The Bromodomain of Gcn5 Regulates Site Specificity of Lysine Acetylation on Histone H3*

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In yeast, the conserved histone acetyltransferase (HAT) Gcn5 associates with Ada2 and Ada3 to form the catalytic module of the ADA and SAGA transcriptional coactivator complexes. Gcn5 also contains an acetyl-lysine binding bromodomain that has been implicated in regulating nucleosomal acetylation in vitro, as well as at gene promoters in cells. However, the contribution of the Gcn5 bromodomain in regulating site specificity of HAT activity remains unclear. Here, we used a combined acid-urea gel and quantitative mass spectrometry approach to compare the HAT activity of wild-type and Gcn5 bromodomain-mutant ADA subcomplexes (Gcn5-Ada2-Ada3). Wild-type ADA subcomplex acetylated H3 lysines with the following specificity: H3K14 > H3K23 > H3K9 ~ H3K18 > H3K27 > H3K36. However, when the Gcn5 bromodomain was defective in acetyl-lysine binding, the ADA subcomplex demonstrated altered site-specific acetylation on free and nucleosomal H3, with H3K18ac being the most severely diminished. H3K18ac was also severely diminished on H3K14R, but not H3K23R, substrates in wild-type HAT reactions, further suggesting that Gcn5-catalyzed acetylation of H3K14 and bromodomain binding to H3K14ac are important steps preceding H3K18ac. In sum, this work details a previously uncharacterized cross-talk between the Gcn5 bromodomain “reader” function and enzymatic HAT activity that might ultimately affect gene expression. Future studies of how mutations in bromodomains or other histone post-translational modification readers can affect chromatin-templated enzymatic activities will yield unprecedented insight into a potential “histone/epigenetic code.” MS data are available via ProteomeXchange with identifier PXD001167. Molecular & Cellular Proteomics 13: 10.1074/mcp.M114.038174, 2896–2910, 2014.

Eukaryotic DNA is packaged around the histone proteins H3, H4, H2A, and H2B to form the nucleosomal core particle that is the fundamental building block of chromatin (1, 2). Post-translational modifications (PTMs) on histones such as lysine acetylation and methylation are added to (or “written” on) specific histone residues by acetyltransferase and methyltransferase complexes, respectively (3, 4). Histone PTMs are found clustered in distinct combinations across the genome, where they regulate chromatin-templated processes such as transcription, replication, and DNA repair (5–7). Histone PTMs have the potential to introduce structural variation into chromatin through charge or steric effects, but many PTMs are also recognized or “read” by relatively small protein motifs that interact with particular PTMs. These PTM “reader” motifs include the acetyl-lysine binding bromodomains and the methyl lysine binding PHD fingers and tudor domains, which are thought to target or stabilize the enzymatic activity of chromatin-associated complexes along specific chromosomal regions (8–11). Most enzymes that catalytically add (“write”) or remove (“erase”) histone PTMs exist in complexes that contain multiple histone PTM readers, leading to the postulation of a “histone code” or “epigenetic code.” However, the molecular rules for reading and writing combinations of histone PTMs are poorly defined (6, 12, 13).

Gcn5 is a well-studied, highly conserved histone acetyltransferase (HAT) that has been shown to acetylate multiple histone lysines in vitro, primarily lysine 14 of histone H3 (H3K14), but also H3K9, H3K18, H3K23, H3K27, H3K36, and additional lysines found in histones H4 and H2B (14–18). In addition to the catalytic HAT domain, Gcn5 also contains a bromodomain that binds acetyl-lysine, making Gcn5 both a

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Received February 4, 2014, and in revised form, August 4, 2014.
Published, MCP Papers in Press, August 8, 2014, DOI 10.1074/mcp.M114.038174


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reader and writer of histone acetylation (19–22). In vivo, Gcn5 is the histone acetyltransferase subunit of chromatin-associated transcriptional activator complexes SAGA and ADA (23–27). Notably, point-mutation experiments with yeast Gcn5 suggest that disrupting the ability of the Gcn5 bromodomain to interact with acetyl-lysine can lead to a decrease in Gcn5-dependent transcription and histone acetylation at promoter regions in vivo, as well as a decrease in total nucleosome acetylation on nucleosomes in vitro (19, 28). Nevertheless, how the overall HAT activity and lysine specificity of Gcn5 are altered upon disruption of bromodomain acetyl-lysine binding function remains unclear. Increased understanding of how bromodomains or other histone PTM readers can affect chromatin-templated enzymatic activities could yield unprecedented insights into mechanisms of histone PTM cross-talk, and might provide important parameters for epigenetic drug design, such as how reader domains might contribute to substrate specificity and processivity of catalytic activity. Importantly, the human homolog of yeast Gcn5 is found mutated in several disease states, such as cancers and spinocerebellar ataxia, and is thus a potential therapeutic target (29, 30).

In order to elucidate the interplay between Gcn5 reader and writer functions, a sensitive and quantitative approach that detects subtle changes in acetylation on specific lysines is required. HAT assays utilizing acetyl CoA radioisotopes primarily evaluate bulk lysine acetylation within a target polypeptide or a mixture of proteins, but they do not yield information about site specific acetylation. In contrast, mass spectrometric approaches yield detailed information describing the specificity of a HAT reaction regarding acetylation rates for specific lysines (31). However, previous mass spectrometric studies of Gcn5 HAT kinetics predominantly analyzed combined populations of the histone acetylation states generated during a particular reaction, so it is difficult to interpret information about HAT site specificity (the acetylation of a specific lysine relative to that of a proximal lysine) (16, 32). Because we wanted to examine the site specificity of Gcn5 HAT activity on histone substrates, we first needed to develop an alternative method for following acetyl-lysine addition on distinctly acetylated isoforms of histone proteins. Here, we employed a coupled approach using acid-urea gels and quantitative mass spectrometry to monitor in vitro ADA subcomplex HAT activity. Our findings reveal that the Gcn5 bromodomain contributes to lysine specificity and is necessary for processive acetylation on histone H3.

**EXPERIMENTAL PROCEDURES**

**Cloning, Recombinant Expression, and Purification of Full-length ADA (Gcn5/Ada2/Ada3) Subcomplex**—The pST44-yAda2/H11003-yAda2 × 3-yGcn5 × 5 polycistronic vector containing yeast GCN5, ADA2, and ADA3, obtained courtesy of Dr. Song Tan (Penn State, PA), originally encoded a truncated form of Ada3 (missing N-terminal residues 1–184) (33). Full-length Ada3 was generated using nested primers to extend the truncated ADA3 fragment, which was cloned into the pST44 vector (supplemental Fig. S1). The Gcn5 Y413A bromodomain mutant and the Gcn5 P371T/M372A double point mutant were generated using QuikChange site-directed mutagenesis (LaJolla, CA) on the plasmid containing full-length ADA3. The expression of full-length Gcn5/Ada2/Ada3 subcomplex and subsequent purification with Talon metal affinity resin (Clontech) and an FPLC SourceQ column (GE Healthcare) were performed as previously described (33). Concentrations of the recombinant wild-type and mutant Gcn5/Ada2/Ada3 subcomplexes were normalized using SDS-PAGE gels and Coomassie Blue staining.

**Cloning, Recombinant Expression, and Purification of the Gcn5 Bromodomain—Wild-type (WT), Y413A, and P371T/M372A yeast Gcn5 bromodomains (residues 329–438 of Gcn5p) were cloned from the respective pST44-yAda2/H11003-yAda2 × 3-yGcn5 × 5 polycistronic vectors containing yeast GCN5, ADA2, and ADA3 from WT, Y413A, and P371T/M372A. Bromodomains were cloned into an N-terminal thioredoxin-His6-S tag tag (pET32a vector, Darmstadt, Germany). Proteins were recombinantly expressed in chemically competent BL21 Escherichia coli (Invitrogen) after overnight induction with 1 mM isopropyl 1-thio-β-D-galactopyranoside at 20 °C in LB medium. Bacteria were pelleted, freeze-thawed, and resuspended in purification buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 10% glycerol, 2 mM B-ME, 1 mM PMSF, 2 mM benzamidine) and lysed by sonication (Thomas Scientific, Swedesboro, NJ). Lyssate was incubated with nickel-nitriotriacetic acid agarose resin (Invitrogen) for at least 1 h at 4 °C. Resin was washed with purification buffer, and protein was eluted with purification buffer containing 150 mM imidazole. Protein was aliquoted, flash-frozen in liquid nitrogen, and stored at −80 °C.

**Nucleosome Reconstitution**—Recombinant histones from Xenopus laevis were expressed in E. coli, purified from inclusion bodies, and assembled into histone octamers as described previously (34). DNA for recombinant mononucleosomes was obtained via EcoRV digestion of pSTS5–16xNCP601, courtesy of Dr. Song Tan (Penn State, PA), which contained 16 tandem copies of a 147-bp fragment with the 601 positioning sequence (35). Prior to nucleosome reconstitution, the 147-bp DNA fragments were purified as described previously (36). Nucleosome core particles were assembled using the salt gradient dialysis method followed by HPLC purification on a TSKgel DEAE-5PW column with 13-μm particle size (34). Nucleosomes were dialyzed into low-salt buffer (10 mM Tris-HCl, pH 7.5, 5 mM KCl, 1 mM DTT) and concentrated to 25 to 50 μM for storage at 4 °C, and they were used within a month of preparation.

**In Vitro Histone Acetyltransferase Assays**—HAT assays were performed with full-length recombinant Gcn5/Ada2/Ada3 subcomplex using 1 μg of recombinantly expressed Saccharomyces cerevisiae free histone H3 and 30 μM acetyl CoA in HAT reaction buffer (20 mM Tris, pH 7.5, 50 mM NaCl, 5% glycerol) in a total volume of 60 μl. For HAT assays with variable enzyme concentrations, samples were incubated for 30 min at 30 °C and then flash-frozen in liquid nitrogen to stop the reaction. Samples were then lyophilized. Acetyl CoA was omitted from the control reactions.

HAT assays run over a time course were first incubated at 30 °C for 2 min prior to the addition of enzymatic subcomplex at a concentration of 50 nM, except for the Time 0 assay, which was flash frozen immediately upon the addition of enzyme. Reactions were carried out for the respective time course at 30 °C and flash-frozen and lyophilized upon completion. A non-enzymatic reaction was carried out for 8 h at 30 °C to control for spontaneous acetyl CoA acetylation or histone degradation (supplemental Fig. S4).

HAT reactions analyzed via quantitative mass spectrometry were performed in triplicate using 150 nM of WT or Y413A ADA subcomplex and carried out at 30 °C for 30 min.

Assays containing nucleosomal substrates consisted of 0.55 μM nucleosome, 150 nM of either WT or Y413A subcomplex, and 30 μM baculovirus.
membrane as previously described (38). Membranes were blocked, transferred to a PVDF membrane using a semi-dry transfer system. Subsequent mass spectrometric analysis.

Bands were individually excised using a clean scalpel and frozen for nano-pure water and stained with SimplyBlue Safe Stain (Invitrogen). All lyophilized samples were resuspended in Laemmli loading buffer. Running chambers were washed with methanol to prevent keratin contamination. NuPAGE SDS-PAGE 12% gels (Invitrogen) were used to resolve histone H3. Acid-urea gels were assembled and run as previously described (38). Gels were washed with nano-pure water and stained with SimplyBlue Safe Stain (Invitrogen). Bands were individually excised using a clean scalp and frozen for subsequent mass spectrometric analysis.

Western Blot Analysis of Site-specific Histone H3 Lysine Acetylation—Histone H3 samples were resolved by means of SDS-PAGE and transferred to a PVDF membrane using a semi-dry transfer system. Samples resolved on acid urea gels were transferred to a PVDF membrane as previously described (38). Membranes were blocked overnight in 5% milk at 4 °C and washed in Tris-buffered saline (TBS). Primary antibodies were diluted in 1% milk in TBS and 0.1% Tween as follows for nucleosomes (Fig. 6 and supplemental Fig. S2): anti-H3 (Cambridge, MA ab1791, 1/50,000), anti-H4 (Abcam ab10158–25, 1/5000), anti-H2B (Abcam ab1790–25, 1/20,000), anti-H3K9ac (Abcam, 1/5000), anti-H3ac (Millipore, 1/5000), anti-H3K14ac (ab52946, Abcam, 1/5000), anti-H3K18ac (Carlsbad, CA 91930, 1/10,000), anti-H3K23ac (Carlsbad, CA 91930, 1/10,000), anti-H3K18ac (Carlsbad, CA 91930, 1/10,000), anti-H3K9ac (Millipore, Darmstadt Germany, 1/10,000). The following antibodies were used for histone K to R mutants: anti-H3K14ac (ab46984, Abcam, 1/5000), anti-H3K18ac (Millipore 07–354, 1/7500), anti-H3K23ac (Millipore 07–355, 1/5000). Each primary antibody was applied for 1 h at room temperature and then washed in TBS and 0.1% Tween. Goat anti-rabbit IgG-horse-radish peroxidase secondary antibody (Amersham Biosciences) was diluted to 1/4000 in 1% milk and TBS~0.1% Tween, applied for 1 h at room temperature, and washed in TBS and 0.1% Tween. Blots were developed using Pierce ECL Western Blotting Substrate (Thermo Scientific) and exposed using film.

Bromodomain Peptide Pulldown Assays—Peptide pulldowns were essentially performed as by Taverna et al. (10), with the following modifications. Briefly, streptavidin-coupled dynabeads (20 µl per sample) (Invitrogen M-280) were incubated with biotinylated histone peptides (1 µg per sample) in PBS and washed in PBS. Peptide-coated beads were then incubated with purified bromodomains (1 µg) in the presence of BSA competitor (1 µg) in binding buffer (20 mM HEPES, pH 7.9, 100 mM NaCl, 0.2% Triton X-100, 0.5 mM DTT, 10% glycerol) for 3 h at room temperature. Beads were washed three times for 5 min each time with high-salt wash buffer (20 mM HEPES, pH 7.9, 300 mM NaCl, 0.2% Triton X-100, 0.5 mM DTT, 10% glycerol) and one time with low-salt wash buffer (4 mM HEPES, pH 7.9, 20 mM NaCl). Peptide bound protein was eluted off beads with boiling 1X SDS-PAGE sample buffer, resolved on 12% SDS-polyacrylamide gels, transferred to PVDF, and probed with antibodies recognizing S-tag (ab18588, Abcam, 1/500) and Streptavidin-HRP (Molecular Probes S-911, 1/1000). Input lanes represent 0.1% bromodomain protein used in the pulldown.

H3 peptides:


H3K14ac 1–20: biotin: ARTKQTKSTGK(ac)APRKQL-K(Biotin)-NH2.

Mass Spectrometry—Gel bands excised from acid-urea gels were de-stained (serially washed with 50 mM ammonium bicarbonate in 50% methanol), treated with 30% d6-acetyl anhydride in 100 mM ammonium bicarbonate to chemically acylate lysines (39), and subjected to in-gel trypsin digestion (100 ng of trypsin at 37 °C for 15 h). Treatment with d6-acetyl anhydride adds isotopically heavy acetyl groups (+45 Da) to unmodified and monomethylated lysines, which serves to prevent trypsin digestion at lysine residues with a distinguishable synthetic modification. This heavy acetylation enhances the identification of histone peptides (39, 40). Tryptic peptides were separated by reverse phase Jupiter Proteo resin (Torrance, CA) on a 100 × 0.075 mm column using a nanoAcquity UPLC system (Milford, MA). Peptides were eluted using a 45-min gradient from 98:2 to 40:60 buffer A:buffer B. (Buffer A = 0.1% formic acid, 0.05% acetonitrile; buffer B = 0.1% formic acid, 75% acetonitrile.) Eluted peptides were ionized by electrospray (1.9 kV) and subjected to MS/MS analysis using collision-induced dissociation on an LTQ Orbitrap Velos mass spectrometer (Thermo) (41–43). MS data were acquired using the FTMS analyzer in profile mode at a resolution of 60,000 over a range of 375 to 1500 m/z. MS/MS data were acquired for the top 15 peaks from each MS scan using the ion trap analyzer in centroid mode and normal mass range with a normalized collision energy of 35.0. Mass spectrometric data were database searched with Mascot using heavy and light acetylation of lysines as variable modifications. The percentage of site-specific lysine acetylation was determined with spectral count comparisons of peptides with light or heavy acetylation at the given lysine (% light acetylation for a lysine in a given peptide = (light spectral counts/light + heavy spectral counts) × 100). The standard deviation was calculated from triplicate measurements of spectral counts. Spectral counting provided a robust, semi-quantitative approach for initial analysis of site-specific lysine acetylation.

Tandem mass spectra were extracted by Thermo ExoExtractMSn version 1.0.0.8. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (version 2.4.1, Matrix Science, London, UK). Mascot was set up to search the Con uniprot sprot 1 database (selected for S. cerevisiae, version 4, 7034 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 ppm. Acetyl of lysine and acetyl-2H(3) of lysine were specified in Mascot as variable modifications. Scaffold (version Scaffold 4.0.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 50.0% probability by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (44). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (45) with the dataset identifier PXD001167 (46–48).
RESULTS

Design of a Coupled Acid-Urea Gel and Mass Spectrometry Approach to Quantitate Site Specific Lysine Acetylation Levels on Histone H3—Fig. 1 diagrams our in vitro approach for measuring the site specific HAT activity of the ADA subcomplex on histone H3, a primary endogenous target of Gcn5 (Fig. 1A) (15, 24). Importantly, to increase sensitivity, samples for mass spectrometry were first resolved using acid-urea gels to separate distinctly acetylated isoforms of histone H3 generated during HAT reactions (Fig. 1B). Acid-urea gels separate many proteins, including histones, by both charge and size (38, 49). The ablation of charge that occurs when a single lysine is acetylated (from positive to neutral) results in a specific decrease of migration rate on acid-urea gels. Thus, different charge states within a population of heterogeneously acetylated H3 will cause the isoform mixture to resolve as a “ladder” ranging from a hypoacetylated to hyperacetylated state, where each H3 polypeptide within a specific band contains the same number of acetylated lysines (Fig. 1B). To accurately determine site specific lysine acetylation, one can treat samples with deuterated $d_6$-acetic anhydride, which converts all unmodified lysines to a “heavy,” deuteroacetylated form (Figs. 1C and 1D) (39, 40, 50, 51). The amount of acetylation catalyzed by a HAT enzyme on a specific lysine is calculated using the ratio of “light” (enzymatic) and “heavy” (chemical) acetylation signal detected at that lysine position (Fig. 1E) (39, 40, 50–54).

Determining ADA Subcomplex Site Specificity for Histone H3 Lysines—The minimal form of Gcn5 required for HAT activity on the physiological nucleosome template is the ADA subcomplex (Gcn5, Ada2, and Ada3), and accordingly, we used full-length recombinant yeast ADA subcomplex for our studies (supplemental Fig. S1) (55–58). ADA subcomplex reacted with histone H3 under steady-state reaction conditions for 30 min yielded seven distinct H3 bands when resolved by acid-urea gels (Fig. 2A). In vitro studies have shown that Gcn5

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**Fig. 1. Outline of experimental design and validation of mass spectrometry approach.** Schematic depicting the quantitative mass spectrometry approach to determine Gcn5/Ada2/Ada3 lysine-specific acetylation on histone H3 beginning with A, an in vitro assay consisting of recombinantly expressed ADA subcomplex, recombinantly expressed histone H3, and acetyl CoA. B, various acetylation states of histone H3 are resolved on an acid-urea gel where bands are excised and C, subjected to deutero-acetic anhydride chemical acetylation to modify all unacetylated lysine residues with a chemically equivalent but isotopically heavier acetyl group. D, tryptic digestion of histone H3 yields a mixture of peptides containing native “light” acetylation catalyzed by Gcn5 (white) and chemical “heavy” acetylation (black). E, peptides were resolved and analyzed using LC-MS/MS, and the abundance of acetylation on a given lysine residue was calculated by comparing the ratio of light versus heavy signal.
targets up to six of the N-terminal H3 lysines (H3K9, H3K14, H3K18, H3K23, H3K27, and H3K36) (15, 16, 18). Utilizing our semi-quantitative mass spectrometry approach, we determined the percent acetylation of each lysine on H3 polypeptides extracted from each of the individual acid-urea gel bands (Figs. 2B and 2C). As expected, we observed acetylation on H3K9, H3K14, H3K18, H3K23, H3K27, and H3K36, whereas no detectable acetylation had occurred on H3 polypeptides purified from either acid-urea gel band 0 in the negative control or HAT reaction band 0. H3 in bands 1–6 all contained acetylation on H3K14 at levels near 100% (Figs. 2A and 2B), in accordance with previous studies showing that H3K14 is a major site of acetylation by Gcn5 (15, 16, 59, 60).

Our data provide additional insight into the site specificity of the ADA subcomplex. We posited that these seven bands represented distinct reaction intermediates as H3 proceeded from an unmodified state (band 0) to a monoacetylated state (band 1) and eventually to a hexa-acetylated/hyperacetylated state (band 6) in our HAT assay. Under steady-state conditions, Gcn5 HAT activity has also been shown to occur in a progressive and unidirectional manner, where acetylation of different lysines on H3 occurs in single increments (16). For example, if the six lysine targets on an H3 polypeptide were acetylated simultaneously, the “ladder” of seven bands that we do observe in Fig. 2A would not be present, and instead only a single band at the band 6 position in Fig. 2A would be detected. Therefore, a comparison of the acetylation levels on specific H3 lysines as H3 polypeptides progress from hypoacetylated to hyperacetylated reaction intermediates can be used to gauge the site specificity of Gcn5, where site specificity is defined as the ability of Gcn5 to acetylate one lysine residue relative to another lysine on the same H3 histone polypeptide (16, 32). Because 100% of the acetylation detected on H3 polypeptides from band 1 (the first reaction intermediate) is on H3K14, and because H3K14ac is present at 100% levels in the other five acetylated H3 bands, we can extrapolate that H3K14 is also the initial site of acetylation, a result that agrees with previous kinetic studies (16). Our data also suggest that H3K23ac directly follows H3K14ac, in that we noted H3K23ac as the only acetylation besides H3K14ac.
in the di-acetylated H3 polypeptides that were confined to band 2 (Figs. 2A and 2B), rather than the potential multitude of combinatorial acetylation events. In band 4, H3K14ac, H3K23ac, H3K18ac, and H3K9ac were present at levels near 100% (a small amount of acetylation was also detected on H3K27) (Fig. 2B). These data suggest that H3K18 and H3K9 acetylation events occur after H3K14 and H3K23 acetylation of individual H3 polypeptides. H3 in band 5 was fully acetylated on lysines H3K14, H3K23, H3K18, H3K9, and H3K27, and H3 in band 6 was fully acetylated on lysines H3K14, H3K23, H3K18, H3K9, H3K27, and H3K36 (Fig. 2B). These data suggest that the fifth and sixth acetylation events by the ADA subcomplex were catalyzed on H3K27 and H3K36, respectively. This predicted hierarchy of acetylation adds novel information about the HAT activity of the Gcn5 ADA subcomplex. For example, a recent kinetic study found that monomeric Gcn5-catalyzed H3K9ac and H3K23ac occur at a similar rate, and the sensitivity afforded by our approach of combining acid-urea gel separation and mass spectrometry might help elucidate the order of H3K9 and H3K23 acetylation (16).

To complement our mass spectrometry data, we used well-characterized anti-acetyl antibodies to analyze our HAT reactions, although these affinity reagents can cross-react with off-target epitopes and yield false positive or negative results (supplemental Fig. S2) (61, 62). Western blots on SDS-PAGE and acid-urea resolved HAT reactions supported our interpretation of the mass spectrometry data and showed that ADA subcomplex-catalyzed H3 acetylation was confined within the N-terminal tail region (Figs. 1A and 1B; supplemental Fig. S2). In sum, our data imply that the ADA subcomplex acetylates histone H3 with the following site specificity: H3K14 > H3K23 > H3K9 ≈ H3K18 > H3K27 > H3K36 (Fig. 2C).

Mutation of the Gcn5 Bromodomain Disrupts Lysine Acetylation on Histone H3—The evolutionarily conserved bromodomain module, often found in HATs and HAT-associated complexes, directly binds acetyl-lysine (10, 21, 63). A variety of in vitro biophysical assays have shown that recombinant Gcn5/PCAF bromodomains interact with H3K9ac, H3K14ac, H3K36ac, H4K16ac, and H4K8ac, as well as non-histone lysines like K50ac on HIV-1 Tat (19, 20, 22, 63–68). Mutations that disrupt binding of the Gcn5 bromodomain, or other HAT bromodomains, to acetyl-lysines have been shown to alter histone acetylation in vitro and in vivo and decrease transcription at HAT responsive genes (19, 28, 69–71). Although these data suggest that the bromodomain may help Gcn5 to both read and write lysine acetylation on histones, the impact of the bromodomain on Gcn5 acetylation remains poorly characterized. We hypothesized that the bromodomain plays a role in directing the site specificity of Gcn5, and we sought to generate a point mutation within the bromodomain that disrupted acetyl-lysine binding but maintained the structural integrity of the ADA subcomplex. Mutation of Y413 in yeast Gcn5 (or the homologous Y809 in PCAF) disrupts acetyl-lysine binding, but not the structural integrity of the bromodomain or the stoichiometry of the ADA subcomplex (Fig. 3A, supplemental Fig. S3) (19, 20, 28, 67). Introducing the Y413A Gcn5 mutation into the ADA subcomplex has also been shown to reduce HAT activity (28). Accordingly, we introduced the Gcn5 Y413A mutation into the ADA subcomplex to assess changes in HAT site specificity as measured via our combined acid-urea gel and mass spectrometry approach (Fig. 3B).

To better understand how loss of Gcn5 bromodomain function impacts ADA subcomplex activity, we first performed HAT reactions using increasing concentrations of either WT or Y413A ADA subcomplex and resolved the acetylated H3 products using acid-urea gels (as in Fig. 2A). At enzyme concentrations of 50 nM and greater, the pattern of H3 acetylation became noticeably different between the WT and mutant ADA subcomplex, and the Y413A ADA subcomplex reactions showed a relative lag in the generation of hyperacetylated H3 isoforms (Fig. 3C). In particular, at an enzyme concentration of 200 nM, the Y413A ADA subcomplex predominantly catalyzed di-acetylated on H3 polypeptides (see band 2, Fig. 3C), compared with the WT ADA subcomplex HAT reaction, which was enriched for hexa-acetylated H3 polypeptides (see band 6, Fig. 3C). Because the staining intensity of band 6 (hexa-acetylated) generated by the WT ADA subcomplex was relatively comparable with the intensity of band 2 (di-acetylated) from the Y413A subcomplex reaction, it can be inferred that bromodomain acetyl-lysine binding function contributes to a 3-fold increase in acetylation. These results suggest that when the ADA subcomplex contains Gcn5 with a bromodomain defective in acetyl-lysine binding, total H3 acetylation is significantly decreased.

To further examine how the overall H3 acetylation rate was disrupted by mutation of the Gcn5 bromodomain, we examined the evolution of differential acetylation patterns in kinetic time course experiments comparing WT and Y413A ADA subcomplexes at a concentration of 50 nM (Fig. 3D). Consistent with the acetylation differences using increasing enzyme concentrations (Fig. 3C), we observed a reduction in total acetylation of H3 with the Y413A ADA subcomplex at later time points (60 min, 4 h, and 8 h) (Fig. 3D, supplemental Fig. S4). Densitometry quantitation of acid-urea resolved H3 band intensity underscored the differences in H3 acetylation between the WT and mutant subcomplex (Fig. 3E and data not shown). Interestingly, at earlier time points (1 min, 5 min, and 15 min) the Y413A mutant exhibited greater acetylation activity (Fig. 3E), suggesting a previously unappreciated role for the bromodomain in restricting the initial lysine acetylation events on H3. Taken together, these results suggest that the Gcn5 bromodomain helps regulate histone H3 acetylation catalyzed by the ADA subcomplex.

The Gcn5 Bromodomain Regulates Site Specificity of Acetylation on Histone H3—Because our acid-urea gel analysis of the Gcn5 bromodomain mutant HAT reactions sug-
The Gcn5 Bromodomain Guides Site Specific Acetylation on Histone H3

A

B

ADA subcomplex

WT   Y413A

Gcn5
Ada2
Ada3

(Coomassie)
SDS-PAGE

C

Histone H3 (Coomassie)
Acid-urea

Band# 1 2 3 4 5 6

10 nM 25 nM 50 nM 100 nM 200 nM

Band

WT   Y413A   WT   Y413A   WT   Y413A   WT   Y413A   WT   Y413A

D

Histone H3 (Coomassie)
Acid-urea

Band# 1 2 3 4 5 6

0 min 1 min 5 min 15 min 60 min 4 hrs

WT   Y413A   WT   Y413A   WT   Y413A   WT   Y413A   WT   Y413A

E

Relative band intensity

0 min 1 min 5 min 15 min 60 min 4 hrs

Relative band migration in acid-urea gel

Band# 0 1 2 3 4 5

WT   Y413A
gested an alteration in the overall abundance and rate of H3 acetylation (Fig. 3), we sought to examine differences between the acetylation levels on specific H3 lysines catalyzed by Gcn5 Y413A and WT ADA subcomplexes. First, we used mass spectrometry on SDS-PAGE-resolved reactions to quantify absolute acetylation on H3 for the Gcn5 Y413A and WT ADA subcomplex HAT reactions (Fig. 4A). We determined that the Gcn5 bromodomain mutant ADA subcomplex was able to acetylate H3K14 and H3K23 to WT levels (Fig. 4A). In contrast, the bromodomain mutant ADA subcomplex demonstrated reduced acetylation of residues H3K9 and H3K18, suggesting a role for the Gcn5 bromodomain in the proper acetylation of these sites (Fig. 4A). Although the SDS-PAGE approach allowed us to directly compare the overall ability of WT and mutant Gcn5 to acetylate specific H3 lysines, information about changes in site specificity was difficult to discern. As reported by other groups, we had difficulty detecting acetylation events beyond H3K9ac, H3K14ac, H3K18ac, and H3K23ac under steady-state conditions using an SDS-PAGE approach, particularly in the Gcn5 bromodomain mutant re-
action, suggesting this method was arguably less sensitive than our analysis using acid-urea gels (Fig. 4A) (16). Regardless, our data implied that mutation of the Gcn5 bromodomain did not result in significant expansion of acetylation sites beyond those lysines acetylated by WT Gcn5.

To gain insight into bromodomain-related changes in site specificity, we compared the WT and mutant HAT reactions using the combined acid-urea gel and mass spectrometry approach as in Fig. 2 (Figs. 2, 4B, and 4C). This approach indicated that mutation of the Gcn5 bromodomain did not detectably alter sites of H3 acetylation, as we observed that the same six H3 lysines were acetylated by both mutant and WT ADA subcomplexes (Figs. 4B and 4C). However, although H3K14ac, H3K23ac, and H3K9ac were the preferred lysine acetylation targets in both WT and Gcn5 Y413A HAT reactions, significant differences were detected when we compared acetylation levels among the remaining lysines (Fig. 4C). In contrast to the pattern of H3 acetylation observed with WT ADA subcomplex, H3K18ac was not detected until band 5 in Y413A (Fig. 4C). Additionally, the Y413A mutation of Gcn5 resulted in relatively increased H3K27ac and H3K36ac levels within band 4 (Fig. 4C). Interestingly, a similar trend of decreased H3K18ac and increased H3K27ac and H3K36ac was observed for the double P371T/M372A Gcn5 bromodomain within band 4 (Fig. 4C). Together, these findings indicate that general disruption of the acetyl-lysine binding capacity of the Gcn5 bromodomain will change Gcn5 site specificity, such that H3K27 and H3K36 are preferred over H3K18 as sites of acetylation.

Next, we aimed to better understand the reduction of H3K18ac observed in Y413A mutant subcomplex HAT reactions. Because H3K14ac and H3K23ac were not significantly affected by mutation of the Gcn5 bromodomain, and because the bromodomain has been shown to bind acetylated H3, we hypothesized that H3K18ac could be dependent on an interaction of the Gcn5 bromodomain with either H3K14ac or H3K23ac. To test whether H3K18ac is affected when H3K14 or H3K23 is unable to be acetylated, we generated histone H3 point mutants containing either H3K14R or H3K23R. Next, the acetylation patterns catalyzed by ADA subcomplex on these histone point mutants and the WT H3 were compared (Fig. 5A). In both H3K14R and H3K23R histone mutants, only five lysines were detectably acetylated due to the loss of either H3K14 or H3K23 as a Gcn5 target (Fig. 5A). We observed the acetylation rate to be reduced in both histone mutants relative to WT H3, particularly in the H3K14R reaction, which seemed to be acetylated at a substantially slower rate, indicated by a greater intensity in the lowest, unacetylated H3 band, band 0 (Fig. 5A). To determine whether either H3K14 or H3K23 acetylation might affect K18 acetylation levels, we employed acetyl-lysine specific antibodies. We first tested the anti-H3K18ac, anti-H3K14ac, and anti-H3K23ac antibodies on WT H3, H3K14R, and H3K23R that had been chemically acetylated at each lysine residue with acetic anhydride to ensure that the K to R mutations did not disrupt antibody epitope recognition (Fig. 5B, first column). We then performed Western blotting on SDS-PAGE-resolved HAT reactions to determine the total acetylation level catalyzed by the ADA subcomplex on WT or mutant histone H3 (Fig. 5B, second column). Here we observed a significant decrease of H3K18ac on the H3K14R mutants, whereas H3K18ac was to be minimally affected on the H3K23R mutant. When these reactions were resolved on acid-gels and subjected to the same Western blot analysis, a striking decrease of H3K18ac was again observed on the H3K14R mutant relative to that of WT H3 or H3K23R, as H3K18ac was only observed on H3K14R histone in the highest acetyl-H3 band, band 5 (Fig. 5C). Together, these data suggest that acetylation of H3K18 by the ADA subcomplex is dependent upon the presence of an acetylatable lysine at H3K14, but not H3K23.

Bromodomain-dependent Site Specific Acetylation on Nucleosomal H3 Substrates—A previous study using the Gcn5 Y413A ADA subcomplex identified bromodomain-dependent cooperativity of acetylation on nucleosome substrates, although alterations to site specificity were not identified (28). Having established a link between the Gcn5 bromodomain and site specificity of acetylation on free histone H3, we sought to examine HAT activity of the Y413A Gcn5 mutant on the physiologically relevant nucleosome template. We analyzed HAT reactions using antibodies against histones and site specific acetylation events as in supplemental Fig. S2 (Figs. 6A and 6B, respectively). Consistent with previous reports, the majority of WT Gcn5-dependent acetylation within the nucleosome occurred on histone H3 (Fig. 6A) (27). Just as we observed with free H3, acetylation on nucleosomal H3 was strikingly diminished in the Y413A ADA subcomplex reactions relative to the wild type, with the majority of H3 in the bromodomain mutant reaction resolving as a mono- (band 1) or di-acetylated (band 2) species (Fig. 6A). In contrast, H4 and H2B, which have also been reported as Gcn5 histone targets, showed no significant change in acetylation patterns when we compared the WT and mutant HAT reactions (Fig. 6A) (15, 17, 18). Using anti-acetyl antibodies, we found that H3K14ac was enriched in the lower states of acetylation catalyzed by the Gcn5 bromodomain mutants, suggesting a loss of acetylation processivity relative to the wild type (Fig. 6B). Interestingly, in Y413A ADA subcomplex reactions, H3K18ac was almost undetectable, and although H3K27ac remained detectable at WT levels, this modification was now enriched in relatively lower acetylation states than in the wild type (Fig. 6B). Together with the considerable alteration of acetylation patterns observed in bromodomain mutant HAT reactions on free histone H3, the altered nucleosomal acetylation patterns in Y413A ADA subcomplex reactions suggested that the interaction between the Gcn5 bromodomain and acetyl-lysine is crucial in determining the site specificity on histone H3.
DISCUSSION

In our study, we optimized an approach to better understand how the site specificity of HAT enzymes may be regulated by histone PTM reader modules within the same protein (or complex). We used a combined approach of acid-urea gel resolution and quantitative mass spectrometry to investigate the interplay between histone acetyltransferase activity and bromodomain function of the highly conserved transcriptional co-activator Gcn5 within the biologically relevant ADA subcomplex on recombinant histones and nucleosomes. We observed that within the ADA subcomplex, Gcn5-dependent acetylation of H3 occurred with a processivity of H3K14 > H3K23 > H3K9 ≈ H3K18 > H3K27 > H3K36 (Fig. 2C). Furthermore, we found that ablation of the acetyl-lysine binding function of the Gcn5 bromodomain both diminished the amount of total acetylation and disrupted the order in which lysines were acetylated on free and nucleosomal histone H3. Ultimately, ADA subcomplex acetylation of lysines H3K14 and H3K23 was shown to be independent of bromodomain function, whereas downstream acetylation of lysines H3K9, H3K18, H3K27, and H3K36 was altered upon Gcn5 bromodomain mutation.

With respect to the order of H3 lysine acetylation catalyzed by Gcn5, our data suggest that the ADA subcomplex clearly preferred H3K14, as it was found to be 100% acetylated in each of the acetylated histone H3 bands, immediately followed by acetylation at H3K23ac on the same polypeptide. However, another group recently observed that the acetylation...
tion of histone H3 lysines H3K9 and H3K23 demonstrated similar rates of acetylation following H3K14ac (16). One possible reason for this difference in the apparent order of acetylation catalyzed by monomeric Gcn5 and the ADA subcomplex might be interactions of Gcn5 with the Ada2 and Ada3 adaptor proteins. Indeed, previous studies that directly compared HAT activity among monomeric Gcn5, the 0.8-MDa intact ADA complex, and the 1.8-MDa SAGA complexes indicate an expanded repertoire of lysine acetylation sites as the enzyme becomes organized into complexes with additional adaptor proteins, and they also noted distinct acetylation kinetics between ADA and SAGA (15). Alternatively, it is possible that our approach of using acid-urea gels to fractionate distinct acetylation states prior to mass spectrometry enabled greater resolution of individual H3 species than was possible with the methods used in the previous study (16). Although this previous study provided valuable information regarding the specificity of Gcn5 by examining the rates of acetylation on individual lysine residues, it might have been difficult to accurately distinguish the order of H3K9 and H3K23 without first purifying distinct acetylation states (16).

The contributions of the Gcn5 bromodomain to HAT site specificity within histone polypeptides remain poorly characterized. Prior in vivo studies of Gcn5 bromodomain mutants (including Y413A and P371T/M372A) suggest that the acetyl-lysine binding capacity of bromodomains acts to regulate HAT activity at Gcn5-targeted promoters, and loss of this function can impair nucleosome remodeling (19). Furthermore, the interaction of the Gcn5 bromodomain with acetylated lysine residues has been suggested to underlie the cooperative acetylation that Gcn5 complexes exhibit on nucleosomal substrates (28, 70). Here, our comparison of WT...
ADA subcomplex HAT activity to that of a bromodomain Y413A mutant shows that disruption of the acetyl-lysine binding capacity of the Gcn5 bromodomain clearly altered the order of acetylation events on H3K18, H3K27, and H3K36 (Figs. 4C and 6B). As initial H3K14, H3K23, and possibly H3K9 acetylation events are likely dominated by residues surrounding the HAT domain (73, 74), the subsequent acetylation events of H3K18, H3K27, and H3K36 might be aided by bromodomain interactions with previously deposited acetyl-lysine “marks.” Interestingly, the data of our group and others suggest the H3K14 residue is the preferred substrate for Gcn5 HAT activity and an acetyl-lysine binding target for the Gcn5 bromodomain (15, 16, 18, 59, 60, 75). Considering this and our data showing that H3K18ac was greatly diminished in HAT reactions involving bromodomain mutants or H3K14R mutants (Figs. 5B and 5C), we propose a two-step “reader/writer” model wherein Gcn5 first acetylates H3K14; then the bromodomain would recruit (or “tether”) Gcn5 at H3K14ac, thus stimulating HAT activity on H3K18 (supplemental Fig. S7). Given the changes in site specific HAT activity we observed in both the non-disruptive (Y413A) and disruptive (P371T/M372A) Gcn5 bromodomain mutants, other Gcn5 bromodomain mutations such as Gcn5 Y364 and N407, which are predicted to be important for acetyl-lysine binding in structural studies, might display a similar loss in H3K18-directed HAT activity (20, 65–67, 76). We also noted that mutant ADA subcomplex catalyzed moderately higher H3 acetylation states early in the reaction (Fig. 3D), which supports a role for the bromodomain in restricting initial acetylation events along H3. Although these findings suggest a mechanistic role for the bromodomain in processive acetylation along the H3 polypeptide and extend the utility of the bromodomain beyond a simple tethering function, future biochemical and structural studies on full-length Gcn5 will likely be needed to determine how the HAT domain and bromodomain synergize to promote specific acetylation patterns (77).

Cross-talk among histone PTMs like acetylation, methylation, and phosphorylation is widely appreciated as a regulatory mechanism to foster or prevent interactions between chromatin effector complexes and substrates (78, 79). Phosphorylation of threonine 11 on histone H3 reduces the ability of Gcn5 to acylate H3K14 (80). NuA3, a yeast acetyltransferase complex containing the HAT Sas3, binds trimethylated H3K4 using the PHD finger of the Yng1 subunit, which stimulates H3K14 acetylation on the same H3 polypeptide (8, 81). Interestingly, Gcn5-containing ADA and SAGA are also capable of binding H3K4me3 through the tandem tudor domain of their subunit Sgf29, although the impact of this interaction on Gcn5 HAT specificity is poorly understood (82, 83). In the context of prior in vivo studies of bromodomain mutants, our work also suggests the existence of endogenous pathways that target the acetyl-lysine binding capacity of bromodomains as a means to regulate HAT activity (65). Many studies have linked mutation of histone binding proteins and misregulation of histone PTMs to human disease (84, 85). Relevant to our observation that H3K18 acetylation was dependent on bromodomain function (Figs. 4A, 4C, and 6B), it was recently shown that H3K18ac is linked to increased grades of prostate cancer (86). In addition, hyperacetylation of H3K18 is associated with cellular transformation by the viral oncoprotein E1A (87). Our findings contribute to potential therapeutic relevance given the recent focus on pharmacological targeting of the epigenome (84, 88). For example, the potent small molecule inhibitors JQ1 and I-BET disrupt interactions between acetyl-lysines and the dual bromodomains of the BET family proteins (89–91). Although JQ1 and I-BET have shown therapeutic promise against midline carcinoma and excessive inflammatory response, much like we observed with mutation of the Gcn5 bromodomain, these drugs can disrupt histone acetylation profiles in ways that are poorly understood (89). Indeed, the large number of histone binding motifs within acetyltransferases and other chromatin-targeted enzyme complexes that represent potential drug targets only further emphasizes the importance of comprehending the tenets of a “histone” or “epigenetic code” (6, 10, 12, 92).

**Acknowledgments**—We thank the UAMS Proteomics Facility for mass spectrometric support; the laboratory of G.D. Bowman for recombinant yeast histones; S. Tan for the pST44-Ada32His3-yAda2x3-yGcn5x5 polycistronic vector containing yeast GCN5, ADA2, and ADA3; B. D. Strahl for the H3 unmodified and H3K14ac histone peptides; P. A. Cole for critical reading of the manuscript; and the PRIDE team for assistance in dissemination of the mass spectrometry data.

*This work was supported by National Institutes of Health grants R01DA025755 (S.D.T. and A.J.T.), R01GM106024 (S.D.T. and A.J.T.), R01GM095822 (C.W.), U01GM094588, U54 RR020839, P30GM103450, P20GM103429, and U11TR000039 and National Science Foundation grant DGE-1232825 (A.M.C.). 
[S] This article contains supplemental material.

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