 1A). Every fraction was subsequently concentrated in a SpeedVac.

Different acetylation states of H3 were further separated by acid urea gel electrophoresis using gels composed of 15% Triton X-100, acetic acid, urea (typically 1.5-mm thick and 13 × 14 cm²) according to Mullen et al. (32), except that gels were run at 250 V for 20 h to obtain better resolution of H3. Proteins were visualized by Coomassie Blue staining. A duplicate gel was transferred to polyvinylidene difluoride and probed with anti-Lys4 trimethylation antibodies (Abcam).

**H3 Glu-C Digestion and RPLC Separation**—H3 purified from RPLC was dried down and resuspended in 50 μL of 25 mM NH₄HCO₃ buffer (pH 4). Glu-C (Roche Applied Science) was added at a ratio of Glu-C/H3 of 1:10 (w/w) and incubated at room temperature for 4–5 h. The reaction was stopped by freezing the sample. H3 digests were loaded to a 4.6 mm × 250 mm C18 Vydac column pre-equilibrated with 0.2% trifluoroacetic acid. Peptides were fractionated in 60 min from 0 to 60% B (90% acetonitrile with 0.188% trifluoroacetic acid) and detected by absorbance at 214 nm (Fig. 3A).

**Mass Spectrometry: ESI/Quadrupole-FTMS**—Purified and dried histone samples were resuspended in 50 μL of ESI solution (50% acetonitrile, 49% H₂O, and 1% formic acid), and 10 μL was loaded to a nanospray robot (Advion BioSciences, Ithaca, NY). The system used in this study was a custom 8.5 T quadrupole-FTMS hybrid described elsewhere (24). Ions from ESI were directed through a heated metal capillary and multiple ion guides into the ion cell (~10⁻¹⁰ torr) of the FTMS system. Theoretical isotopic distributions were generated using IsoPro version 3.0 and fit to experimental data by least squares to assign the most abundant isotopic peak. Typically, 10–50 scans were used to collect broadband data. Samples were also analyzed using a quadrupole-based selection strategy. In short, targeted peaks were selected by a quadrupole filter before accumulation (1–2 s) in an external octupole and transfer to the ion cell. Ions were fragmented by ECD (23). Most spectra were stored as 512,000 data points and processed using zero or one truncation, no zero fill, and Hanning apodization. MS/MS data were analyzed by a modified version of the THRASH algorithm. Individual histone proteins were identified and characterized by uploading data onto the prosightptm World Wide Web server to search the yeast data base.

**Statistical Comparison with ChIP-on-chip Data**—To cross-validate our findings, we compared our results with publicly available data. In a recently published paper, genome-wide histone acetylation levels of H3 at Lys⁹ and Lys¹⁴ and methylation levels of H3 at Lys⁴ of wild type yeast in rich medium (YPD) were measured using ChIP-on-chip (33). The average expression ratios of immunoprecipitated DNA to control DNA across experimental replicates were downloaded from the World Wide Web. After a log₂ transformation of Lys⁹ and Lys¹⁴ acetylation level and Lys⁴ trimethylation level, a plot comparing the acetylation (Lys⁹ and Lys¹⁴) and methylation (Lys⁴) levels at 3,581 intergenic regions was obtained for subsequent analysis (34).

**RESULTS**

**Histone Extraction and Purification**—Extraction of histones from yeast cells is widely known to be more problematic than from mammalian cells. Waterborg and colleagues (35) have
described a quick method for yeast histone preparation, but in our hands the yield is less than 50% of expected (data not shown). To address this limitation, we designed a simplified procedure that relies on the observation that yeast chromatin is not readily solubilized when the cell wall is intact. Thus, we used a mixture of nonionic detergents to deplete cells of the nonhistone proteins and then used conventional acid extraction in the presence of 8 M urea to prepare crude histone from the residual cell pellet. By using this method, more than 95% histone recovery was confirmed through Western blot analysis using an antibody to hyperacetylated H4 (data not shown). Oxidation of samples before RPLC separation helped to resolve each histone to a single chromatographic peak (Fig. 1A), with H3 giving two peaks later during the RPLC run (see below). The PTM profiles obtained from intact histones did not change appreciably upon repeated sampling, confirming that differentially modified forms of intact histones co-elute during reversed phase separation (35); this enables quantitative determination of PTM profiles using top down MS (25–27). For WT and mutant cells described below, results are presented for intact profiles first, followed by site-specific PTM localization using MS/MS.

Effects of Gcn5 Deletion on H4 Acetylation—To date, there have been inconsistent results when assessing the effect of Gcn5 on histone H4. By using antibodies specific for each acetylated site, Roth and co-workers (14) showed that deletion of Gcn5 caused a diminution of acetylation at each of the four acetylation sites in H4. Other studies have shown that Gcn5 does not acetylate H4 in vivo and in vitro (9, 13). In order to clarify these issues, we compared H4 modification forms from wild type and gcn5Δ cells. To begin, we acquired high resolution, intact mass spectra of histone H4 (Fig. 1B). The H4 modification profile showed that the majority of H4 (>85%) is acetylated with di- and triacetylated forms as the dominant modification forms, which is consistent with a much larger fraction of the yeast genome actively being transcribed versus mammalian histone H4 (22). Gcn5 deletion results in very slight reduction in global H4 acetylation (Fig. 1B versus D). This result is consistently reproducible (n = 5) and appears to be near the limit of the FTMS-based assay to reliably detect changes in PTM patterns. These results indicate that Gcn5 deletion has only a marginal effect on the global H4 acetylation pattern in vivo.

To determine which lysine sites were acetylated, we performed ECD on each acetylation state (states 1–5) from WT and gcn5Δ cells. In WT cells, the 1 Ac form was N-terminally acetylated (data not shown). Modifications on the 2 Ac form were localized to the N terminus and Lys16. For the 3 Ac form, Lys12 was acetylated in addition to the initial two acetylations (supplemental Fig. S1, A and B). The 4 Ac form was acetylated at Lys8 in addition to acetylation sites in the 3 Ac form (supplemental Fig. S1C). In the 5 Ac form, N terminus, Lys16, Lys12, Lys8, and Lys5 were all acetylated (data not shown). All acetylation forms were found to be pure species; no acetylation isoforms were detected (28). The directionality of acetylation is rigorously a C→N-terminal zipper (i.e. Lys16→Lys12→Lys8→Lys5) in both WT and gcn5Δ cells. We conclude that Gcn5 deletion only slightly reduces the relative abundance of each acetylation form on H4 and does not affect residue specificity of acetylation.

Gcn5 Deletion: Effect on H2A—There are two H2A gene products, H2A.1 and H2A.2, and one H2A.Z variant in yeast cells. H2A.1 and H2A.2 have exactly the same mass and the same protein sequence, apart from a C-terminal switch of an Ala-Thr in H2A.1 to a Thr-Ala in H2A.2. Combining the
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Figure 3. Change of modification profiles of H3 assessed by MS, acid-urea electrophoresis, and Western blotting. A and B, MS of intact H3 modification profiles in WT and gcn5Δ cells. C, acid urea gel of intact histone H3 from WT and gcn5Δ. D, Western results from using an anti-Lys6 trimethylation antibody to visualize an analogous acid urea gel analogous to that in C.

intact protein profile (Fig. 1C) and ECD data of each form reveals that the most abundant form is N-terminally acetylated, followed by the form with N-terminal and Lys2 acetylation. The form with three acetylations is present at very low abundance, and after digestion with Glu-C, the site was localized to Lys5 (data not shown). Most studies suggest that Gcn5 has no effect on H2A acetylation, but others have suggested that Gcn5 may have some role in H2A acetylation (9). Our data show no detectable changes in the acetylation level of H2A upon Gcn5 disruption (Fig. 1, C versus E). H2A.Z was shown to be present in two adjacent nucleosomes in most yeast gene promoters (36), but H2A.Z levels are too low for detection by the MS approach employed here. However, Milar et al. have shown that Gcn5 affects acetylation on Lys14 of H2A.Z (37).

H2B from WT and gcn5Δ Cells—There are two H2B forms present in yeast, H2B.1 and H2B.2, with a mass difference of 15 Da. Previous studies revealed that 6 lysines can be acetylated on each gene product, with the specific HATs responsible for individual acetylations not yet all known. Using top down MS, up to five acetylations were observed on H2B.1 and H2B.2 (Fig. 2A). Due to the low abundance of the forms with six acetylations, they were not obvious in the “broadband” FT mass spectrum. However, after signal enhancement using selected ion accumulation, which increases the dynamic range by ~50-fold (21, 38), these species were detectable (data not shown). For both H2B.1 and H2B.2, monoacetylation was the most abundant form, and the abundance of other forms decreased with increasing acetylation. Using top down MS to monitor the relative abundance of all forms at once, a spectrum of H2B from gcn5Δ cells showed strikingly that all forms with >1 acetylation were undetectable with 60% occupancy on Lys22 and 40% occupancy on Lys16 (with the first acetylation 100% on the N terminus). The same method applied to triacetylated H2B showed that the N terminus and Lys16 were acetylated at 100% occupancy, whereas the third acetylation was split 60% at Lys11 and 40% at Lys22 (Supplemental Fig. S2B). In the tetra-acetylated forms, all of the Lys11, Lys16, and Lys22 were acetylated (supplemental Fig. S2C). Due to the low abundance of the 5 Ac and 6 Ac forms, reliable ECD data have not been acquired to date. These results indicate that acetylation of Lys6, Lys11, Lys16, Lys17, and Lys22 in H2B are lost upon deletion of Gcn5.

WT H3 and Effects of Gcn5 Deletion—For WT S. cerevisiae extracted with our new procedure, two H3 peaks were consistently observed in all of our RPLC chromatograms (Fig. 1A, far right), whereas other histone extraction methods only yield one H3 peak (35). FT mass spectra showed that the modification profiles of these two HPLC peaks are different (supplemental Fig. S3). The reason for the elution differences between these two peaks is unknown at the current time. Considering that the nominal mass of three methylations is the same as one acetylation (+42 Da), these peaks could differ in the levels of different modifications and/or their site isomers. Thus, to simplify sample comparisons, we combined these two H3 HPLC peaks (displayed separately in supplemental Fig. S3) for all sample comparisons reported below.

As shown in Fig. 3, A and B, the modification profiles for H3 in gcn5Δ cells showed that the relative abundance of peaks with ∆m values of +126, +168, and +210 Da above the unmodified form decreased detectably. Consistent with a large body of literature on Gcn5 deletion, it seemed likely that these decreases were attributable primarily to H3 hypoacetylation. However,
recently Zhang et al. (39) showed that histone methylation is also correlated with acetylation, suggesting that the change could be caused by decreased methylation on Lys\(^4\) or Lys\(^79\) as well. In order to assess this with an orthogonal method, we compared these MS profiles with the resolution achieved on acid urea gels, well known for the ability to resolve histones according to acetylation state (32). Based on the Coomassie-stained gel of Fig. 3C, H3 from gcn5\(\Delta\) cells clearly has less acetylation \textit{versus} that from WT cells. To correlate these acetylation states with trimethylation at Lys\(^4\) (K4me3), the anti-K4me3 antibody was used to probe a transfer from an analogous gel. This suggested that less K4me3 occurred in gcn5\(\Delta\) cells (Fig. 3D), but extracting quantitative information about the specific acetylation states was challenging. In order to quantify the relationship between acetylation and methylation states, we performed ECD on the bulk H3 from both wild type and gcn5\(\Delta\) cells.

With direct ECD fragmentation of all intact H3 isoforms \textit{en masse}, each acetylation site is difficult to assess in isolation from one another (27). Therefore, we used endoproteinase Glu-C to liberate the H3 peptides corresponding to the 1–50 aa (40), 51–59 aa, and 74–94 aa segments, which collectively contain almost all known modification sites. Mass spectrometry identified one major peak in the chromatogram corresponding to H3 1–50 aa in both WT and gcn5\(\Delta\) H3 digests (Fig. 4A). In addition to the major HPLC peak at \(~\)30 min, two “satellite” peaks at 30.5 and 31.5 min were identified to be heavily acetylated forms of the 1–50 aa piece of WT H3 (see Fig. 4B). In Glu-C digests of H3 from gcn5\(\Delta\) cells, both satellite peaks were typically missing, with the 30.5 min peak observable only if we doubled our conventional loading. Although these two satellite peaks are low in abundance (<10 milliabsorbance units at 214 nm), the data are reproducible. To further characterize the modifications on those heavily modified species, we combined peaks 2 and 3 and performed bulk ECD on all of these forms together. Bulk ECD results on the hypermodified forms in these fractions showed that 55% of Lys\(^4\) and 20% of Lys\(^36\) were trimethylated, 75% of Lys\(^{18}\) was acetylated, and all of the other lysine residues (Lys\(^{7}\), Lys\(^{14}\), Lys\(^{23}\), and Lys\(^{27}\)) were 100% modified (supplemental Fig. S5).

Fig. 4C shows the change of modification profiles on the 1–50 aa piece are similar to those observed for intact H3 upon Gcn5 deletion (Fig. 3, A and B). The relative abundance of peaks with +84, +126, and +168 Da were decreased dramatically in gcn5\(\Delta\) cells, whereas the +42 Da peak remains much the same. ECD of the +42 Da peak of WT and gcn5\(\Delta\) cells showed that it
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To streamline analysis of H3 1–50 aa from gcn5Δ cells, we combined all HPLC peaks of 1–50 aa and did bulk ECD. This showed that the acetylation levels on Lys⁹ (Fig. 5A) and Lys¹⁴ (supplemental Fig. S4A) each decreased by ~40%. The acetylation levels on Lys⁹ and Lys¹⁴ were determined through analyses of the c₁₀ and c₁₇ fragment ions, respectively. Although the mass changes due to trimethylation and acetylation are very close, they are readily distinguished by accurate mass measurements on the high resolution FTMS. As seen in Fig. 5B, the +42 Da peak of c₁₀ showed peak splitting. The low m/z peak represents acetylation from Lys⁹, and the high m/z peak represents trimethylation from Lys⁹. Overall, there is ~10% acetylation occupancy for Lys¹⁴ and Lys²⁷ (supplemental Fig. S4, B and C) in WT cells, but neither of them were observed in gcn5Δ cells. A change in the level of methylation at Lys⁴ and Lys⁷⁹ was also observed. Trimethylation on Lys⁴ decreased about 30% (Fig. 5C), whereas trimethylation on Lys⁷⁹ increased about 25% (Fig. 5D). Acetylation on Lys⁵⁶ (Fig. 5E) and methylation on Lys⁸⁶ and Lys⁷⁷ remained the same in gcn5Δ cells (supplemental Fig. S4, D and E). The quantitative change of acetylation and methylation levels on each lysine residue is summarized in Fig. 6. Since Gcn5 is a HAT, the decrease of overall acetylation level on H3 is expected in Δgcn5 cells. Here, the observed decrease of Lys⁴ trimethylation level also demonstrates that Lys⁴ methylation and acetylation levels on H3 are regulated in reciprocal fashion in vivo.

Set1 Mutants—To further characterize the relationship between trimethylation at Lys⁴ and acetylation on the same H3 molecule, we explored the changes in PTM patterning on H3 from different Set1 mutant cells. Set1 has been shown to be the sole methyltransferase responsible for H3 Lys⁴ mono-, di-, and trimethylation in yeast (29, 41–44). Its N-terminal truncated form (829–1080 aa) lacks trimethylation ability on Lys⁴ but still maintains normal levels of mono- and dimethylation (30).

Fig. 7, A and B, shows mass spectra of intact H3 and Glu-C-digested H3 (1–50 aa), respectively, from the parental strain and Set1 mutants. Although the broadband MS showed dramatic changes of modifications on H3 for the mutants, ion fragmentation was required to localize the changes to specific sites. Bulk ECD was performed on the 1–50 aa piece of H3 as described above, and the results from signature fragment ions reporting on lysines 4, 9, 14, 18, and 23 are summarized graphically in Fig. 7D and supplemental Fig. S6. The c₄ frag-

is a mixture of 20% of trimethylation on Lys⁴ and 80% trimethylation on Lys⁸⁶. We further characterized the +84, +126, and +168 Da peaks in WT cells by ECD. The results showed that there is a mixture of “PTM isomers” in each peak. From the +42 Da peak to the +168 Da species, we observed that Lys⁴ trimethylation increased from 10 to 80% (Fig. 4D). Specifically, the MS/MS-based assay quantified the amount of Lys⁴ trimethylation increased from 10 to 80% (Fig. 4D). Specifically, the MS/MS-based assay quantified the amount of Lys⁴ trimethylation on the 0, 1, 2, and 3 acetylation states as 10, 55, 75, and 80%, respectively (Table 1). In going from 0 to 3 acetylations, the distribution of acetylation occupancies for each discrete state was semiquantitatively determined (Table 1). These results revealed that higher degrees of trimethylation level on Lys⁴ are associated with higher degrees of acetylation.

### Table 1

| Modifications | +42 Da (0 Ac) | +84 Da (1 Ac) | +126 Da (2 Ac) | +168 Da (3 Ac) |
|--------------|--------------|--------------|--------------|--------------|
| K⁴me⁵      | %           | %            | %            | %            |
| Lys⁹⁴ Ac    | 10           | 55           | 75           | 80           |
| Lys¹⁴⁴ Ac   | 0            | 20           | 35           | 45           |
| Lys¹⁴⁵ Ac   | 0            | 40           | 50           | 100          |
| Lys¹⁴⁶ Ac   | 0            | 10           | 10           | 35           |

FIGURE 5. Comparison of the modifications at different lysine residues of H3 in WT and gcn5Δ cells.

A, change of modification on fragment ion c₁₀, which contains modification information on Lys⁴ and Lys⁹.

B, peak splitting of the c₁₀ +42 Da peak in A due to the presence of both acetylation (42.0106 Da) and trimethylation (42.047 Da). The low m/z peak represents acetylation from Lys⁴, and the high m/z peak represents trimethylation from Lys⁴.

C–E, change of modification on Lys⁴, Lys⁷⁹, and Lys⁵⁶, respectively.
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Although in WT type cells, Lys$^{23}$ is 100% acetylated when Lys$^4$ is highly trimethylated (Table 1), we did not obtain enough information about the correlation between them from these bulk ECD results because of the excessive complexity of the data. In order to increase the dynamic range for the detection of minor forms, we compared the ECD results of the +126 Da peak of 1–50 aa of H3 from WT and set1Δ cells. As shown in supplemental Fig. S7, in set1Δ cells, the peak with Lys$^4$ trimethylation plus Lys$^{14}$ and Lys$^{23}$ acetylation disappeared. This result showed that with the loss of Lys$^4$ trimethylation, Lys$^{23}$ cannot be added to this particular modification form.

Gcn5-containing Protein Complexes—In vitro studies have shown that Gcn5 alone does not acetylate H2B; however, in the context of protein complexes such as SAGA, it might be capable of directly acetylating H2B or affecting the function of other HATs, which could acetylate H2B. In addition to SAGA, several other protein complexes, such as ADA and SLIK, have also been discovered to contain Gcn5 (45). Gcn5 may have different or overlapping functions in those protein complexes through protein interactions. In order to verify that, we compared the change of histone modification profiles upon disruption of each protein complex. Previous studies have shown that Spc7 is an essential component of SAGA (46). Other proteins, such as Aci1 and Rtg2, are found to be required for the integrity of ADA and SLIK protein complexes (47). Using the method described here, histone profiles were obtained from each of these mutants. Fig. 8 reveals that although the relative abundance of different modification forms was altered compared with WT, H2B and H3 were still highly modified after knocking out one protein complex. This result suggests that considerable redundancy exists between Gcn5-containing protein complexes or possibly other types of HATs that can compensate for the loss of HAT function of these specific types of Gcn5 complexes.

N-terminal Acetyltransferases—With only N-terminal acetylation of H2B left unaffected by Gcn5 knock-out, we investigated the effect of different N-terminal acetyltransferases on histones. Previous studies have shown that Ard1 and Nat1 are H2B N-terminal acetyltransferases, with Nat4 known to acetylate H4 and H2A (32, 48). Top down MS data of histones from set1Δ cells showed no change in acetylation levels on H2B and other histones (data not shown). MS data from ard1Δ cells revealed a dramatic increase of unmodified H2B over the monoacetylated form (Fig. 8A, second row from top). MS/MS analysis of this monoacetylated form of H2B by ECD showed residual N-terminal acetylation exclusively (data not shown). This indicates that Ard1 is the major H2B N-terminal acetyltransferase, and there exists one or more unknown N-terminal acetyltransferases. The H3 modification pattern was also dramatically changed in ard1Δ cells (Fig. 8B, second row from top). MS data of histones from nat1Δ cells showed no change in acetylation levels on H2B and other histones (data not shown). MS data from ard1Δ cells revealed the increase of H4 and H2A acetylated forms, consistent with prior studies (32, 48).

DISCUSSION

Histone Detection and MS/MS with Minimal Bias—This is the first implementation of high resolution “top down” mass spectrometry on yeast histones and allowed characterization of more than 50 distinct molecular forms arising from multiple
modifications of the yeast core histones from wild type and mutant cell lines. In addition to establishing a definitive "basis set" of expressed yeast histones with minimal bias (i.e. detection of intact histone forms generally), this global view of bulk chromatin allows quantitative assessment of all major forms of each histone upon mutation (or other perturbation), with MS/MS allowing direct connection of intact forms with a quantitative readout of PTM occupancy at specific sites (28). Since this particular approach determines both the changes of abundance for multiply modified forms and the localization of each modification to individual amino acid residues, this study overcomes some of the limitations of antibodies by using MS of intact histones (and the N-terminal 1–50 aa piece of histone H3). Antibodies are good for PTMs in isolation, but they generally cannot accurately account for changes involving combinations of modifications. In addition, since different antibodies have different affinities, accurate quantitation of changes in the relative abundance of different PTMs is not possible. Although top down mass spectrometry can overcome these problems, one limitation relative to antibodies is that of limit of detection. The methodology employed here has the ability to detect species at 0.1–1% relative abundance in bulk chromatin, with the quantitative picture of histones precise to ~5% using MS/MS data of moderate quality (28). For the direct comparison of histone H3 from WT and gcn5Δ cells, the MS results qualitatively match those from acid urea gels and Western analysis of selected PTM combinations (Fig. 3).

**Effects of Gcn5 on All Core Histones**—Due to the inherent limitations of antibodies discussed above, previous studies that were mostly based on antibodies did not show a comprehensive view of the effects of Gcn5 on all core histones. By using top down strategies described in this paper, we reexamined its effect on all core histones with minimal bias. The results showed that knock-out of Gcn5 had slight effects on H4 and H2A but dramatically decreased acetylation levels on histone H2B and H3. Further investigation using tandem mass spectrometry localized the change of modification to individual amino acid residues. The results demonstrated that for H2B, Gcn5 is the major HAT that affects all acetylation sites except for the N terminus. Ard1 was shown to be the major N-terminal acetyltransferase for H2B. On H3, not only acetylation on Lys9, Lys14, Lys18, and Lys23 were affected by Gcn5 but also the methylation levels on Lys4 and Lys79. The correlation of methylation and acetylation on H3 is discussed further below.

Since Gcn5 exists in different protein complexes, such as SAGA, SLIK, and ADA, it may have different effects on histone proteins in different protein complexes. However, disruption of each individual protein complex did not show a dramatic change of modification on H2B and H3. This may due to the redundancy of function among different Gcn5-containing complexes or possibly other HATs. Here, a "global" view prevents us from determining whether PTM changes are confined to only a portion of the genome. However, with Gcn5 knocked out, HAT function of all of the protein complexes was lost, resulting in a dramatic decrease of acetylation on H2B and H3.

**Linking Methylation with Acetylation on the Same H3 Tail**—There are three lines of evidence that reveal the interplay of acetylation and Lys4 trimethylation on the H3 tail, with the quantitation of this reciprocal relationship afforded by our approach a novel feature of this work. For WT yeast histone H3, the FTMS-based assay quantified the direct link between increases of Lys4 trimethylation and the 0, 1, 2, and 3 acetylation states of histone H3. The results reveal a swing from 10, 55, 75

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**FIGURE 7. Comparison of H3 modification at intact, Glu-C-digested and fragment level from WT, Set1-(829–1080), and set1Δ cells.** A and B, change of modification pattern on intact H3 and Glu-C-digested H3, respectively. C, change of Lys4 methylation in WT and Set1 mutants. D, representation of the change of modifications on Lys4, Lys9, Lys14, Lys18, and Lys23 detected in MS/MS experiments from WT and set1Δ cells. In set1Δ cells, mono-, di-, and trimethylation on Lys4 disappeared. The forms that contain Lys5 trimethylation, Lys14 acetylation, and Lys18 or Lys23 acetylation were not observed in set1Δ cells.
to 80% trimethylation levels on the 0, 1, 2, and 3 acetylation states, respectively. This work therefore meshes well with assertions in recent reviews (49–52) and other work (39, 53) proposing a high correlation between Lys4 trimethylation and hyperacetylation on H3.

Our results from deletion of gcn5Δ and set1Δ further characterize the interplay between acetylation and Lys4 trimethylation on H3 and are largely consistent with previous studies. In gcn5Δ cells, the expected decrease of acetylation levels (primarily on Lys9, Lys14, Lys18, and Lys23 of H3) were accompanied by an overall drop of 30% in trimethylation level on Lys4. Zhang et al. (39) has shown that a higher level of methylation of Lys4 and Lys79 is associated with hyperacetylation on H3. Since the absence of Gcn5 results in decreased acetylation, lower methylation levels on Lys4 and Lys79 are expected. However, our observation of a 20% decrease of trimethylation on Lys79 in gcn5Δ cells is inconsistent with the study of Zhang et al. (39). The results from Set1 mutants also showed that loss of Lys4 trimethylation, not mono- or dimethylation, caused the loss of detectable Lys14 and Lys18 or Lys23 acetylation on the same H3 tail. Interestingly, previous studies have shown that some of the Lys4 methyl readers reside within different chromatin remodeling complexes that also have acetyltransferase activities. For example, Chd1 and Yng1, two major trimethyl Lys4 binding proteins in yeast, are components of SAGA/SLIK and NuA3, respectively (54, 55). Loss of Lys4 trimethylation may cause the loss of binding sites for those protein complexes, which can acylate Lys14 and Lys18 or Lys23 to further activate transcription. These results are consistent with a more potent role for Lys4 trimethylation (versus mono- or dimethylation) in transcriptional activation.

Integrating Diverse Data Types: Linking K4me3 with Acetylation at Thousands of Loci—Correlation of recent ChIP-on-chip results (32) with our global histone modification readouts by mass spectrometry was initiated by creating the scatter plot between genome-wide acetylation levels at Lys14 and trimethylation levels at Lys4 of H3 in 3,581 intergenic regions of WT yeast cells (Fig. 9). The acetylation level of Lys14 and trimethylation level of Lys4 on H3 exhibit a strong positive linear relationship (Pearson correlation coefficient 0.72) after taking log2 transformation of their values. From our studies of acetylation site usage, we found that Lys14 and Lys23 are the most occupied
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acetylation sites and correlate tightly with the increase in Lys4 trimethylation level (Table 1). The results showed that Lys9 acetylation correlates less tightly with Lys4 trimethylation (Table 1); indeed, an analogous plot to Fig. 9 using Lys9 acetylation gives a Pearson correlation coefficient at 0.42. Since Lys73 acetylation has not been studied in these ChIP-on-chip experiments, the statistical results are unavailable, but from our mass spectrometry results, we believe that it should also correlate well with Lys4 trimethylation. The reciprocal relationship between Lys14 acetylation and Lys4 trimethylation has also been shown in the gen5Δ and set5Δ cells (discussed above). One novel feature of our work is that data in Fig. 7D and supplemental Fig. S7 showed that Lys14 acetylation and Lys4 trimethylation co-exist on the same H3 tail and that the loss of Lys4 trimethylation caused the linked disappearance of acetylation on Lys14.

Yeast provides an excellent system for studying the nature of transcriptionally active chromatin, as a large percentage of the chromatin is in a transcriptional competent conformation (86% of the genome encodes genes). This makes it especially interesting to examine the levels of histone acetylations. In yeast cells besides Gcn5, there are many other HATs which also exhibit site and histone specificity. Currently, because of the advantage of yeast genetics, the knock-out library makes it convenient to study the effects of all yeast histone modifying enzymes. In combination with the mass spectrometry and histone extraction methods described here, it enables us to investigate the substrate specificity of all the known HATs on histones, which will unravel how HATs work together to produce specific acetylation patterns. The acetylation levels are maintained by the balance of HATs and histone deacetylases. However, until now the interplay of HATs and histone deacetylases has remained unclear. The study of histone deacetylase substrate specificity and the changes of acetylation patterns after double mutation of certain HATs and histone deacetylases will elucidate the correlations between them.

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