CHARACTERIZATION OF GCN5 HISTONE ACETYLTRANSFERASE ACTIVITY AND BROMODOMAIN ACETYL-LYSINE BINDING FUNCTION

By

Anne M. Cieniewicz

A dissertation submitted to Johns Hopkins University in conformity with the requirements for the degree of Doctor of Philosophy

Baltimore, MD

February 2015

Abstract

In eukaryotes, DNA is packaged into chromatin, which consists of the DNA wrapped around an octameric core of histone proteins (H2A, H2B, H3, and H4) which collectively comprise the nucleosome. Gene activation and silencing determined by the access of transcriptional machinery to DNA can be dictated in part by dynamic post-translational modifications (PTMs) on histone proteins. These PTMs can be added, removed, and interpreted by chromatin effector complexes that interact with chromatin. In yeast, the highly 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. Consistent with our *in vitro* results, when the Gcn5 bromodomain was impaired *in vivo* we observed a global decrease in H3K18 acetylation.

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. Through our development of *in vitro* assays that contain selectively modified histones and intact epigenetic complexes and modules, we can gain biologically relevant insight into the activity and chromatin interactions of effector protein complexes. 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."

Dissertation Referees

Graduate Advisor: **Sean D. Taverna, Ph.D.**,
Assistant Professor, Department of
Pharmacology and Molecular Sciences

Dissertation Reader: **Heng Zhu, Ph.D.,**
Professor, Department of Pharmacology and
Molecular Sciences

Acknowledgements

I would like to thank all of those teachers and individuals who have influenced my decision to pursue an education and career in science. Dr. Zimniski, Dr. Casotti, Dr. Pagan, and Dr. Knabb from West Chester University have facilitated my undergraduate education and prepared me for a Ph.D. in the biomedical sciences. I have to express my utmost gratitude to my undergraduate mentor, Dr. Richard Woodruff, who cultivated my passion, appreciation and expertise for scientific research. I am extremely fortunate to have had the opportunity to learn from someone as gifted, supportive, and kind as Dr. Woodruff.

I am very grateful to have been accepted into the Department of Pharmacology and Molecular Sciences and to have had the opportunity to continue my education at an institution as reputable as The Johns Hopkins University School of Medicine. I would like to express my gratitude to my graduate mentor, Dr. Sean Taverna, for granting me the opportunity to perform my thesis research in his lab. I thank him for his training, mentorship and enthusiasm that have allowed me to grow as a scientist during my Ph.D. The entire Taverna lab, both past and present members, has been an absolute pleasure to work with and I am very thankful for their support and guidance. I would like to thank my thesis committee of Dr. Philip Cole, Dr. Heng Zhu, and Dr. Greg Bowman for their valuable scientific insight and professional guidance. I am

very grateful to Dr. Namandje Bumpus for her generous support and advice during my time in graduate school.

I would like to acknowledge our scientific collaborators who contributed to our scientific and experimental progression of this work. I am extremely grateful to the Lab of Dr. Alan Tackett and his students, Linley Moreland and Samuel Mackintosh, for performing the mass spectrometry processing and analysis. I was fortunate to have worked with Alison Ringel and Dr. Cynthia Wolberger who have been insightful and generous collaborators in deciphering Gcn5 activity and localization on chromatin.

I would like to express my gratitude to my friends and family who have been an immense support and positive influence in my life. My peers in the Pharmacology and Molecular Sciences department have been such a joy to have as colleagues through graduate school. I especially would like to thank my fellow lab member, Dr. Tonya Gilbert, for her friendship and dedication. I am very grateful to my fiancé, Dr. Romeo Papazyan, for his continued love and support. Finally, I would like to thank my incredible siblings, grandparents, and my parents, Barbara and Paul Cieniewicz, for their unconditional love, support and sacrifices.

Table of Contents

List of Figures

Chapter 1: General Introduction

1.1 Epigenetics

Epigenetics is classically defined as heritable changes in gene expression and phenotype that are independent of DNA nucleotide sequence (1-3). There are approximately 250 different types of cells in the human body that can vary phenotypically in both appearance and function, yet each of these cell sub-types arises from the same DNA template (4). Alterations in phenotype from the same DNA sequence is established through elaborate control of gene expression, that is, whether a gene is actively expressed or whether it is transcriptionally silenced (1-3, 5, 6). Epigenetic mechanisms that determine gene expression without altering the DNA sequence of a gene include DNA methylation, histone modifications, and RNA interference (1-3, 5, 6). These mechanisms of epigenetic regulation are involved in fundamental biological processes of eukaryotes such as cell differentiation, stem cell renewal, cell aging, and when they are misregulated, disease states arise (7-9).

1.2 The Nucleosome and Chromatin

Gene expression is dictated in part by the accessibility of underlying DNA to transcriptional machinery (5). In eukaryotes, DNA is packaged into chromatin, which consists of 146 base pairs of DNA wrapped around an octameric core of histone

Figure 1

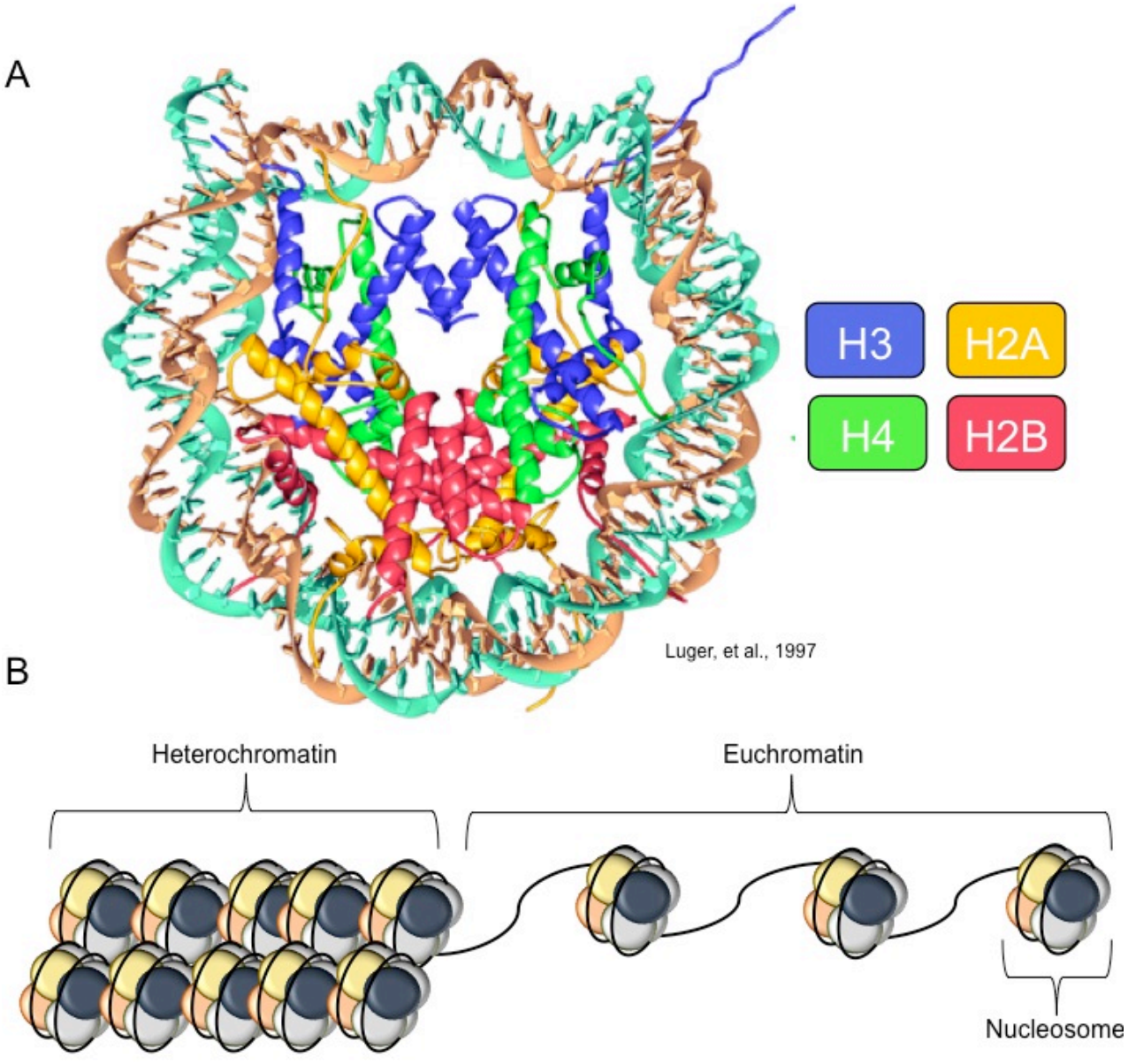


Figure 1. The nucleosome is the fundamental repeating unit of chromatin. (A) The nucleosome consists of a histone core octamer with two copies each of histones H2A, H2B, H3, and H4 wrapped with ~146 base pairs of DNA. (B) Chromatin can be arranged into higher ordered states such as the loosely associated euchromatin which is transcriptionally permissive, in part, because underlying genes can be accessed by transcriptional machinery. Additionally, heterochromatin is a more tightly packed chromatin state in which underlying genes are typically silenced due to the restriction of transcriptional machinery to DNA.

proteins (10-12). This octamer contains two copies each of histones H2A, H2B, H3, and H4 where histones H3 and H4 interact as a tetramer and histones H2A and H2B fold into the octamer as two dimers **(Figure 1A)** (10, 11). This arrangement of DNA and histone proteins collectively comprises the nucleosome, which is the fundamental repeating unit of chromatin **(Figure 1A)** (5, 10, 11). Nucleosome linkage is facilitated by histone H1 that is outside of the core octamer and acts to stabilize nucleosome arrangement into chromatin fibers (13). Histones themselves are relatively small proteins (~ 11 – 18 kDa) that contain a globular core region in addition to flexible N-terminal extension or "tail" that extends outward from the nucleosome, through the gyres of the nucleosomal DNA (10, 11). Histones contain many arginine and lysine residues, especially within the tail region, which bestows an overall basic charge which is thought to play a significant role in the regulation of nucleosomal and chromatin structure (10, 11).

Chromatin can exist in higher-ordered structures known as euchromatin and heterochromatin **(Figure 1B)** (12, 14, 15). Euchromatin is considered a transcriptionally active state where DNA is less densely packed with histone proteins, thereby allowing access of transcription factors to underlying genes (13, 14, 16-18). In contrast, heterochromatin is a tightly compact state to which transcriptional machinery is restricted access to DNA and thereby gene transcription is inaccessible (5, 16). The maintenance of chromatin organization can depend on the biological state of the cell, and the region and the function of the chromosomal locus, and is dictated by several factors such as histone chaperones, chromatin remodelers, and histone effector protein complexes (19-23).

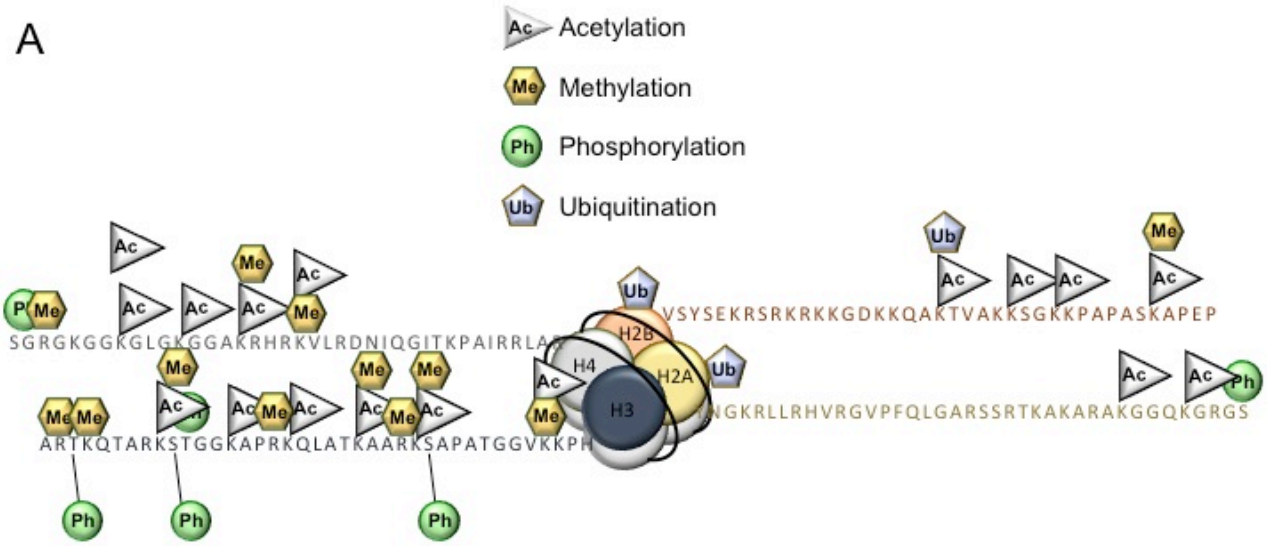
1.3 Histone Post-Translational Modifications

1.3A Histone modifying enzymes

Euchromatin and heterochromatin states can be distinguished in part by various post-translational modifications (PTMs) present on histone proteins (5, 23-25). These modifications include but are not limited to acetylation, methylation, phosphorylation, and ubiquitylation **(Figure 2A)** (17, 25-27). PTMs are found to a high degree on the flexible, N-histone tails, and are also found to a lesser extent within the globular region of histone proteins (5, 17, 23-28). These PTMs are extremely dynamic in nature, which is due to histone modifying enzymes that can add, or remove PTMs **(Figure 2B)** (17, 24, 25, 28). Histone modifying enzymes that add PTMs to histone proteins, such as histone acetyl-transferases (HATs) which enzymatically add acetyl moieties to lysine residues, and histone methyl-transferases (HMTs), which catalyze the addition of methyl groups to lysine or arginine residues, are termed “writers” (29-31). Those enzymes that remove histone PTMs, or “erasers”, include histone deacetylases (HDACs) that remove lysine acetylation and histone demethylases (DMTs), which remove methyl groups from lysines and arginines (29-31).

Additionally, there are non-enzymatic domains that can bind to or interact with modified residues known as “readers”. Readers can recognize the PTM and surrounding residues in the histone sequence, which allows these reader-containing proteins and complexes to distinguish between similar PTMs. These reader domains can vary in both their recognition motifs and structure. For example, the reader known as a bromodomain, is an acetyl-lysine binding motif that consist of a four alpha-helical

Figure 2



Euchromatic PTMs		Heterochromatic PTMs	
Lysine acetylation	Ac	Hypoacetylation	Ac
H3K4 trimethylation	Me	H3K9 trimethylation	Me
H3K36 trimethylation	Me	H3K27 trimethylation	Me

Figure 2. Dynamic histone post-translational modifications are regulated by histone modifying enzymes and binding domains. (A) Histone proteins are subject to a great degree of post-translational modifications (PTMs), particularly on the N-terminal tail portions. These modifications include acetylation, methylation, phosphorylation, and ubiquitination. (B) Histone PTMs are dynamically added, removed and interpreted by histone interacting proteins respectively termed "writers", "erasers", and "readers".

structure (32, 33). In contrast, the reader domain known as a PHD (plant homeodomain) finger is capable of binding to di- and tri-methylation states on both H3K4 and H3K9 and are structurally characterized by a two-stranded antiparallel β-sheet and three loops stabilized by Zn^{2+} molecules (34-39).

1.3B The histone code hypothesis

PTMs, either individually or in combination, can act to determine chromatin structure by serving as a docking site for chromatin interacting proteins that bind to modified histones through protein-protein interactions via reader domains **(Figure 2B)** (25, 27, 28, 33, 43). The existence of an epigenetic or histone "code" has been hypothesized and states that a distinct PTM or set of PTMs can specifically recruit or occlude effector protein complexes to chromatin, which can thereby elicit a downstream biological effect within the cell (25, 27, 28, 33, 43). This hypothesis is supported by the association of specific PTMs with cellular processes. For example, during gene transcription, histone H3 lysine 4 trimethylation (H3K4me3) is associated with transcription initiation as it is enriched at the gene's 5'-end at the transcription start site, while histone H3 lysine 36 trimethylation (H3K36me3) is associated with transcriptional elongation and enriched within the gene body (34, 44-46).

This hypothesis is further supported by the presence of distinct combinations of readers, writers, and erasers present within various effector complexes that could allow for interpretation of a histone code (16, 25, 28, 36, 43). For example, bromodomains are often found within the same protein or protein complex as HATs, but are also found in a diverse range of proteins including chromatin remodelers, helicases, and transcriptional coactivators (41, 42). Because chromatin effector complexes are often found with many

subunits that contain multiple effector and reader functions, such as the 19-subunit SAGA complex, combinatorial PTMs may act to specify localization and activity of these complexes (33, 36, 43, 47).

1.3C Lysine acetylation

PTMs can also determine chromatin structure by altering the affinity of DNA to histone proteins(18). For example, when a positively charged histone lysine residue is acetylated that charge is neutralized and in turn decreases the affinity of the histone protein to the negatively charged DNA backbone. Therefore, regions of chromatin that contain a high degree of lysine acetylation are typically transcriptionally active **(Figure 2A)** (16, 18, 48-51). Genes found within areas of the genome that contain a low degree or hypoacetylation are usually silenced **(Figure 2A)** (16, 26, 51). The discovery of histone lysine acetylation dynamics and the writers, readers, and erasers responsible for this modification have led to groundbreaking studies that have shaped our understanding of epigenetic regulation and gene expression.

Lysine acetylation was hypothesized to play a role in gene expression in the 1960's when it was observed that acetate was incorporated and deposited on histone proteins from isolated nuclei (52-54). Furthermore, this same group suggested the reversibility of lysine acetylation and linked it to regulation of RNA synthesis (52, 53). Subsequent studies using acetyl-lysine specific antibodies on polytene chromosomes indicated that specific acetylation sites occurred at various regions within the chromosome suggesting histone acetylation may act to impact chromatin structure and function (53, 55, 56). This relationship was further emphasized by mutations to histone lysine residues in yeast

and their impact on transcription. However, it was not until the discovery of the first nuclear HAT, Gcn5, and its acetylation of histone H3 that a clear link between histone acetylation and its transcriptional activation of genes was established (53, 57). With advances in proteomics, lysine acetylation has been implicated on a global level within the cell, as there have been at least 1,750 proteins and 3,600 acetylation sites identified (53, 58). In regard to gene expression, lysine acetylation is carefully regulated through the interplay of HATs and HDACs and has been known to play a role in several biological processes in addition to transcriptional activation such as DNA repair, histone deposition, and chromosome condensation (18, 29, 48, 50, 51).

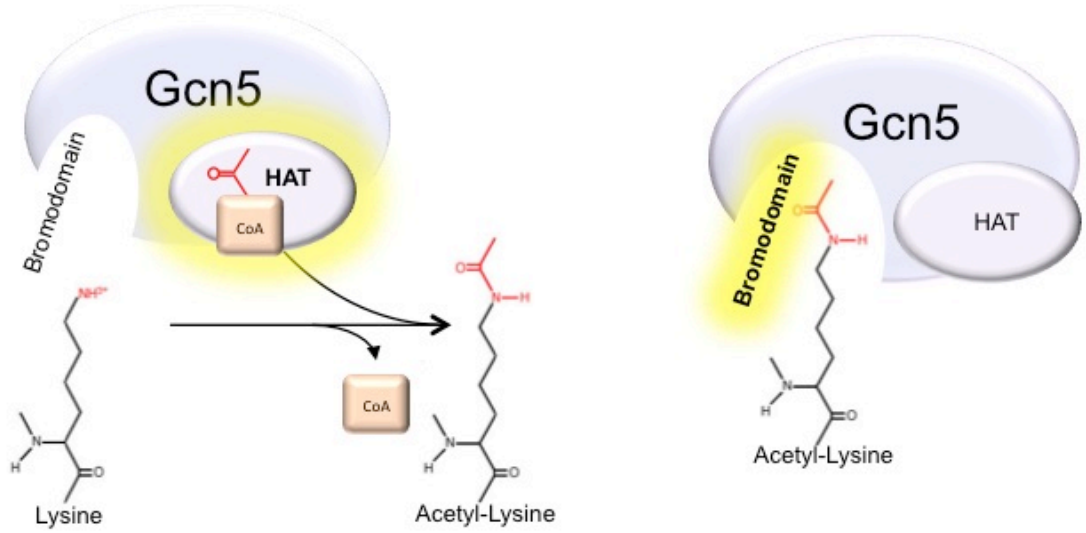
1.4 The Histone Acetyltransferase Gcn5

1.4A Gcn5 is a reader and writer of lysine acetylation

Gcn5 (general control non-derepressible) was the first nuclear HAT discovered, purified from nuclear extracts of the eukaryotic protozoan, *Tetrahymena thermophila,* and linked to histone acetylation and gene transcription in yeast (57). Gcn5 is highly conserved from yeast through humans and has the ability to write lysine acetylation via its catalytic HAT domain and also bind to acetyl-lysine residues through its adjacent bromodomain **(Figure 3A)** (32, 40, 73, 74). While Gcn5 has been primarily associated with transcriptional activation through its acetylation of histone proteins, it also acetylates non-histone substrates such as CDK5 and PGC-1α, which implicate Gcn5 in in cell cycle progression and metabolic pathways **(Figure 3B)** (75-77). Since the hallmark discovery of Gcn5 linking histone acetylation to gene transcription, many

Figure 3

A



B

Substrate	Function
Histone H3	Transcriptional activation
Histone H2B	Transcriptional activation
Histone H4	Transcriptional activation
CDK5	Alters kinase activity
PGC1-α	Inhibits activity and gluconeogenesis

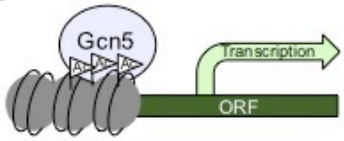
Figure 3. Gcn5 is both a writer and reader of lysine acetylation. (A) Gcn5 is a "writer" of lysines acetylation as it contains a catalytic histone acetyltransferase domain. Additionally, Gcn5 also contains an adjacent bromodomain which interacts with acetyl-lysines thereby making Gcn5 a "reader" of lysine acetylation. (B) Gcn5 contains several histone and non-histone protein targets where it contributes primarily to transcriptional activation, cell cycle progression, and gluconeogenesis.

elegant studies deciphering Gcn5 structure, enzymatic activity, inhibition, and transcriptional regulation have been published which are described in detail below **(Figure 4)** (57, 78-84).

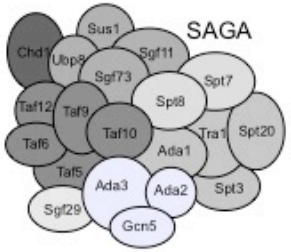
1.4B Gcn5 is found in several multi-subunit protein complexes

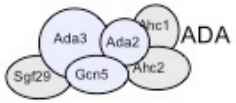
Gcn5 has been found to exist in several multisubunit protein complexes, such as the SAGA complex, that contribute to transcriptional activation **(Figures 5A, 5B)** (79). SAGA plays a pivotal role in transcriptional as it has been found to act on the entire transcribed genome with functions that include initiation, elongation, RNA pol II recruitment, TBP interaction, histone acetylation and ubiquitination (47, 85). Additionally, SAGA has been shown to act in maintaining DNA integrity and is involved in mRNA export (47). While the 1.8 MDa SAGA complex, which contains about 19 protein subunits, remains highly conserved from yeast through humans, Gcn5 is found in several other effector protein complexes that diverge throughout evolution **(Figure 5B)** (47). In addition to SAGA, several additional Gcn5-containg complexes exist **(Figures 5A, 5B)** (47). Yeast contain a related SAGA complex, known as SLIK (or SALSA) which contains a truncated version of the Spt7 protein and has the additional protein Rtg2 linking it to the mitochondrial retrograde response pathway (47, 86). The function of the yeast-specific ADA complex, which is a smaller Gcn5-containing complex (0.8 MDa), has yet to be determined and its Ahc1 and Ahc2 components do not appear to be conserved in higher eukaryotes (87, 88). The ~14 subunit Gcn5-containing ATAC complex is not found in yeast but is found exclusively in multicellular organisms (47, 64, 89). In mammals, the emergence of a related but distinct HAT, known as PCAF evolved and is highly homologous to Gcn5 with 75% identity to Gcn5 at the protein level

Figure 4

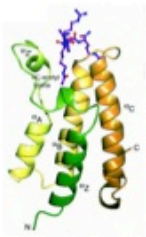


Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation.
Brownell JE, et al. Cell. 1996.

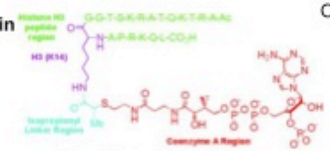




Yeast Gcn5 functions in two multisubunit protein complexes to acetylate nucleosomal histones: characterization of an Ada complex and the SAGA complex.
Grant PA, et al. Genes Dev. 1997.



The structural basis for the recognition of acetylated histone H4 by the bromodomain of histone acetyltransferase gcn5p.
Owen DJ, et al. EMBO J. 2000



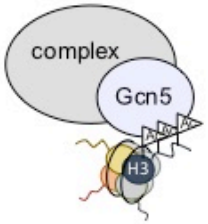
Structure of the Gcn5 histone acetyltransferase bound to a bisubstrate inhibitor
Poux AN, et al. Proc Natl Acad Sci USA. 2002.

K14 > K23 = K9 > K18 > K27 = K36

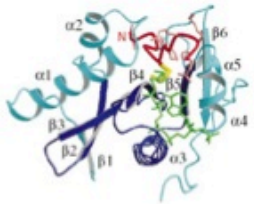
Quantitating the specificity and selectivity of Gcn5-mediated acetylation of histone H3.
Kuo YM and Andrews AJ. PLoS One 2013.

1996 1997 1999 2000 2002 2013

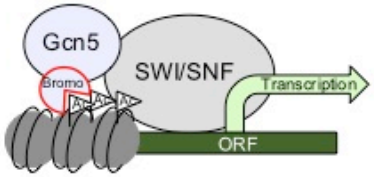
Gcn5 is involved in the acetylation of histone H3 in nucleosomes.
Ruiz-Garcia AB, et al. FEBS Lett. 1997.



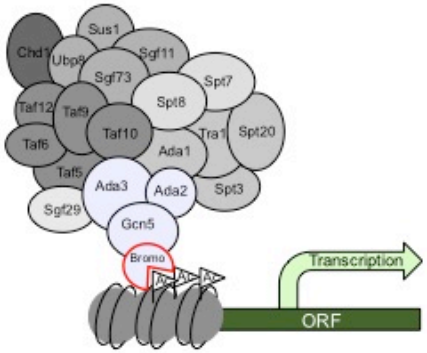
Structure of Tetrahymena GCN5 bound to coenzyme A and histone a H3 peptide.
Rojas, JR et al. Nature. 1999.



The Gcn5 bromdomain co-ordinates nucleosome remodeling.
Syntichaki P, et al. Nature. 2000



Function and selectivity of bromodomain in anchoring chromatin-modifying complexes to promoters nucleosomes.
Hassan AH, et al. Cell. 2002



(47, 90-92). Both Gcn5 and PCAF interact with the Ada2 and Ada3 proteins to form a trimeric subcomplex (47, 79, 93-97). Therefore, humans contain two versions of SAGA and two versions of ATAC with either Gcn5 or PCAF acting as the HAT (47, 89, 98). Interestingly, the *in vitro* HAT activities showed that SAGA primarily acetylates histone H3 while ATAC had specificity toward histone H4 in nucleosomes (47, 89, 98, 99).

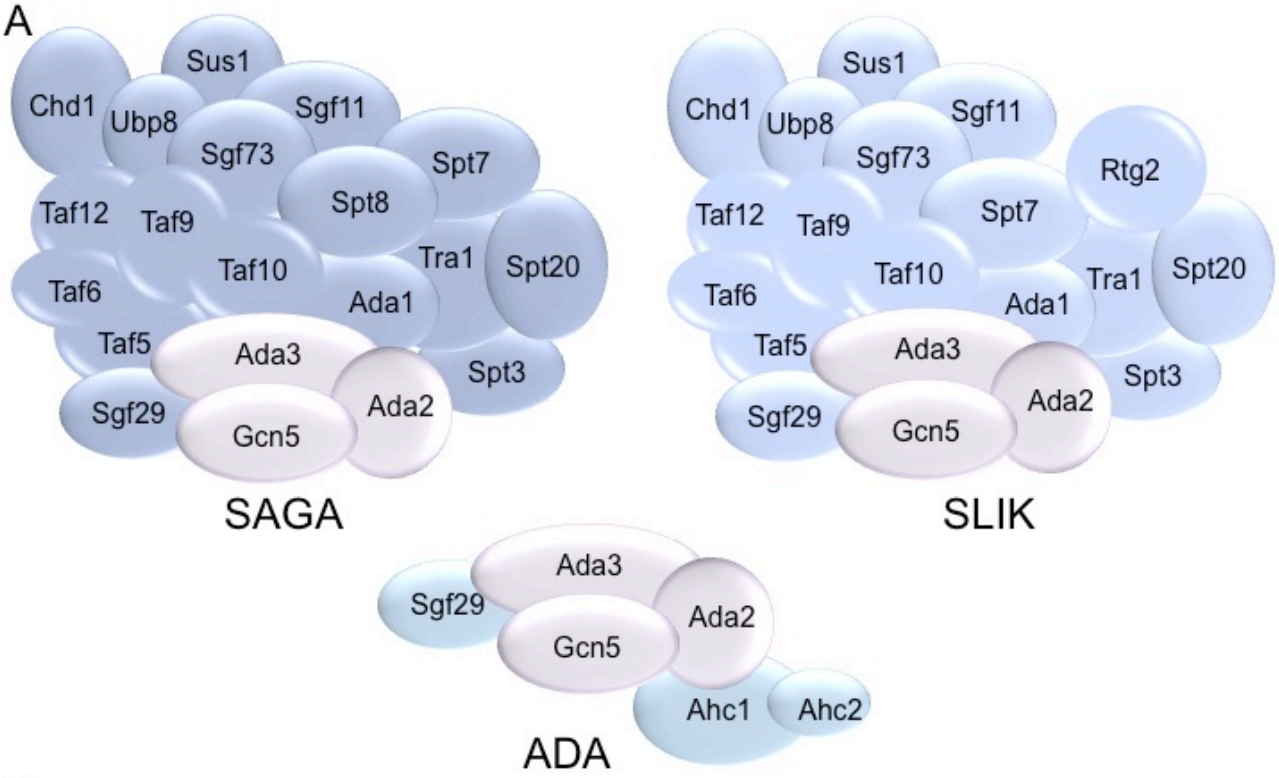
1.4C Functions of Gcn5 in development

Gcn5 plays a very crucial role in mammalian development requiring proper Gcn5 expression levels for normal development (8, 100-102). Deletion of Gcn5 is embryonic lethal in mice with extreme growth defects and cell death occurring at 8.5 days. Interestingly, mice with a catalytically inactive Gcn5 HAT domain survive longer than those with a null Gcn5 allele suggesting Gcn5 plays a role in early development that may be independent of its enzymatic histone acetyltransferase activity (64, 100). Furthermore, deletion of both Gcn5 and PCAF resulted in more severe defects, but deletion of PCAF alone results in no change in developmental phenotype (64, 100). This suggests that Gcn5 and PCAF have may have distinct but overlapping functions within the cell. Interestingly, PCAF has been reported to contain E3 ubiquitin ligase activity that affects downstream degradation of p53 levels (103, 104). It is currently unknown whether Gcn5 is also able to carry out E3 ubiquitin ligase activity.

When Gcn5 was deleted in embryonic stem cells, they were still able to differentiate undergo normal growth conditions (64, 102). However, as differentiation progressed in Gcn5 null cells it resulted in aberrant gene expression of pluripotent stem cell specific transcription factors such as Nodal and Oct4, which were found to be

Figure 5

A



B

Complex	Species	# of subunits
SAGA	Yeast through humans	19
ADA	Yeast	6
SLIK/SALSA	Yeast through humans	~19
ATAC	*Drosophila* through humans	9 - 10
HAT-A2	Yeast	3

Figure 5. Gcn5 is a component of several multi-subunit protein effector complexes. (A) Gcn5 is conserved from yeast through humans and comprises the catalytic HAT subunit of several protein complexes *in vivo*, such as the SAGA, ADA, and SLIK present in yeast. (B) Protein complexes, such as SAGA and SLIK, are evolutionarily conserved from single cell eukaryotes to mammals. Gcn5-containing complexes, such as ATAC, has emerged later in evolution possibly due to added complexity of multicellular organisms. The cellular function of the ADA complex, found exclusively in *S. cerevisiae* is currently unknown.

decreased upon deletion of Gcn5 (64, 102). Therefore the ability of stem cells to self-renew depends on the presence of Gcn5.

1.4D Implications of Gcn5 in disease

Gcn5 has been linked to several disease states including neurological disease, cancer, respiratory disease, and metabolic conditions (9, 60-62, 64, 105). The genetic and incurable disease known as spinocerebellar ataxia type 7 is caused by a polyQ expansion in the SAGA subunit, Atxn7 (60, 62, 106, 107). This disease was found to be accelerated upon Gcn5 deletion in mice and when the expanded polyQ-Atxn7 was incorporated in SAGA, nucleosome acetylation was shown to impaired implicating a role for Gcn5 in this disorder (60, 108). Additionally, because Gcn5 regulates cell cycle progression and growth it has been associated with cancer and oncogenic transformation. Gcn5 has been shown to interact with the oncoprotein, Myc, where it is not only recruited to acetylate Myc-targeted genes, but Myc itself is stabilized upon acetylation by Gcn5 (109, 110). It has recently been shown that Gcn5 is upregulated and overexpressed in non-small cell lung cancer tumors in which it contributes to cell growth by increasing the expression of cyclins D1 and E1 and the protein E2F1 (64, 75, 105). Additionally, Gcn5 has been shown to play a role in respiratory diseases such as asthma and COPD and could be a potential target in metabolism disorders as it is tightly tied to regulation of the transcription factor, PGC-1α, which regulates gluconeogenesis (65, 76, 77). The implication of Gcn5 in these disorders merits further understanding of how its HAT activity and substrate specificity might be impaired or altered within these disease states. In addition to identifying Gcn5 acetylation sites and substrate targets,

understanding how these substrates might be acetylated in a site specific or processive order could lend insight into targeting Gcn5 catalytic activity in a therapeutic manner.

1.5 Drugging the Epigenome

1.5A Therapeutic rationale

Epigenetics has emerged as an attractive field for therapeutic intervention. Because epigenetics plays such a fundamental role in many disease states such as cancer, metabolic disease, cardiovascular, and neurological diseases the impact of targeting proteins involved in epigenetic regulation holds great promise (7, 9, 39, 59-64). Moreover, it is the potentially reversible nature of epigenetic states that makes this field so promising to pharmacology (9). Small molecule inhibitors designed to block certain histone interacting proteins have already proved effective against certain cancers and neurological diseases (9, 65).

1.5B HDAC inhibitors

The HDAC family of epigenetic proteins is structurally diverse and they are divided into four classes, which begs the need for specific inhibitors when targeting these enzymes. HDAC inhibition has been successful in treating cancer and neurological disease. Vorinostat (also known as SAHA) targets Class I and II HDACs and has been approved in 2006 for the treatment of cutaneous T-cell lymphoma (65-67). Additionally, valproic acid, a general HDAC inhibitor, is an anticonvulsant that is also used to stabilize mood (65). HDAC inhibition is currently being studied for

diseases like Alzheimer's disease and holds promise for further disease intervention (65).

1.5C Bromodomains as drug targets

There are 61 known bromodomains in humans that are found in 46 bromodomain-containing proteins (65, 68, 69). These bromodomain-containing proteins are involved in oncogenic rearrangements, the transcriptional regulation of viral genomes, and mediation of the inflammatory transcription factor NF-kB (65, 68-70). The small molecule bromodomain inhibitors, JQ1 and I-BET, target the bromodomain of the BET protein, Brd4, show extreme promise for therapeutic potential (65, 70-72). JQ1 has been used to treat midline carcinoma while I-BET has been shown effective against inflammation associated with septic shock (65, 71). Both JQ1 and I-BET are currently under active investigation in areas such as cardiovascular disease, cancer, and male contraception (65, 71).

The success of BET bromodomain inhibition sets the stage for the development of small molecules against "reader" domains that act by disrupting protein-protein interactions instead of ablating enzymatic activity. Because bromodomains and bromodomain-containing proteins are often found adjacent to HATs within the same protein or within the same complex as HATs, determining if the bromodomain acts to affect catalytic activity could further establish this reader as a druggable target. Therefore, understanding if reader domains, such as the bromodomain, act to regulate catalytic activity of writers could provide unprecedented insight into pharmacological targeting of the epigenome.

Chapter 2: Characterization of the Gcn5 subcomplex HAT activity on histone H3

This research was originally published in Molecular and Cellular Proteomics. Anne M. Cieniewicz, Linley Moreland, Alison E. Ringel, Samuel G. Mackintosh, Ana Raman, Tonya M. Gilbert, Cynthia Wolberger, Alan J. Tackett, and Sean D. Taverna. The Bromodomain of Gcn5 Regulates Site Specificity of Lysine Acetylation on Histone H3. *Molecular and Cellular Proteomics*. 2014; 13: 2896-2910. © The American Society for Biochemistry and Molecular Biology. (111)

2.1 Introduction

Gcn5 has been shown to acetylate multiple histone lysine residues *in vitro*, with its primary target being lysine 14 of histone H3 (49, 57, 112-114). However, histone H3 lysines K9, K18, K23, K27, and K36 have also been reported to be acetylated by Gcn5 in addition to lysine residues found on histone H4 and H2B (39, 61, 101, 129, 130). When regarding substrate specificity of Gcn5, it must be taken into consideration that *in vivo* Gcn5 is most always found in a complex with other protein subunits and does not exist as a monomer in the cell (79, 86, 88, 93, 115). *In vitro*, monomeric Gcn5 is unable to acetylate nucleosomal substrates, but Gcn5 nucleosomal acetylation is achieved when Gcn5 is in the presence of its binding partners Ada2 and Ada3 (95, 96, 116-118). The minimal form of Gcn5 reported *in vivo* is the trimeric Gcn5-Ada2-Ada3 subcomplex also referred to as the HAT A2 complex, in which the Gcn5 trimeric subcomplex is incorporated into even larger complexes such as SAGA and ADA (68,

76, 78, 83, 131). Gcn5 interacts with Ada2 through an Ada2-binding domain, and Ada2 in turn binds to Ada3 (95, 96, 116-118). While Ada2 and Ada3 are nonenzymatic, they have been shown to affect Gcn5 substrate specificity and also its ability to engage higher-ordered substrates, such as the nucleosome, through DNA and histone binding domains **(Figure 6A)** (95, 96, 116-118). Ada2 contains SANT, SWIRM, and Zinc-finger domains and it has been shown to potentiate Gcn5 catalytic activity on histone H3 (116, 118). Ada3 was found to expand Gcn5 lysine specificity and is necessary for Gcn5 nucleosome acetylation (96, 118). Therefore, when studying Gcn5 *in vitro* HAT activity, working with Gcn5 in the context of its binding partners, Ada2 and Ada3, is critical to determine biologically accurate substrate specificity.

Utilizing quantitative mass spectrometry it has been reported that Gcn5 acetylates histone H3 lysines in the order of K14 > K23 > K9 = K18 > K27 > K36 (113). However, this study was performed using monomeric Gcn5. Testing how Ada2 and Ada3 might affect enzymatic Gcn5 HAT activity would provide a more biologically relevant interpretation of histone acetylation. In addition to histone H3, Gcn5 has been reported to target lysines on histone H4 and H2B, but how this acetylation occurs in the context of its binding partners as opposed to monomeric Gcn5 is currently unknown (49, 114). Therefore, analyzing the Gcn5 catalytic site specific acetylation on histone substrates will provide valuable insight as to how Gcn5 functions in the context of biologically relevant complexes.

Here, we aim to better understand Gcn5 HAT activity on histone proteins in the context of its binding partners Ada2 and Ada3. An *in vitro* assay with recombinant Gcn5 subcomplex and recombinant histones was developed to assess enzymatic

Figure 6

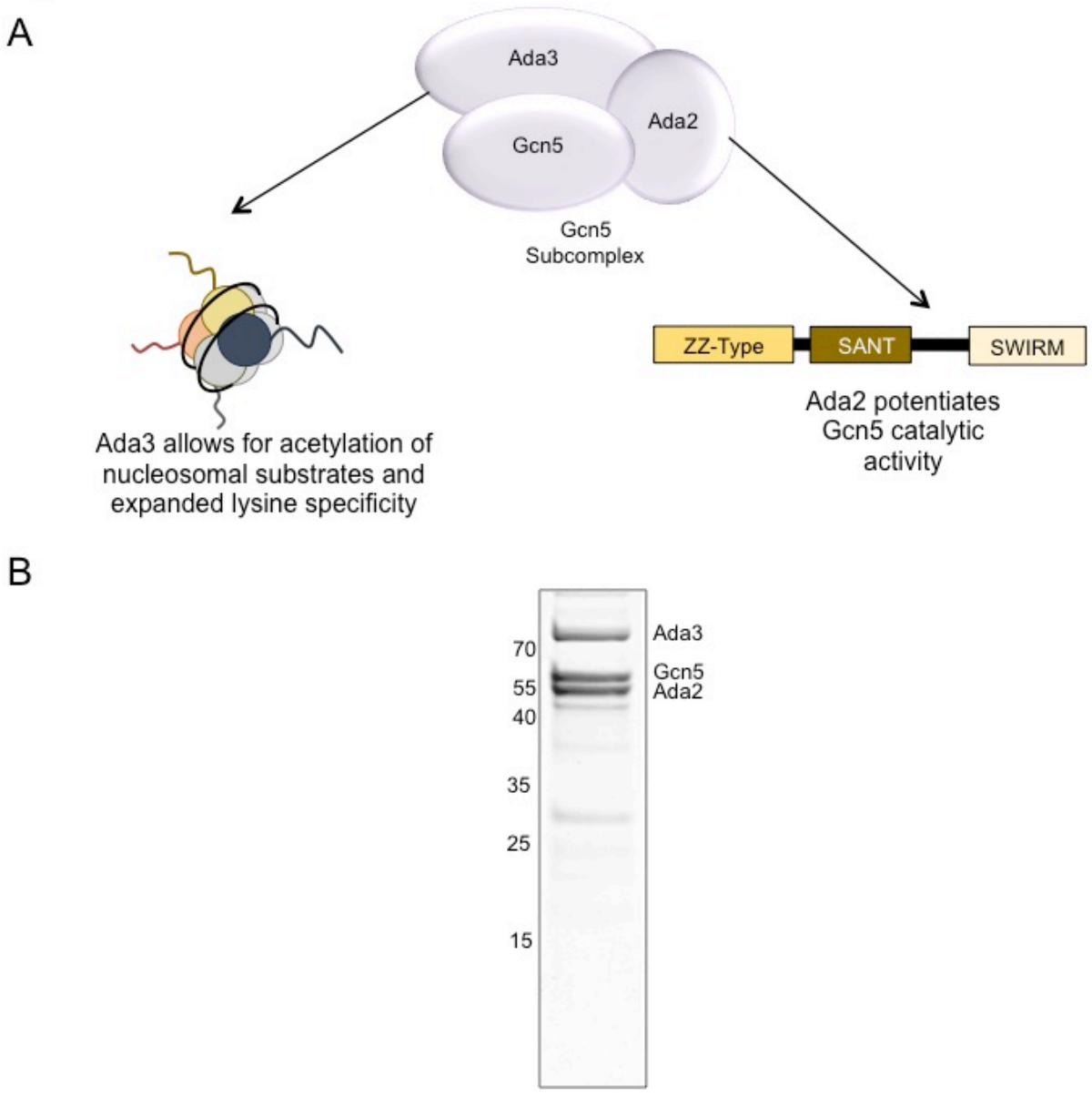


Figure 6. The Gcn5 subcomplex consists of Gcn5, Ada2, and Ada3. (A) The Gcn5 subcomplex consists of Gcn5, Ada2, and Ada3. Ada2 is known to potentiate Gcn5 catalytic activity through its Zinc-like domain, SANT, and SWIRM domains. Ada3 promotes Gcn5 acetylation of nucleosomal substrates and expands lysine specificity. (B) Recombinantly expressed Gcn5/Ada2/Ada3 subcomplex from pST44-yAda3t2HISx3-yAda2x3-yGcn5x5 polycistronic vector containing yeast GCN5, ADA2, and ADA3 obtained courtesy of Dr. Song Tan (Penn State, PA).

activity. Utilizing acid-urea gels, we were able to determine how many lysines were acetylated on each histone and allowed for the comparison of overall acetylation pattern on different substrates such as free histones vs. histone octamers. Finally, semi-quantitative mass spectrometry identified which lysines were acetylated and when used in combination with acid-urea gels, which separated distinct acetylated isoforms, Gcn5 subcomplex site specificity on histone H3 was uncovered where we define site specificity as the acetylation of a specific lysine relative to that of a proximal lysine.

2.2 Methods

2.2A Cloning, recombinant expression, and purification of full-length Gcn5 (Gcn5/Ada2/Ada3) subcomplex

The pST44-yAda3t2HISx3-yAda2x3-yGcn5x5 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 the 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 (Figure S1). The expression of full-length Gcn5/Ada2/Ada3 subcomplex and subsequent purification with Talon metal affinity resin (Clontech) and a FPLC SourceQ column (GE Healthcare) was performed as previously described (117).

2.2B *In vitro* histone acetyltransferase assays

HAT assays were performed with full-length recombinant Gcn5/Ada2/Ada3 subcomplex using 1 µg of recombinantly expressed *S. cerevisiae* free histone H3 or 1ug histone H3 in the context of the histone octamer, 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, reactions were incubated for 30 mins at 30˚C, followed by flash freezing in liquid nitrogen to stop the reaction. Samples were then lyophilized. Acetyl CoA was omitted from the control reactions. HAT reactions analyzed by quantitative mass spectrometry were performed in triplicate using 150 nM of WT or Y413A ADA subcomplex and carried out at 30˚C for 30 mins.

2.2C Acetic anhydride treatment of recombinant histone H3 for positive controls

To obtain maximally acetylated histone H3 control, 200 µg recombinant *S. cerevisiae* histone H3 was subjected to *in vitro* acetylation as previously outlined (119). Acetylated histone H3 was purified and desalted using HPLC purification, and fractions were combined, flash frozen, and lyophilized. Concentrations were normalized using a Ponceau stained dot blot.

2.2D Resolution of histone H3 with SDS PAGE and acid-urea gel electrophoresis

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 (120). Gels were washed with nano-pure

water and stained with SimplyBlue Safe Stain (Invitrogen). Bands were individually excised using a clean scalpel, and frozen for subsequent mass spectrometric analysis.

2.2E Western blot analysis of site-specific histone H3 lysine acetylation

Histone H3 samples were resolved by SDS PAGE and were transferred to a PVDF membrane using a semi dry transfer system. Samples resolved by acid-urea gels were transferred to a PVDF membrane as previously described (106). Membranes were blocked overnight in 5% milk at 4˚C and washed in Tris Buffer Saline (TBS). Primary antibodies were diluted in 1% milk in TBS and 0.1% Tween (TBST) as follows for nucleosomes (Figure 6) and Supplementary Figure 2: anti-H3 (Abcam ab1791, 1/50,000); anti-H4 (Abcam ab10158-25, 1/5,000); anti-H2B (Abcam ab1790-25, 1/20,000); anti-H3K9ac (Abcam, 1/5000); anti-H3ac (Millipore, 1/5,000); anti-H3K14ac (ab52946, Abcam, 1/5000); anti-H3K18ac (Active Motif 39130, 1/10,000); anti-H3K23ac (Active Motif 39132, 1/20,000); anti-H3K27ac (Active Motif 39134, 1/10,000); anti-H3K56ac (Upstate, 1/10,000). The following antibodies were used for histone K to R mutants: anti-H3K14ac (ab46984, Abcam, 1/500); anti-H3K18ac (Millipore 07-354, 1/7,500); anti-H3K23ac (Millipore 07-355, 1/5,000);. Each primary antibody was applied for 1 hour at room temperature followed by washing in TBST. Goat anti-rabbit IgG-horseradish peroxidase secondary antibody (Amersham Biosciences) was diluted to 1/4000 in 1% milk and TBST and applied for 1 hour at room temperature and washed in TBST. Blots were developed using Pierce ECL Western Blotting Substrate (ThermoScientific) and exposed using film.

2.2F 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*-acetic anhydride in 100 mM ammonium bicarbonate to chemically acetylate lysines (121), and subjected to in-gel trypsin digestion (100 ng trypsin, 37°C 15 hr). Treatment with *d6*-acetic anhydride adds isotopically heavy acetyl groups (+45Da) 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 (121, 122). Tryptic peptides were separated by reverse phase Jupiter Proteo resin (Phenomenex) on a 100 x 0.075 mm column using a nanoAcquity UPLC system (Waters). Peptides were eluted using a 45 min gradient from 98:2 to 40:60 buffer A:B ratio. [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) followed by MS/MS analysis using collisioninduced dissociation on an LTQ Orbitrap Velos mass spectrometer (Thermo) (123-125). 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 was 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) x 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 ExtractMSn version 1.0.0.8. Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4.1). 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 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (126). 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 (127) with the dataset identifier PXD001167 (128-130).

Figure 7

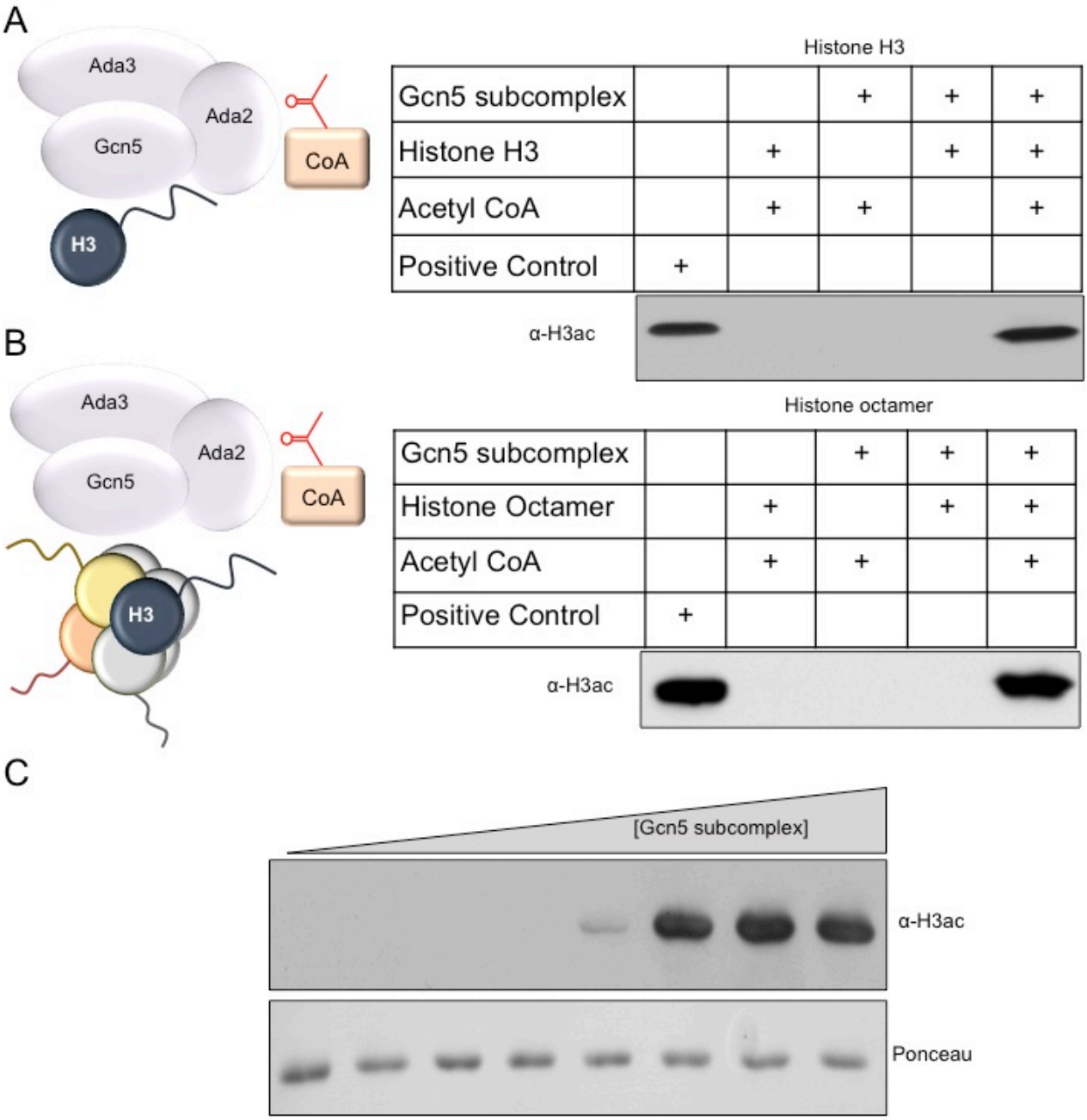


Figure 7. The Gcn5 subcomplex acetylates histone H3 *in vitro.* Western blots against anti-acetyl histone H3 were performed on Gcn5 subcomplex HAT assays containing either free histone H3 (A) or octameric histone H3 (B). A positive control (acetic anhydride treated histone H3) and several negative controls (omission of enzymes, substrate, or cofactor) were used for comparison. (C) Increasing concentrations of enzymatic Gcn5 subcomplex were used on free histone H3 and visualized using a western blot against anti-acetyl histone H3.

2.3 Results

2.3A Development of an *in vitro* histone acetyltransferase assay

The Gcn5 subcomplex was recombinantly expressed and purified using a polycistronic vector **(Figure 6B)**. To test the activity of the Gcn5 subcomplex, it was incubated with acetyl-CoA, the cofactor of Gcn5, and recombinant histone H3 in either free form or in an octamer. An anti-histone H3 antibody was used to detect Gcn5 subcomplex acetylation of histone H3, in which Gcn5 was found to acetylate both free and octameric histone H3 *in vitro* **(Figures 7A, 7B)**. To further test Gcn5 HAT activity on histone H3 we used increasing concentrations of the Gcn5 subcomplex to better understand optimal concentrations of enzyme and substrate for detection using a western blot with anti-acetyl histone H3 antibody **(Figure 7C)**.

2.3B Gcn5 subcomplex acetylation of histone substrates

Acid-urea gels are a valuable tool for studying protein acetylation as they separate proteins, including histones, by both size and charge **(Figure 8)** (120, 131). Because a positively charged lysine residue is neutralized upon the addition of an acetyl group, it is possible to resolve different histone acetylation states on acid-urea gels as the migration rate in the gel is decreased upon acetylation. When different charge states exist within a population of heterogeneously acetylated histones, resolution over an acid urea gel will result in the isoform mixture to separate into a “ladder” of distinct bands based on charge number. Each subsequent band present within the “ladder” represents the addition of an acetyl group. Therefore, the number of lysine residues acetylated or

Figure 8

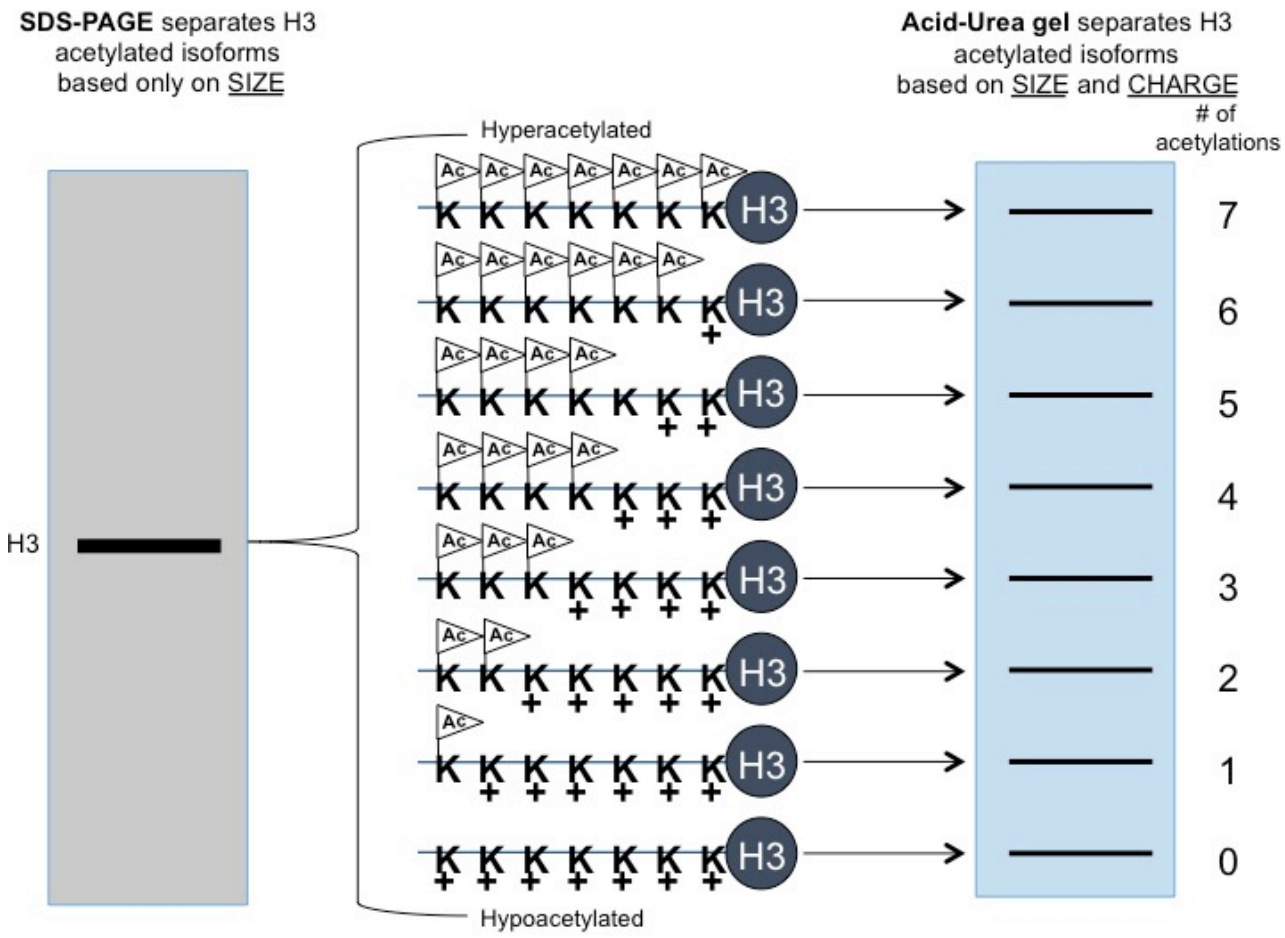


Figure 8. Acid urea gels for studying histone H3 acetylation. Acid urea gels resolve proteins based on both size and charge. When a positively charged lysine residue is acetylated the positive charge is neutralized. Different acetylated states of histone H3 can be resolved over an acid urea gel which results in a "ladder" of bands with each band representing a subsequent number of acetylations. This ladder ranges from a hypoacetylated state which is represented by the fastest migrating band and can range up to a slower migrating hyperacetylated state.

Figure 9

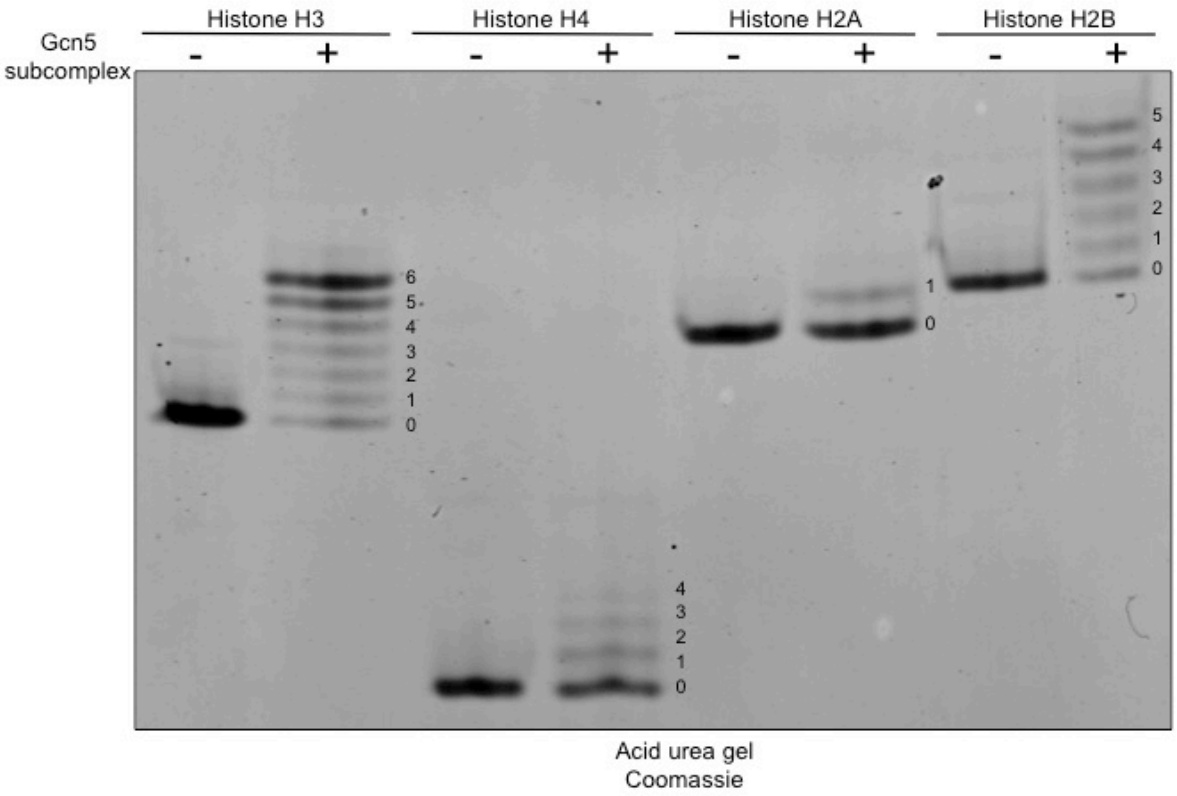


Figure 9. The Gcn5 subcomplex acetylates free histones *in vitro.* Gcn5 subcomplex HAT reactions were reacted individually with each free histone H3, H4, H2A, and H2B (+) or reactions omitting the Gcn5 subcomplex were used as a negative control (-). Reactions were resolved on an acid urea gel and stained with Coomassie to identify the number of acetylated lysines residues occurred on each histone protein.

deacetylated by a particular HAT or HDAC can potentially be determined using an acid-urea gel. Additionally, overall acetylation pattern can be visualized by the formation, loss, and individual band enrichment of resolved histone band laddering. Therefore, acid-urea gels can provide valuable insight into HAT and HDAC enzymes when testing enzyme concentration gradients, time courses, and comparing various conditions.

To determine how many lysine residues the Gcn5 subcomplex acetylates *in vitro* on each histone substrate, each free histone was independently reacted with the Gcn5 subcomplex under steady-state conditions for 30 mins and resolved on an acid-urea gel. This endpoint assay provided us with the number of lysines acetylated on each histone substrate and served to determine which histone substrates are preferred by the Gcn5 subcomplex. From the acid-urea gel we determined the number of lysines the Gcn5 subcomplex was acetylating on each histone. Histone H3 was acetylated on 6 lysine residues; histone H4 was tri-acetylated; histone H2A was mono-acetylated; histone H2B was penta-acetylated **(Figure 9)**. Histone H3 acetylation was further analyzed by resolving HAT assays on an acid-urea gel that contained increasing concentrations of Gcn5 subcomplex with either free histone H3 or octamers. An antibody against histone H3 acetylation was used on the resolved histone H3 isoforms to further analyze Gcn5 subcomplex overall acetylation pattern. These results confirmed that the Gcn5 subcomplex resulted in a total of 7 histone H3 bands when resolved on an acid-urea gel, representing a total of 6 acetylation sites and an unacetylated histone H3 isoform **(Figure 10A)**. Interestingly, the Gcn5 subcomplex histone H3 acetylation pattern differed between free histone H3 and octameric H3. The Gcn5 subcomplex appeared to

Figure 10

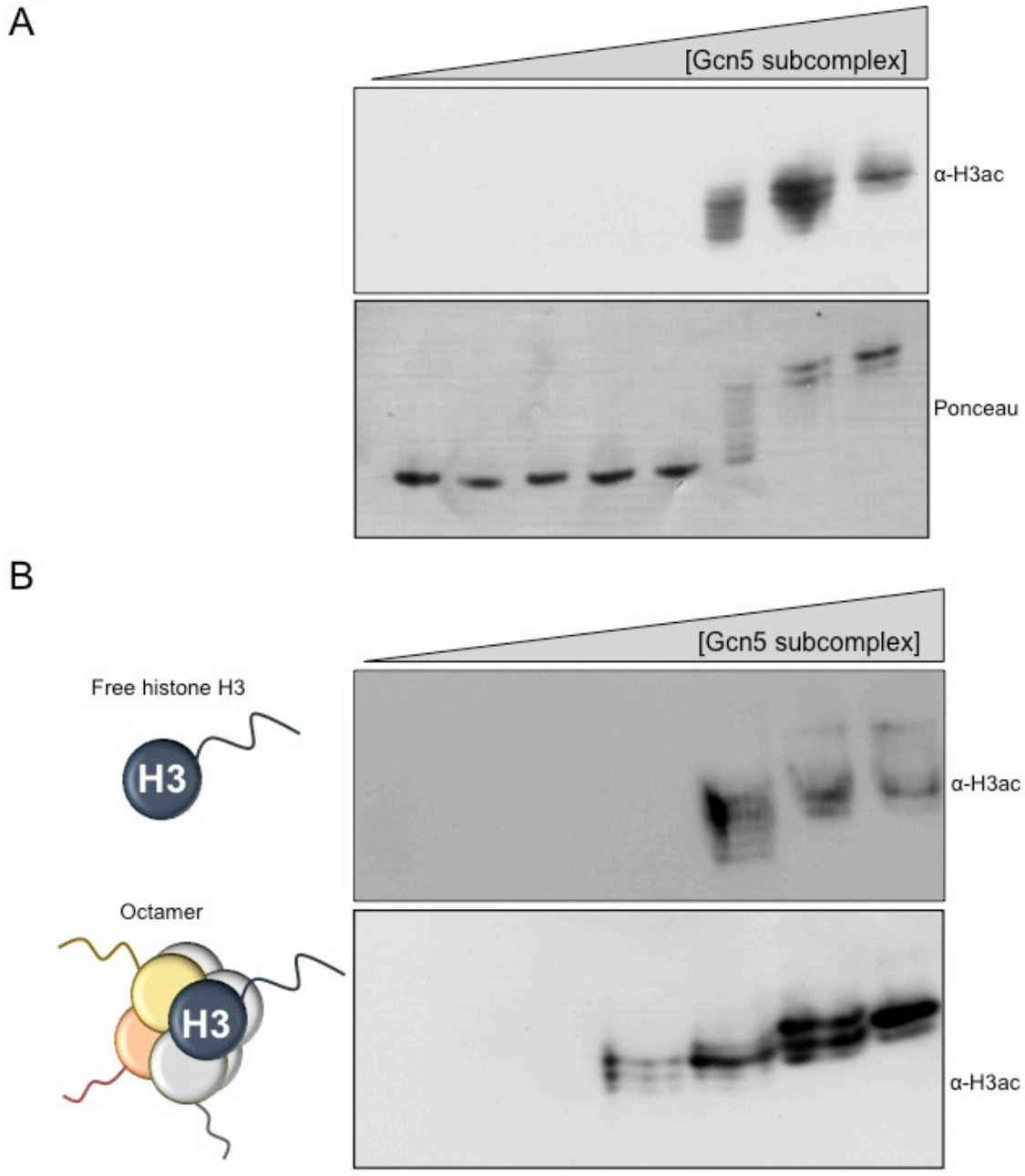


Figure 10. The Gcn5 subcomplex differentially acetylates free histone H3 and octameric histone H3. (A) Free histone H3 was resolved on an acid urea gel from HAT assays using increasing concentrations of the Gcn5 subcomplex. Acetylation was observed using both a Ponceau stain and western blot with an anti-H3 acetyl antibody. (B) Gcn5 subcomplex HAT activity was compared on free histone H3 and octameric histone H3 using increasing concentrations of subcomplex. HAT reactions were resolved on an acid urea gel and subjected to a western blot against anti-acetyl H3.

acetylate octameric histone H3 to a greater degree at lower concentrations suggesting the histone octamer could be the preferred substrate over free histone H3 **(Figure 10B)**.

2.3C Gcn5 subcomplex acetylates histone H3 tail lysine residues with a site specific order *in vitro*

When determining enzymatic activity of chromatin effector proteins, histone substrate specificity on multiple residues within the same protein, and elucidating the interplay between reader and writer functions, a sensitive and quantitative approach capable of detecting subtle changes in modifications on specific residues is required. Assays using radioisotopes of cofactors, such as acetyl CoA and SAM, have been used to evaluate bulk activity within a target polypeptide or mixture of proteins. However, radioisotope assays do not yield insight into site specificity (the modification of a specific residue relative to that of a proximal residue). In contrast, proteomic mass spectrometry approaches can yield detailed information about the specificity of a reaction regarding rates for specific residues (113, 132, 133). To accurately measure PTM abundance among different samples and conditions it is necessary to generate species that are chemically equivalent but isotopically distinct to ensure equivalent time of flight and yet exhibit a discernable mass difference. Utilizing isotopic chemical labeling we were able measure and compare acetylation and methylation on specific lysine residues within histone H3 via mass spectrometry.

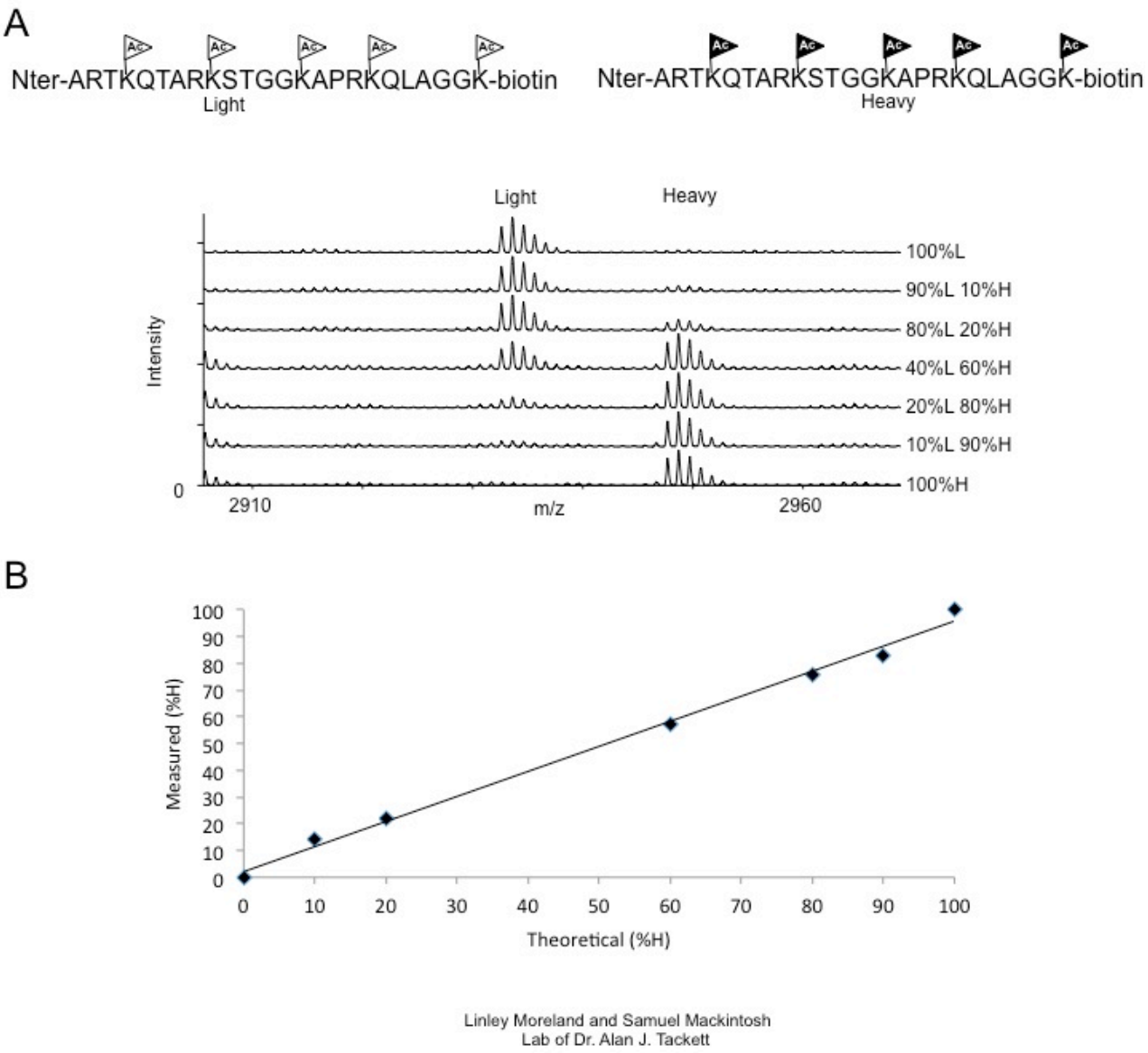
For measuring lysine acetylation we employed acetic anhydride treatment to chemically acetylate all unmodified lysine residues on a given protein. We further exploit this technique by using deutero-acetic anhydride to administer an isotopically heavier

label (121, 122, 134, 135). We tested this technique using histone H3 tail peptides in which we labeled all lysine residues with either acetic anhydride (light) or deutero-acetic anhydride (heavy) and mixing these peptides at different ratios, which were then measured using MALDI-TOF **(Figure 11A)** (121, 122, 134-138). We plotted our known (expected) ratio mixture of light:heavy against what we measured using MALDI-TOF and found our measured data points fit accurately with our expected values **(Figure 11B)**.

We combined this quantitative mass spectrometry approach with acid-urea gel resolution to optimize a technique for determining *in vitro* lysine specific acetylation on histone proteins from a HAT assay using acetyl CoA as a cofactor **(Figure 12 A – E)**. The HAT enzyme will target its given lysine residues depositing 'light" acetyl marks. Because HAT enzymes can often target multiple lysine residues on a single protein, we first resolve these different acetylation states on an acid-urea gel. These bands are excised and subjected to deutero-acetic anhydride treatment where all unmodified lysine residues are now chemically labeled with "heavy" acetyl marks. Histone proteins are broken down into peptides using a tryptic digest that cleaves after unmodified lysines and arginines. Because each lysine residue is acetylated trypsin will cleave only after arginine resulting in an identical tryptic peptide fingerprint for all conditions. LC-MS/MS analysis allows for the comparison of light:heavy signal on each individual lysine residue and we can thereby calculate the amount of acetylation catalyzed in a HAT reaction.

Having established that the Gcn5 subcomplex acetylates six histone H3 lysine residues, we next hoped to identify which lysine residues were acetylated and if the

Figure 11



Linley Moreland and Samuel Mackintosh
Lab of Dr. Alan J. Tackett

Figure 11. Validating quantitative mass spectrometry for histone H3 acetylation. (A) Histone H3 tail peptides were labeled with isotopically light or isotopically heavy acetylation at each lysine residue and mixed at different ratios. Mass spectra were taken and the intensity was measured for each light:heavy ratio. (B) Measured vs. theoretical mass spectra intensities were plotted as a function of percent heavy (%H) acetylation. These experiments were performed by Linley Moreland and Samuel Mackintosh in the laboratory of Alan J. Tackett (UAMS, Little Rock, AR).

Figure 12

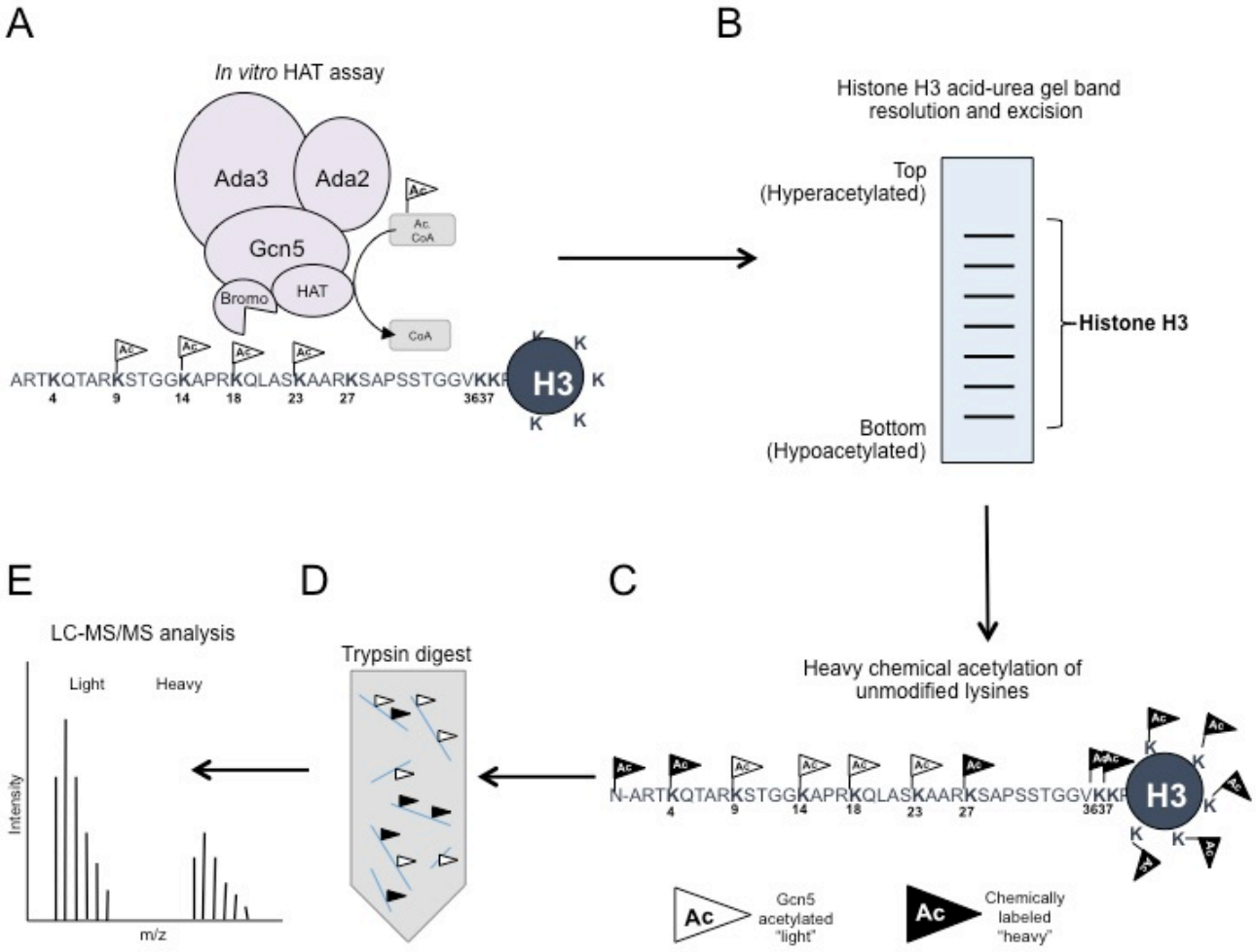


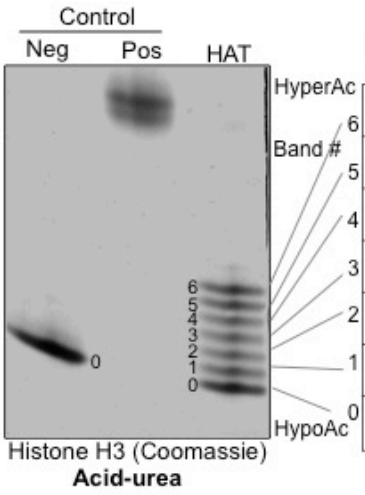
Figure 12. Outline of experimental design and validation of mass spectrometry approach. Schematic depicting the quantitative mass spectrometry approach to determine Gcn5/Ada2/Ada3 lysine-specific acetylation on histone H3 beginning with an (A) *in vitro* assay consisting of recombinantly expressed ADA 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 vs. heavy signal. Mass spectrometry processing and analysis was performed in collaboration with the lab of Dr. Alan Tackett (University of Arkansas, Little Rock, AR)

acetylation of these lysine residues occurred in a site specific sequence or order. Utilizing our semi-quantitative mass spectrometry approach **(Figure 12 A-E)**, we excised histone H3 acetylated isoforms from individual acid-urea gel bands and determined the percent acetylation on each histone H3 polypeptide. In agreement with previous studies, we observed acetylation on residues H3K9, H3K14, H3K18, H3K23, H3K27, and H3K36 (113). Moreover, as expected, no acetylation was detected on band 0 (unacetylated) in the HAT reaction or the negative control reaction, further validating this approach as a way to cleanly separate and identify differentially acetylated histone species **(Figure 13A)**.

Because our dual acid-urea gel and mass spectrometry approach lends additional insight into site specificity of the Gcn5 subcomplex we posited that the 7 histone H3 acetylated isoforms represented distinct reaction intermediates as histone H3 proceeded from an unmodified isoform (band 0), to a monoacetylated isoform (band 1) and eventually to a hexa-acetylated isoform generated in our Gcn5 subcomplex HAT reaction. The observation that the acetylation pattern of Gcn5 subcomplex HAT activity occurred as an even distribution or “ladder” of acetylated H3 isoforms suggests progressive and unidirectional acetylation. Mass spectrometry analysis of acetylation abundance on individual lysine residues revealed K14 was acetylated at levels of 100% in band 1, the first reaction intermediate. K14 was acetylated to levels of 100% in the additional five acetylated histone H3 isoforms indicating K14 is the initial site of acetylation, which is in agreement with previous kinetic studies (113). In the di-acetylated reaction intermediate (band 2) we observed that K23 was acetylated with an abundance of 100% in addition to 100% K14 acetylation, indicating that K23 acetylation

Figure 13

A



Histone H3 lysine residues

Band #	4	9	14	18	23	27	36	37	42	56	64	79	121	122	125
6	-	100 (±0)	100 (±0)	100 (±0)	100 (±0)	100 (±0)	96 (±1)	14 (±6)	-	-	-	-	-	-	-
5	-	97 (±2)	100 (±0)	100 (±0)	93 (±7)	98 (±2)	7 (±4)	-	-	-	-	-	-	-	-
4	-	82 (±4)	100 (±0)	100 (±0)	100 (±0)	35 (±4)	-	-	-	-	-	-	-	-	-
3	-	13 (±5)	100 (±0)	33 (±17)	83 (±8)	-	-	-	-	-	-	-	-	-	-
2	-	-	100 (±0)	-	95 (±5)	-	-	-	-	-	-	-	-	-	-
1	-	-	98 (±2)	-	-	-	-	-	-	-	-	-	-	-	-
0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

B

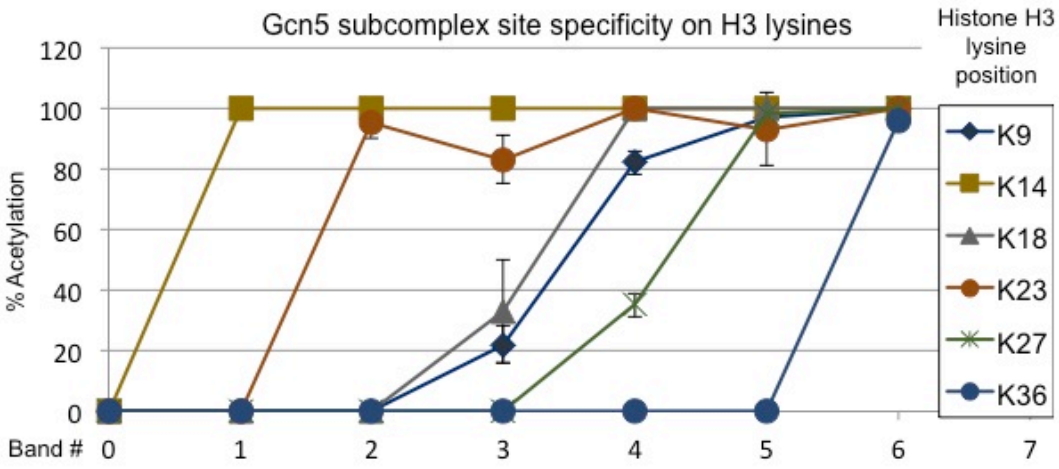


Figure 13. Resolution of acetylated H3 isoforms and quantitation of Gcn5 subcomplex site specificity. (A) Mass spectrometry quantitation of each histone H3 band excised from an acid-urea gel indicating the percent acetylation at each lysine residue. Acetylations on histone H3 catalyzed by the Gcn5 subcomplex *in vitro* (HAT); negative control (Neg) = hypoacetylated histone H3 in the absence of acetyl CoA; positive control (Pos) = acetic anhydride treated hyperacetylated histone H3. Each reaction was run in triplicate. (B) Graphical representation showing lysine site specificity of Gcn5 subcomplex on histone H3 depicting percent acetylation occurring at individual lysine residues within histone H3. Mass spectrometry processing and analysis was performed in collaboration with the lab of Dr. Alan Tackett (University of Arkansas, Little Rock, AR)

occurs directly following K14 acetylation. In band 3, a mixture of H3 acetylated isoforms were present with a tri-acetylated intermediate that contained acetylation on K14, K23, and to a lesser degree K18 in addition to a second intermediate that contained acetylation on K14, K23, and to a lesser degree K9. In band 4, the tetra-acetylated state, K14, K23, K18, and K9 were all acetylated to levels close to 100% indicating that lysines K18 and K9 occur with similar site specificity, but are acetylated after K14 and K23 acetylation. Histone H3 was 100% acetylated on lysines K14, K23, K18, K9, and K27 while the final hexa-acetylated intermediate was entirely acetylated on residues K14, K23, K18, K9, K27, and K36 suggesting K27 and K36 are the fifth and sixth sites acetylated by the Gcn5 subcomplex **(Figures 13A, 13B)**.

To further validate and complement our mass spectrometry data, we used site-specific antibodies against acetylated histone H3 lysine residues resolved on acid-urea gels **(Figures 14A, 14B)**. These western blots indicated that acetylation occurred on the same six histone H3 tail lysine residues detected with mass spectrometry. However, because antibodies may exhibit a degree of cross reactivity and are not quantitative we were unable to conclude an order from western blot analysis alone, which further strengthened the utility of our dual acid-urea gel and mass spectrometry approach.

2.4 Discussion

We developed an in vitro HAT assay utilizing the biologically relevant Gcn5 subcomplex to better understand the activity of the Gcn5 subcomplex on recombinant

Figure 14

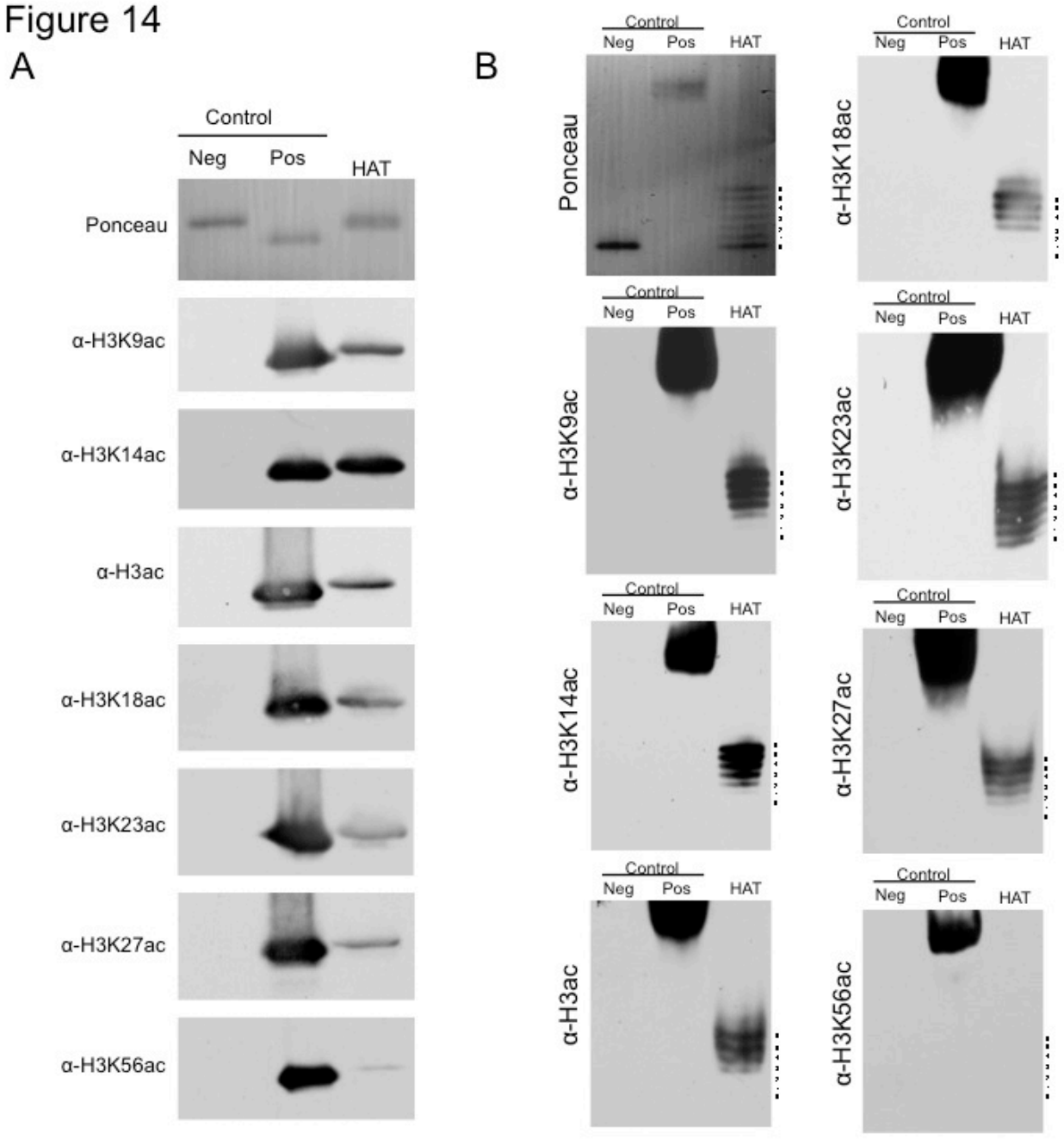


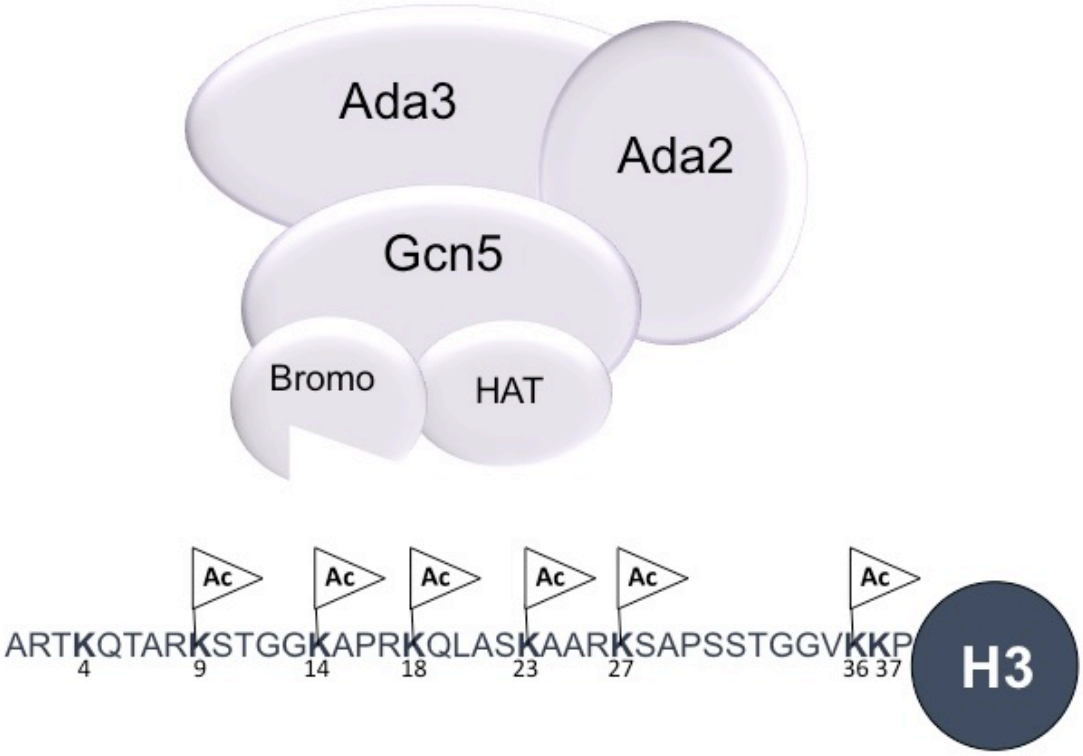
Figure 14. Analysis of ADA subcomplex site-specificity using acetyl-lysine antibodies. Antibodies against specific histone H3 acetyl-lysine residues were used to determine HAT activity of the trimeric Gcn5/Ada2/Ada3 subcomplex (HAT) on histone H3. Western blots against acetylated lysine residues on histone H3 were resolved using both (A) SDS-PAGE and (B) acid-urea gels. Unacetylated histone H3 (Neg) and a chemically treated hyper-acetylated histone H3 (Pos) were used as negative and positive controls.

histone H3. HAT reactions were subjected to in-depth analysis acid-urea gel resolution, western blots using acetyl-lysine specific antibodies, and semi-quantitative mass spectrometry which allowed us to determine the number of lysine residues acetylated, the identity of these residues, and the site-specific acetylation of the Gcn5 subcomplex on histone H3. We observed that Gcn5 in the context of its binding partners, Ada2 and Ada3, acetylates histone H3 on six lysine residues in vitro with the following site specificity: K14 > K23 > K18 = K9 > K27 > K36 **(Figure 15)**.

Regarding the order of histone H3 lysine acetylation catalyzed by the Gcn5 subcomplex, our data suggest that K14 was the preferred lysine residue as it was found to be acetylated at levels of 100% in all reaction intermediates including the mono-acetylated isoform (band 1). Additionally, because subsequent downstream acetylation was found to occur sequentially in single increments it was reasoned that the Gcn5 subcomplex acetylated lysines in a progressive and unidirectional manner. Had Gcn5 acetylated its 6 lysine targets simultaneously, the evenly distributed “ladder” of seven bands would not be present and instead a single band occurring in the band 6 position would be observed. Therefore, a comparison of acetylation abundance on individual histone H3 lysines as histone H3 isoforms progress from a hypoacetylated (band 0) state to a hyperacetylated (band 6) state, reaction intermediates can be used to measure site specific acetylation on one lysine residue relative to another.

Another group recently found that following the acetylation of H3K14, Gcn5 acetylation lysines H3K9 and H3K23 occurred at similar rates, whereas our data clearly showed H3K23ac occurred before H3K9ac (113). In contrast, we observed that it was

Figure 15



Gcn5 subcomplex acetylates histone H3 in the order
K14 > K23 > K9 = K18 > K27 > K36

Figure 15. Histone H3 site specificity of the Gcn5 subcomplex schematic. Model depicting Gcn5 subcomplex acetylation of histone H3. Acetylation occurs on six H3 tail lysine residues with the site specific order of K14 > K23 > K9 = K18 > K27 > K36 as determined in our study.

H3K9ac and H3K18ac that had similar site specificity when reacted with Gcn5. A possible reason for this difference may be due to the fact that our approach enabled greater resolution of histone H3 reaction intermediates due to the separation of histone H3 isoforms using an acid-urea gel prior to mass spectrometry analysis. While this previous study provided valuable kinetic information regarding Gcn5 site-specificity by determining the rates of acetylation on individual lysine residues, it may have been difficult to accurately distinguish the order of H3K9 and H3K23 acetylation without initially purifying distinct acetylation states.

A second possibility that may account for the differences observed between our work and the previous Gcn5 site-specificity study may be due to the form of Gcn5 being utilized in the in vitro reactions. The first study used monomeric Gcn5 alone while our study used the Gcn5-Ada2-Ada3 subcomplex, which could account for slight differences in Gcn5 HAT site specificity since Ada2 and Ada3 were previously shown to affect Gcn5 enzymatic activity (116, 118). For example, previous studies have compared enzymatic HAT activity of monomeric Gcn5, the Gcn5-Ada2-Ada3 subcomplex, the intact ADA complex (0.8 MDa), and the intact SAGA complex (1.8 MDa) which showed an increased repertoire of acetylated histone H3 lysine residues as Gcn5 was able to interact with its binding partners and complex subunits (112, 115, 116). Additionally distinct acetylation kinetics were observed between the ADA and SAGA complexes indicating that Gcn5 enzymatic activity is regulated in part by the protein factors with which it interacts.

It must also be considered, that in addition to biologically relevant enzymatic subcomplexes, using higher-ordered nucleosomal substrates is ideal for performing *in vitro* HAT reactions. Because Ada2 and Ada3 contain nucleosome and DNA interacting domains it is critical to examine HAT activity on histone H3 in the context of the nucleosomal substrate. We observed a variation in acetylation rate between free histone H3 and histone H3 in the context of the histone octamer. While our Gcn5 subcomplex site specific analysis was performed on free histone H3, it is crucial to test that this acetylation pattern can replicated on nucleosomal subunits.

Chapter 3: The Gcn5 bromodomain impacts catalytic acetyltransferase activity

The majority of this research was originally published in Molecular and Cellular Proteomics. Anne M. Cieniewicz, Linley Moreland, Alison E. Ringel, Samuel G. Mackintosh, Ana Raman, Tonya M. Gilbert, Cynthia Wolberger, Alan J. Tackett, and Sean D. Taverna. The Bromodomain of Gcn5 Regulates Site Specificity of Lysine Acetylation on Histone H3. *Molecular and Cellular Proteomics*. 2014; 13: 2896-2910. © The American Society for Biochemistry and Molecular Biology. (111)

3.1 Introduction

The acetyl-lysine binding bromodomain motif is evolutionarily conserved and often found in HATs and HAT associated complexes (32, 33, 41, 139, 140). The bromodomain of Gcn5 and the highly homologous PCAF bromodomain **(Figure 16)** have been shown to interact with acetylated histone lysine residues H3K9ac, H3K14ac, H3K36ac, H4K16ac, and H4K8ac (40-42, 92, 139, 141, 142). Additionally, the Gcn5/PCAF bromodomain has been shown to interact with acetylated lysines on non-histone proteins such as K50ac on HIV-1 Tat (143, 144). The Gcn5 bromodomain has been found to contribute to chromatin remodeling, cooperative nucleosome acetylation, and anchoring complexes to chromatin (141, 145).

Studies that introduced point mutations into the yeast Gcn5 bromodomain which disrupted binding to acetylated lysine residues led to a decrease in Gcn5 dependent

Figure 16

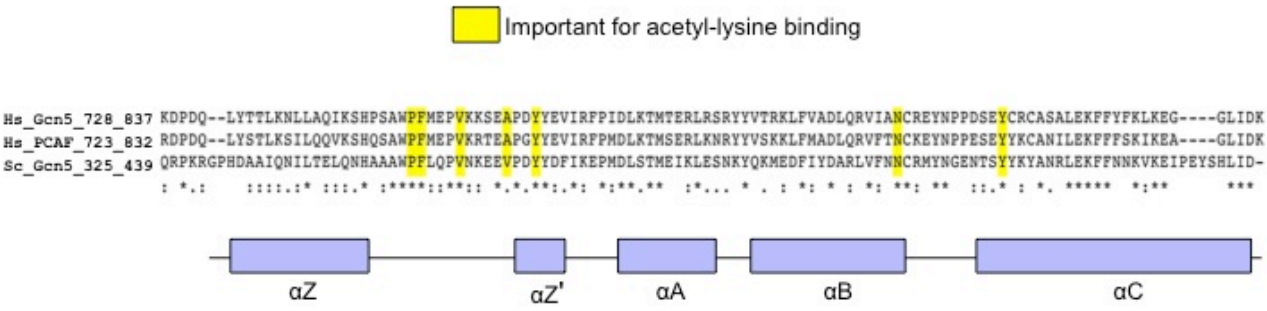


Figure 16. The Gcn5 bromodomain is highly conserved from yeast through humans. Sequence alignment of the *S. cer* Gcn5 bromodomain with the human Gcn5 bromodomain and the bromodomain of human PCAF. Residues important for bromodomain interaction with acetyl-lysine binding are highlighted in yellow. Alignment was made in NCBI protein BLAST.

transcription and histone acetylation at *in vivo* promoters (13, 73, 145-148). Additionally, it was observed that bromodomain mutations that disrupted acetyl-lysine binding led to an overall decrease of nucleosome acetylation *in vitro.* Specifically, the bromodomain mutation of Y413 in yeast Gcn5 (or homologous Y809 in PCAF) disrupts acetyl-lysine binding and was previously introduced into the Gcn5 subcomplex identified bromodomain-dependent cooperativity of acetylation on nucleosome substrates, although alterations in site specificity were not identified (139, 141, 145). Importantly, this mutation maintains the structural integrity of the bromodomain, does not alter the stoichiometry of the Gcn5 subcomplex, and has been shown alter acetylation at Gcn5-targeted promoters *in vivo* (139, 141, 145) **(Figure 17A)**. A double bromodomain mutation within the ZA loop has also been studied in Gcn5. Mutation of P371 and M372 has been shown to completely destabilize the structural integrity of the bromodomain (149) (**Figure 17B)**.

Although these previous studies suggest that the Gcn5 bromodomain may contribute to proper acetylation of Gcn5 HAT activity, the impact of the bromodomain acetyl-lysine binding function on enzymatic HAT activity remains poorly characterized. Specifically, we aim to understand how overall HAT activity and site specificity of Gcn5 are altered upon disruption of the bromodomain acetyl-lysine binding function. We hypothesized that the bromodomain plays a role in directing the site specificity of Gcn5. To test this, we generated bromodomain point mutants in the Gcn5 subcomplex. We generated a Gcn5 complex that contained the Y413A mutation, which disrupts acetyl-lysine binding functions while maintaining bromodomain structural integrity and showed that this mutation disrupts the ability of the Gcn5 bromodomain to bind to acetylated

Figure 17

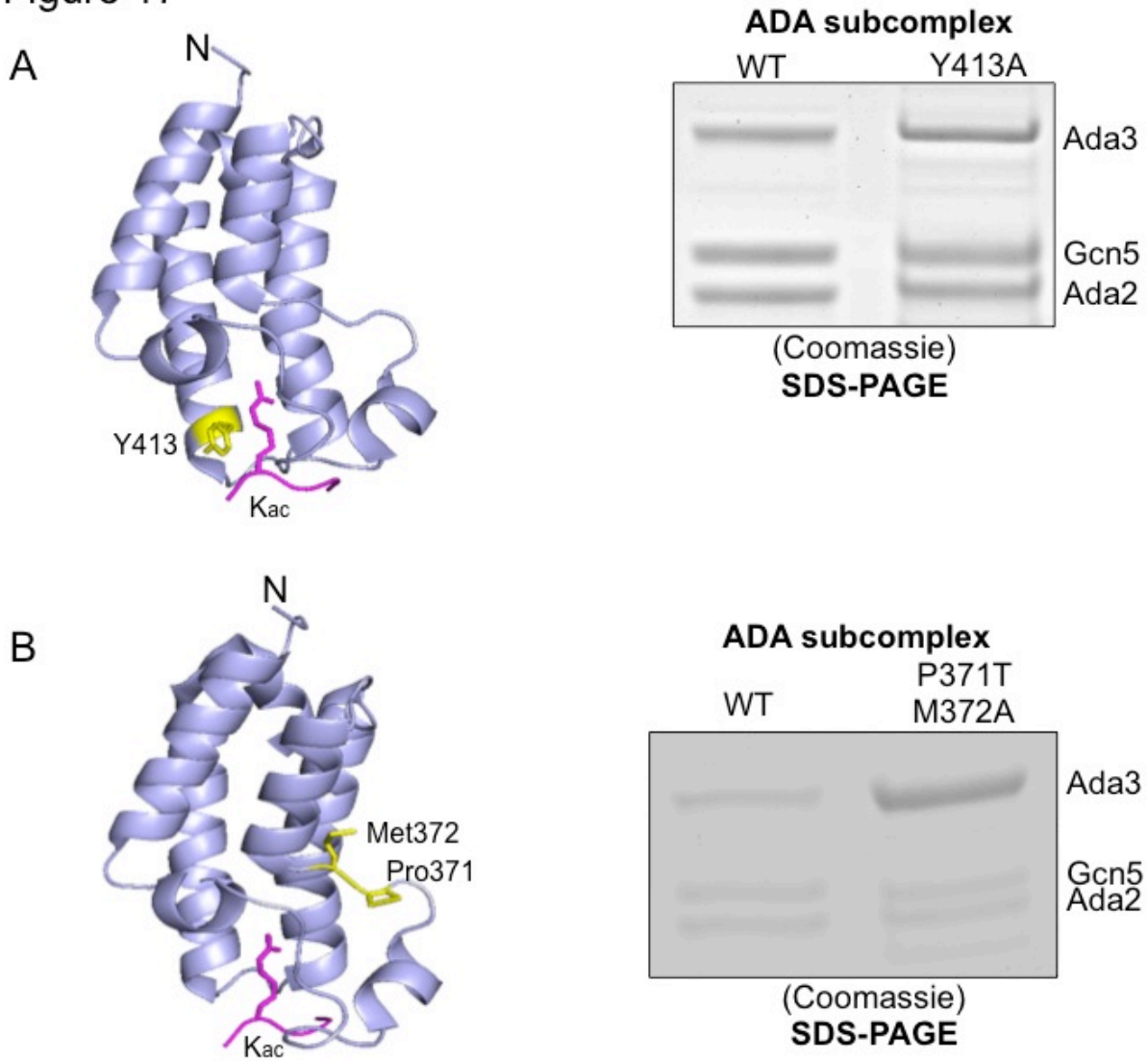


Figure 17. Generation of nondisruptive and disruptive Gcn5 bromodomain point mutations within the Gcn5 subcomplex. (A) The location of Y413 (yellow) within the yeast Gcn5 bromodomain (blue), a residue essential for binding acetyl-lysine (pink) (adapted from PDB structure 1E6I, and rendered in PyMOL). Both the WT and Y413A bromodomain mutant Gcn5/Ada2/Ada3 ADA subcomplex were recombinantly expressed, purified, and normalized for protein amount. (B) The location of P371 and M372 (yellow) within the yeast Gcn5 bromodomain (blue), known to disrupt bromodomain structural integrity upon mutation (adapted from PDB structure 1E6I, and rendered in PyMOL). Both the WT and P371T/M372A bromodomain mutant Gcn5/Ada2/Ada3 ADA subcomplex were recombinantly expressed, purified, and normalized for protein amount.

lysine residues **(Figures 17A, 18)**. Secondly, we introduced the double P371T/M372A mutation into the Gcn5 subcomplex to entirely destabilize the bromodomain **(Figure 17B)**. These bromodomain-mutated complexes were used to assess the impact of the Gcn5 bromodomain on overall HAT activity and site specific acetylation using our combined acid-urea gel and mass spectrometry approach.

Further insight of how bromodomains or other histone readers can affect chromatin-templated catalytic activities may yield greater understanding into mechanisms of histone PTM crosstalk. In-depth characterization of the biologic role of the Gcn5 bromodomain and how it may impact enzymatic activity could provide important parameters for epigenetic drug design. Because the human homolog of Gcn5 is found to be mutated in several diseases and with the success of BET bromodomain inhibitors, better understanding of the Gcn5 bromodomain could refine its use as a potential drug target.

3.2 Methods

3.2A Cloning, recombinant expression, and purification of Gcn5 subcomplex bromodomain mutants

The Gcn5 Y413A bromodomain mutant and the Gcn5 P371T/M372A double point mutant were generated using QuikChange site-directed mutagenesis (Stratagene) 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 a FPLC SourceQ column (GE Healthcare). Concentrations of the

Figure 18

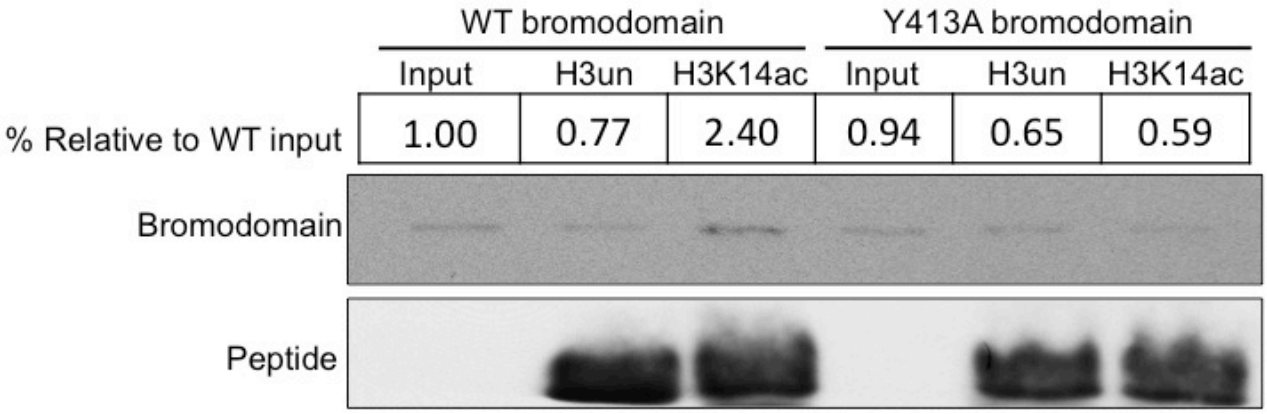


Figure 18. The Y413A mutation in the Gcn5 bromodomain disrupts binding to acetylated histone peptide. Western blot analysis and Image J quantitation of band intensities of histone pull-down assays comparing the binding of recombinant wild type and Y413A Gcn5 bromodomains to H3K14ac peptide.

recombinant wildtype and mutant Gcn5/Ada2/Ada3 subcomplexes were normalized using SDS-PAGE gels and Coomassie blue staining.

3.2B Cloning, recombinant expression, and purification of the Gcn5 bromodomain

Wild type, Y413A, and P371T/M372A yeast Gcn5 bromodomains (residues 329-438 of Gcn5p) were cloned from the respective pST44-yAda3t2HISx3-yAda2x3-yGcn5x5 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, Millipore). Proteins were recombinantly expressed in chemically competent BL21 *E. coli* (Invitrogen) after overnight induction with 1 mM IPTG 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, 40 mM imidazole, 10% glycerol, 2 mM B-ME, 1 mM PMSF, 2 mM benzamidine) and lysed by sonication (Branson). Lysate was incubated with Ni-NTA agarose resin (Invitrogen) for least 1 hour at 4 °C. Resin was washed with purification buffer and protein was eluted with purification buffer containing 150 mM imidazole. Protein was aliquotted and flash frozen in liquid nitrogen and stored at −80 °C.

3.2C Bromodomain peptide pull-down assays

Peptide pull-downs were essentially performed as in Taverna et al., 2007 (10), with the following modifications. Briefly, streptavidin-coupled dynabeads (20 ml per sample) (Invitrogen M-280) were incubated with biotinylated histone peptides (1 mg per sample) in PBS, and washed in PBS. Peptide coated beads were then incubated with purified bromodomain (1 mg) in the presence of BSA competitor (1 mg) 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 hrs at room temperature. Beads were washed 3 times for 5 min each 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 1 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, then 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 pull-down.

H3 peptides:

H3 1-20 unmod biotin: ARTKQTARKSTGGKAPRKQL-K(Biotin)-NH2

H3K14ac 1-20 biotin: ARTKQTARKSTGGK(ac)APRKQL-K(Biotin)-NH2

3.2D Nucleosome reconstitution

Recombinant histones from *Xenopus laevis* were expressed in *E. coli,* purified from inclusion bodies, and assembled into histone octamers as described previously (150). DNA for recombinant mononucleosomes was obtained by *EcoRV* digestion of pST55-16xNCP601, courtesy of Dr. Song Tan (Penn State, PA), which contained sixteen tandem copies of a 147 bp fragment with the 601 positioning sequence (151). Prior to reconstituting nucleosomes, the 147 bp DNA fragments were purified as described previously (152). 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, 1mM DTT) and concentrated to 25-50 μM for storage at 4°C, where they were used within a month of preparation.

3.2E *In vitro* histone acetyltransferase assays

See **Chapter 2.2**

HAT assays run over a time course were first incubated at 30˚C for 2 minutes prior to adding enzymatic subcomplex at a concentration of 50 nM except for Time 0 which was flash frozen immediately upon 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 hours at 30˚C to control for spontaneous acetyl CoA acetylation or histone degradation.

Assays containing nucleosomal substrates consisted of 0.55 μM nucleosome, 150 nM of either WT or Y413A subcomplex, and 30 μM acetyl CoA carried out in a total of 30 μL reaction volume. Reactions were run for 30 mins at 30˚C and flash frozen upon completion. ADA subcomplex was omitted from the non-enzymatic negative controls.

3.2F Cloning, expression, and chemical acetylation of histone H3 point mutants

The pET3a vector containing yeast histone H3 sequence was obtained courtesy of Dr. Gregory Bowman (The Johns Hopkins University, Baltimore, MD). Both H3K14R and H3K23R were generated using QuikChange site-directed mutagenesis (Stratagene). Histones were expressed, purified from inclusion bodies, and further purified over HPLC. To obtain maximally acetylated histone H3 control recombinant *S. cerevisiae* histones were subjected to *in vitro* acetylation as previously outlined (119).

Acetylated histones were purified and desalted using HPLC purification, and fractions were combined, flash frozen, and lyophilized.

3.2G Western blot analysis of histone H3 acetylation

Histone H3 samples were resolved by SDS PAGE and were transferred to a PVDF membrane using a semi dry transfer system. Membranes were blocked overnight in 5% milk at 4˚C and washed in Tris Buffer Saline (TBS). Primary antibody anti-H3ac (Millipore, 1/5,000) was diluted in 1% milk in TBS and 0.1% Tween (TBST) for HAT assay. For histone mutant western blots anti-H3K14ac (ab52946, Abcam, 1/5000); anti-H3K18ac (Active Motif 39130, 1/10,000); anti-H3K23ac (Active Motif 39132, 1/20,000) were used. Primary antibody was applied for 1 hour at room temperature followed by washing in TBST. Goat anti-rabbit IgG-horseradish peroxidase secondary antibody (Amersham Biosciences) was diluted to 1/4000 in 1% milk and TBST and applied for 1 hour at room temperature and washed in TBST. Blots were developed using Pierce ECL Western Blotting Substrate (ThermoScientific) and exposed using film.

3.2H Generation and growth of yeast strains

Δgcn5 and Δgcn5Δyng1 background yeast strains were obtained from the lab of Dr. Brian Strahl University of North Carolina Chapel Hill. WT Gcn5 and Y413A Gcn5 plasmids were introduced into each strain. Yeast were grown in His drop out media and spun down and pelleted with the supernatant removed. Yeast strains were generated by Ana Raman.

3.2I Acid extraction of histones from yeast

Pellets were frozen and thawed on ice and resuspended in 0.5 mL of 20% ice cold TCA. 0.5mLs of glass beads were added and vortexed four times for 30 seconds each in the cold room. Lysate was transferred to a fresh Eppendorf tube. Beads were washed twice with 0.5 mL of %% ice cold TCA and the washes were combined with the lysates. Precipitated protein was spun down at 3krpm for 10 mins at 4°C. Supernatant was removed and pellets were resuspended in 200 uL of 1X sample buffer in which 50 uL of 2 M Tris was added to neutralize the acid. Samples were boiled and loaded onto SDS-PAGE gel.

3.3 Results

3.3A Mutation of the bromodomain disrupts lysine acetylation on histone H3

To better understand how the loss of Gcn5 bromodomain function impacts Gcn5 subcomplex HAT activity, *in vitro* HAT reactions were resolved on acid-urea gels comparing the histone H3 acetylation patterns generated by increasing concentrations of WT vs. Y413A Gcn5 subcomplexes or WT vs. P371T/M372A Gcn5 subcomplexes (**Figures 19A, 19B)**. At enzyme concentrations greater than 50 nM the pattern of histone H3 acetylation became noticeably altered between the WT and bromodomain mutant Gcn5 subcomplexes. A relative lag in acetylated histone H3 reaction intermediates was observed in Y413A and P371T/M372A HAT reactions when compared to histone H3 acetylated by the WT Gcn5 subcomplex. Notably, at higher enzyme concentration (200 nM) bromodomain mutant subcomplexes generated an

Figure 19

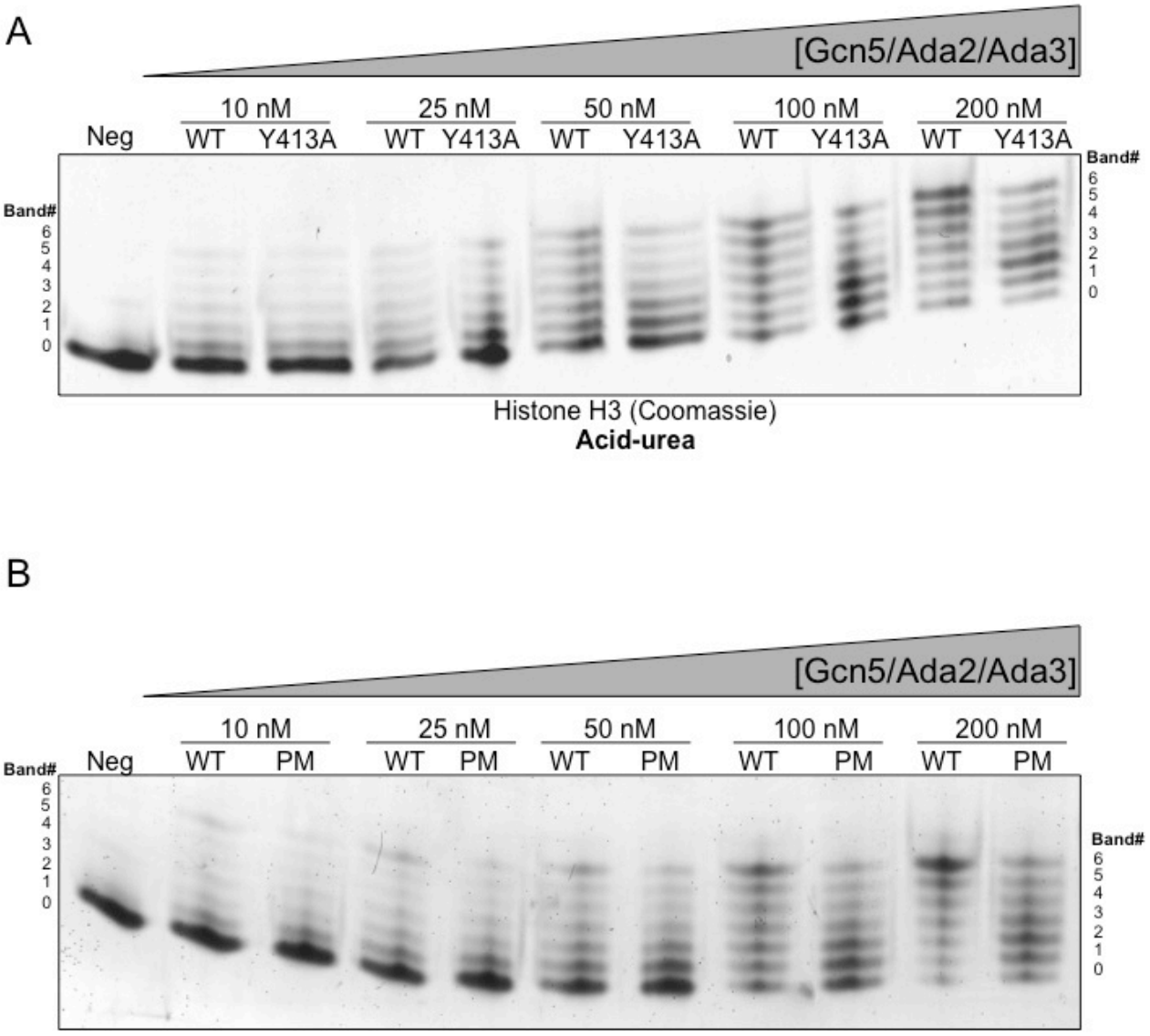


Figure 19. Nondisruptive and disruptive Gcn5 bromodomain point mutations result in altered histone H3 acetylation pattern. (A) Coomassie stain of *in vitro* HAT assays on recombinant H3. Assays were performed using increasing amounts of either wildtype (WT) or Y413A bromodomain mutant (Y413A) Gcn5/Ada2/Ada3 subcomplex, and were resolved with acid-urea gel electrophoresis. Unmodified histone H3 serves as a negative control (Neg). (B) Coomassie stain of *in vitro* HAT assays on recombinant H3. Assays were performed using increasing amounts of either wildtype (WT) or PM bromodomain mutant Gcn5/Ada2/Ada3 subcomplex, and were resolved with acid-urea gel electrophoresis. Unmodified histone H3 serves as a negative control (Neg).

enrichment in a di-acetylated histone H3 isoform instead of progressing to a hexa-acetylated state formed in the WT reaction. Because the staining intensity of the hexa-acetylated histone H3 band (band 6) in the WT reaction was relatively comparable to the di-acetylated histone H3 band (band 2) in the Y413A reaction, it can be inferred that the Gcn5 bromodomain contributes a 3-fold increase in histone H3 acetylation. Taken together, these results suggest that when the Gcn5 bromodomain is defective in acetyl-lysine binding, overall histone H3 acetylation is decreased.

Having established that the bromodomain contributes to changes in histone H3 acetylation pattern over increasing Gcn5 subcomplex enzyme concentrations, we next wanted to further examine how overall acetylation was affected in WT and Y413A reactions. Therefore, we investigated the evolution of differential acetylation patterns in kinetic time course experiments between the WT and bromodomain mutant subcomplexes at a concentration of 50 nM enzymatic subcomplex **(Figures 20A, 20B)**. Consistent with observed acetylation differences using increasing enzyme concentrations, we found a reduction in total histone H3 acetylation with the Y413A Gcn5 subcomplex at later time points of 60 min, 4 hr, and 8 hr. Densitometry quantitation of histone H3 band intensity resolved on acid-urea gels underscored the differences in acetylation pattern between WT and bromodomain mutant Gcn5 subcomplexes **(Figure 20B)**. Interestingly, at earlier time points of 1 min, 5 min, and 15 min the Y413A Gcn5 subcomplex exhibited more robust acetylation activity on initial lysine events. The formation and progression to mono, di, and tri-acetylated histone H3 states occurred faster in the Y413A reactions when compared with the WT reaction.

Figure 20

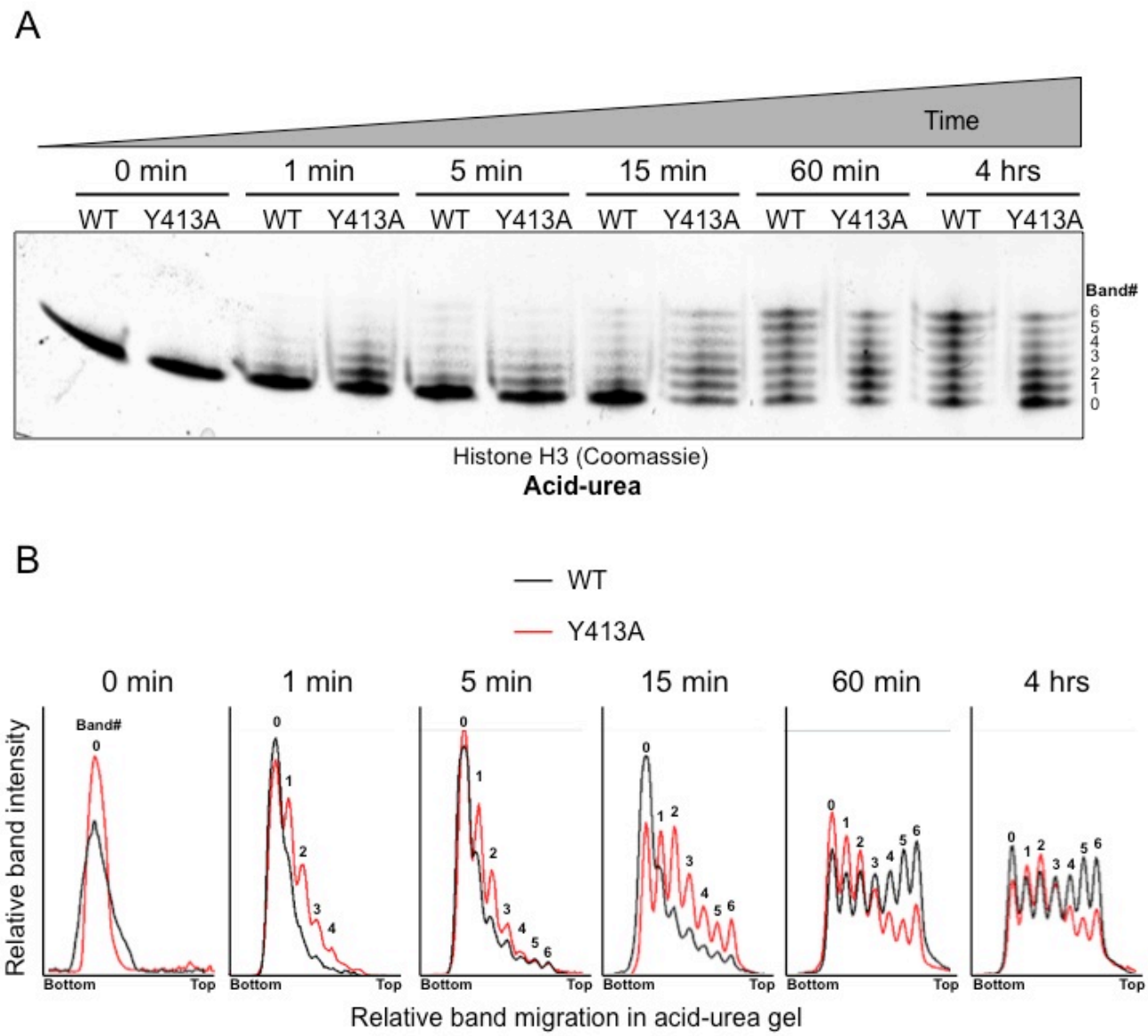


Figure 20. Y413A Gcn5 bromodomain mutation affects the rate of histone H3 lysine acetylation. (A) WT vs. Y413A subcomplex acetyltransferase activity on histone H3 was compared over a time course and analyzed using an acid urea gel to compare banding patterns of histone H3 acetylation states. (B) Acid urea gel histone H3 band quantitation using Image J comparing relative band migration and intensity generated by the WT (black) and Y413A (red) ADA subcomplex at given time points.

3.3B The Gcn5 bromodomain regulates site specificity of acetylation on histone H3

Because our acid-urea gel analysis revealed that disruption of Gcn5 bromodomain acetyl-lysine binding function resulted in an altered histone H3 acetylation pattern we sought to examine differences in acetylation abundance on individual lysine residues and determine if the observed Gcn5 subcomplex site specificity of H3K14 > H3K23 > H3K18 = H3K9 > H3K27 > H3K36 was at all disrupted when the bromodomain was mutated. We first used semi-quantitative mass spectrometry on histone H3 resolved via SDS-PAGE comparing the overall acetylation levels at individual lysine residues generated by either WT or Y413A Gcn5 subcomplexes **(Figure 21)**. We found that the Y413A Gcn5 subcomplex was able to acetylate H3K14 and H3K23 to WT levels. However, the Y413A Gcn5 subcomplex demonstrated reduced acetylation on residues H3K9 and H3K18. Acetylation abundance at H3K27 and H3K36 were undetectable by mass spectrometry when histone H3 was resolved on SDS-PAGE.

While resolution over SDS-PAGE and combined mass spectrometry analysis allowed us to determine total acetylation abundance on individual lysine residues, insight into site specificity requires resolution of differentially acetylated states over an acid-urea gel. Using an acid-urea gel we are able to determine any alterations in acetylation pattern between the WT and Y413A subcomplex reactions **(Figures 22A, 22B)**. To gain understanding into bromodomain related changes in site specificity, we compared WT HAT reactions with that of the Y413A bromodomain mutant reactions using the combined acid-urea gel and mass spectrometry approach **(Figures 22, 23, 24)**. In both the WT and Y413A reactions we found the same six histone H3 lysine

Figure 21

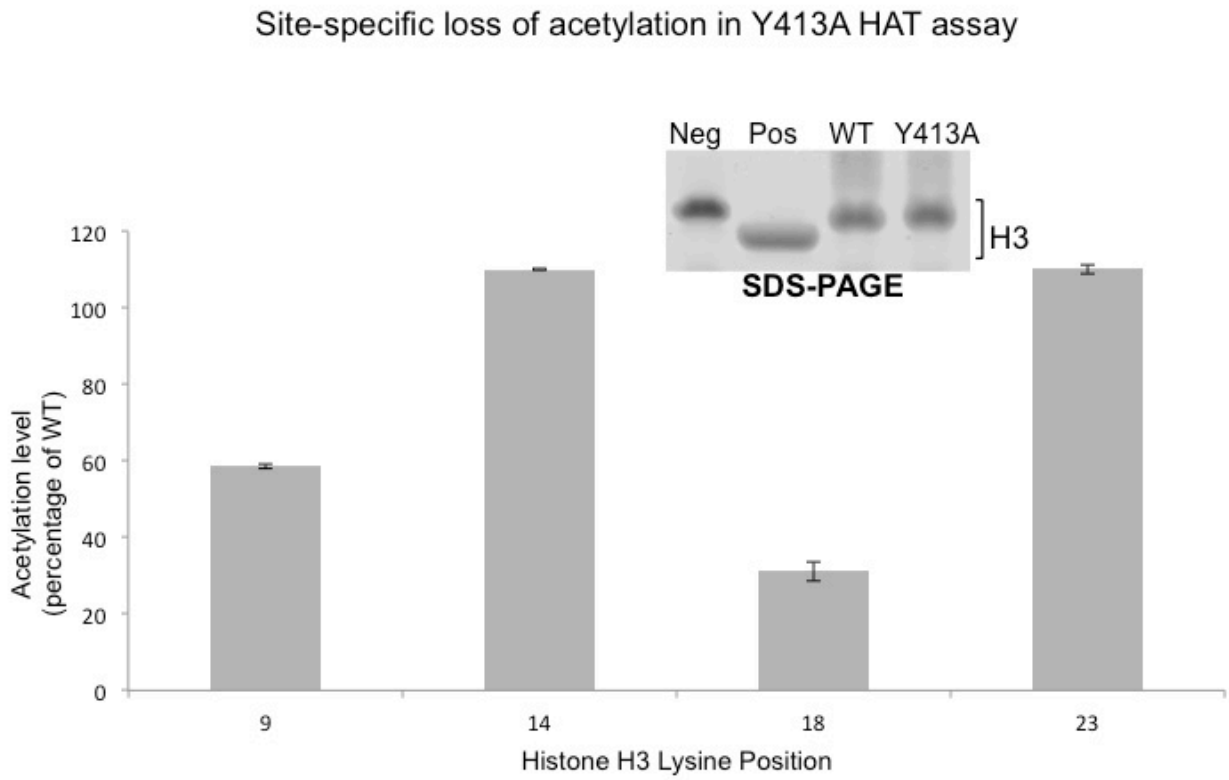
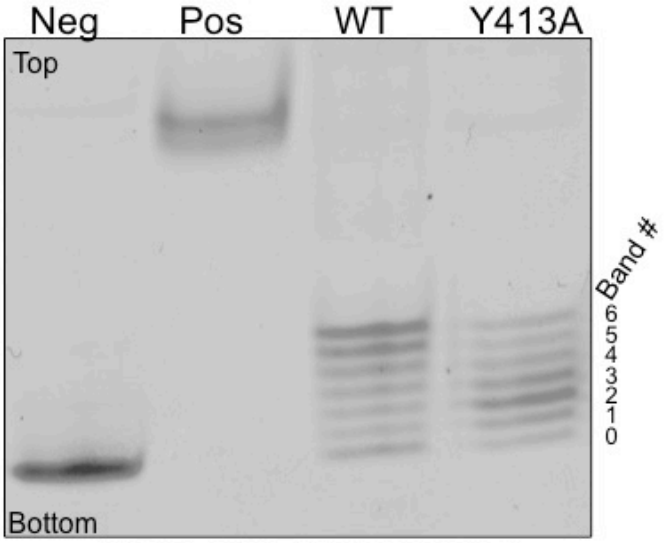


Figure 21. Y413A mutation of the bromodomain results in site specific loss in histone H3 acetylation. Histone H3 from HAT reactions was resolved on an SDS-PAGE gel where bands were excised and subjected to quantitative mass spectrometry analysis. Bar graph depicts the change in acetylation levels on histone H3 lysine residues from the Y413A HAT assay relative to acetylation abundance generated by the WT subcomplex. Only H3 lysines with detectable signal are shown. Each reaction was performed in triplicate.

Figure 22

A



Histone H3 (Coomassie)

Acid-urea

B

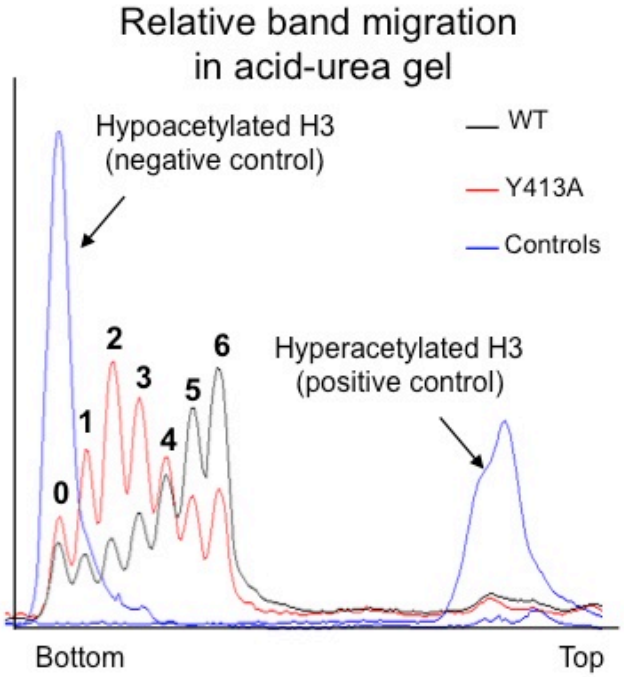


Figure 22. Y413A Gcn5 bromodomain mutation generates altered histone H3 acetylation states. (A) Acetylated histone H3 from WT and Y413A HAT assays was resolved on an acid-urea gel and each band was excised for quantitative mass spectrometry analysis. Negative control (Neg) consisted of HAT assays without acetyl-CoA and positive (Pos) control contains acetic anhydride treated hyperacetylated histone H3. Each reaction was performed in triplicate. (B) Image J band quantitation of histone H3 migration in an acid urea gel.

Figure 23

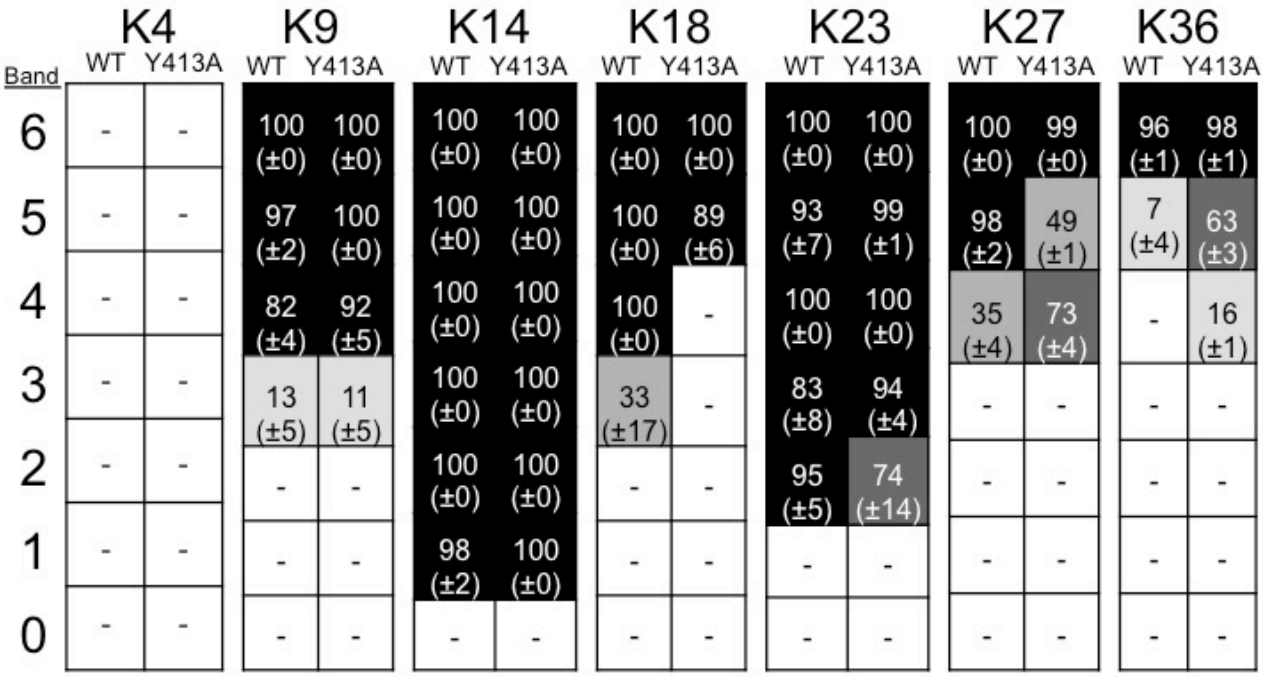


Figure 23. Quantitative mass spectrometry reveals the Y413A bromodomain mutation affects Gcn5 subcomplex histone H3 site specificity . Quantitative mass spectrometry was performed on each histone H3 band resolved by acid-urea gel from either the WT or Y413A reaction. The percent acetylation was calculated for each individual lysine residue present within histone H3 and only those lysines with detectable signal are shown. Mass spectrometry processing and analysis was performed in collaboration with the lab of Dr. Alan Tackett (University of Arkansas, Little Rock, AR)

residues were acetylated. However, although H3K14ac, H3K23ac, and H3K9ac were the preferred lysines acetylated by the WT and with both mutant subcomplexes, significant differences were detected when we compared acetylation levels among downstream lysines. In contrast to the pattern of acetylation observed with the WT subcomplex where H3K18ac occurred in the 3rd acetylation states, in both bromodomain mutants H3K18ac did not occur until the 5th and 6th acetylation states. Furthermore, H3K27ac and H3K36 acetylation generated from the bromodomain mutants occurred earlier when compared histone H3 isoforms from the WT reaction.

We sought to determine if this observed effect could be repeated with additional bromodomain mutations other than Y413A, and therefore tested the disruptive P371T/M372A Gcn5 bromodomain mutation to determine if this would result in the same disruption of histone H3 site-specificity. We resolved the WT and P371T/M372A reactions over an acid-urea gel and excised the bands for mass spectrometry analysis **(Figure 25A)**. Interestingly the histone H3 acetylation pattern generated by the P371T/M372A resembled that generated by the Y413A subcomplex in that there appeared to be an accumulation of mono and di-acetylated states. Quantitative mass spectrometry revealed that histone H3 lysines K9, K14, and K23 were unaffected by the P371T/M372A bromodomain mutation, while the site specificity of lysines K18, K27, and K36 were affected in strikingly similar manner as that of the nondisruptive Y413A mutation **(Figure 25B)**.

3.3C Bromodomain dependent site specific acetylation on nucleosomal substrates

Figure 24

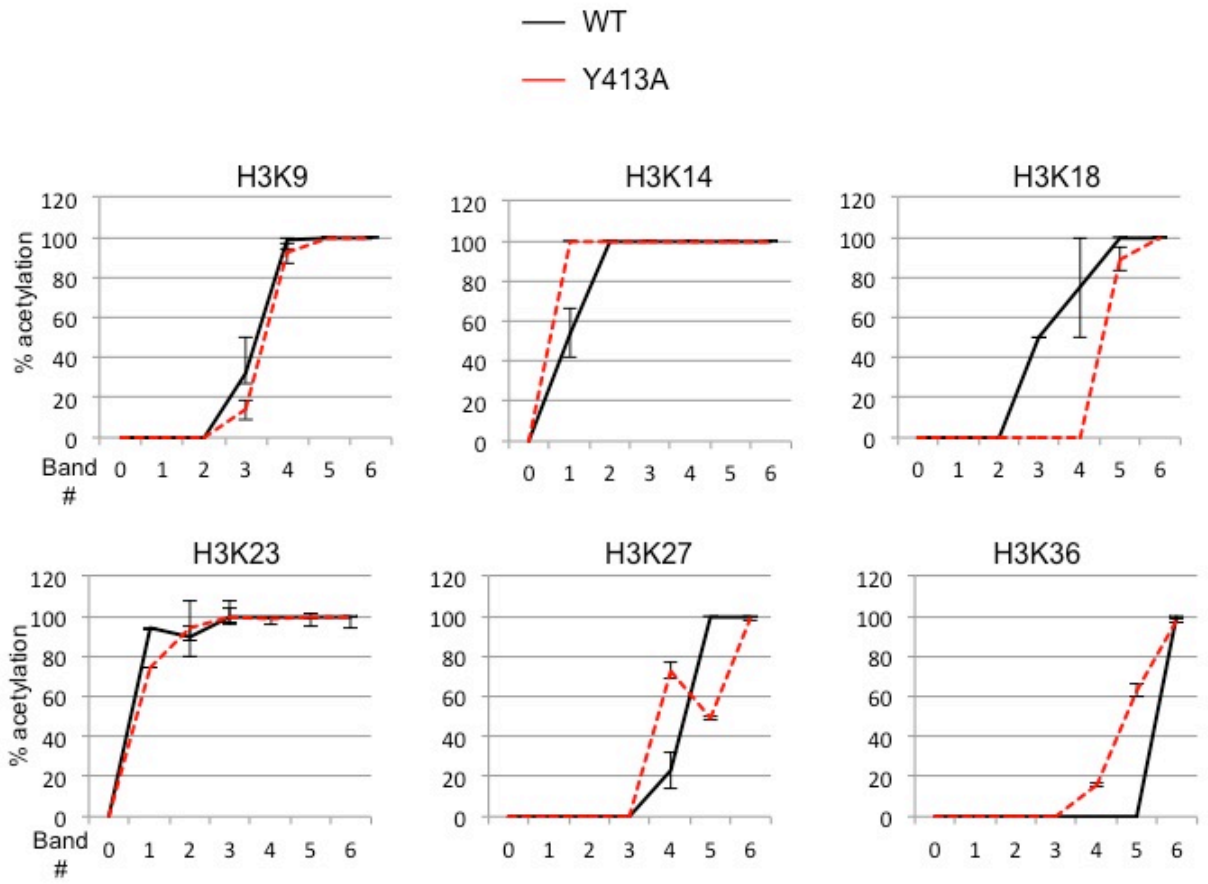
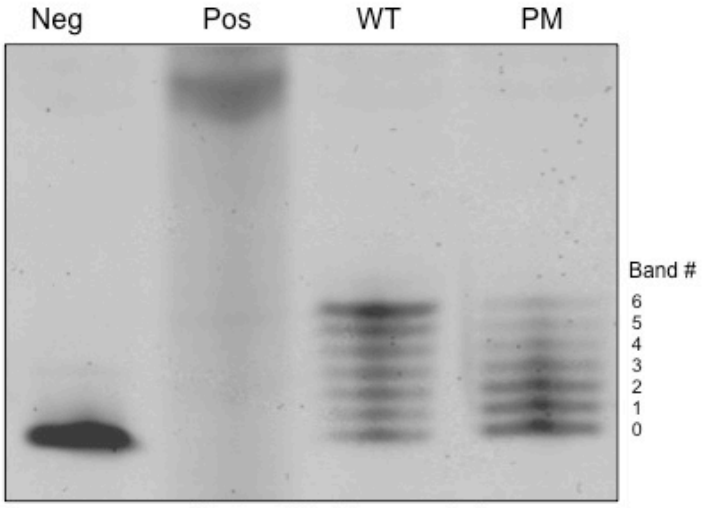


Figure 24. Y413A bromodomain mutation affects Gcn5 subcomplex site specificity of downstream acetylation. Graphical representation of Gcn5 subcomplex percent acetylation on individual targeted histone H3 lysine residues between WT (black) and Y413A (red).

Figure 25

A



Histone H3 (Coomassie)

Acid-urea

B

Band #	K4 WT	K4 Y413A	K4 P371T M372A	K9 WT	K9 Y413A	K9 P371T M372A	K14 WT	K14 Y413A	K14 P371T M372A	K18 WT	K18 Y413A	K18 P371T M372A	K23 WT	K23 Y413A	K23 P371T M372A	K27 WT	K27 Y413A	K27 P371T M372A	K36 WT	K36 Y413A	K36 P371T M372A	K37 WT	K37 Y413A	K37 P371T M372A
6	-	-	-	100 (±0)	100 (±0)	100 (±0)	100 (±0)	100 (±0)	100 (±0)	100 (±0)	100 (±0)	58 (±30)	100 (±0)	100 (±0)	100 (±0)	100 (±0)	99 (±1)	100 (±0)	96 (±1)	98 (±1)	95 (±1)	14 (±6)	29 (±1)	4 (±2)
5	-	-	-	97 (±3)	100 (±0)	100 (±0)	100 (±0)	100 (±0)	100 (±0)	100 (±0)	89 (±6)	8 (±8)	93 (±12)	99 (±1)	100 (±0)	98 (±3)	49 (±1)	82 (±8)	7 (±4)	63 (±3)	74 (±5)	-	-	6 (±4)
4	-	-	-	82 (±4)	92 (±5)	88 (±1)	100 (±0)	100 (±0)	100 (±0)	100 (±0)	-	-	100 (±0)	100 (±0)	100 (±0)	35 (±4)	73 (±4)	92 (±3)	-	16 (±1)	6 (±1)	-	-	-
3	-	-	-	13 (±5)	11 (±5)	14 (±5)	100 (±0)	100 (±0)	100 (±0)	33 (±17)	-	-	83 (±8)	94 (±4)	100 (±0)	-	-	16 (±3)	-	-	-	-	-	-
2	-	-	-	-	-	-	100 (±0)	100 (±0)	100 (±0)	-	-	-	95 (±5)	74 (±14)	92 (±8)	-	-	-	-	-	-	-	-	-
1	-	-	-	-	-	-	98 (±2)	100 (±0)	67 (±33)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Figure 25. Gcn5 bromodomain P371T/M372A (PM) double mutation affects H3 site specificity. (A) Representative acid-urea gel comparing acetylated histone H3 from wild type and PM HAT reactions (as performed in Figure 4).The negative control (Neg) consisted of HAT assays without acetyl-CoA, and the positive (Pos) control contains acetic anhydride treated hyperacetylated histone H3. Each reaction was performed in triplicate and bands from the PM reaction were excised and analyzed by semi-quantitative mass spectrometry. (B) For the PM reaction, the percent acetylation for each individual H3 lysine was calculated, and then compared to the independent data collected on the wild type and Y413A mutant HAT assays. Mass spectrometry processing and analysis was performed in collaboration with the lab of Dr. Alan Tackett (University of Arkansas, Little Rock, AR).

Having established a relationship between the Gcn5 bromodomain and site specific acetylation on free histone H3 we aimed to examine how the bromodomain might affect HAT activity when using the biologically relevant nucleosomal template. We analyzed WT and Y413A HAT reactions using antibodies against histones and histone H3 lysine specific acetylation events that occurred within the nucleosome. Consistent with previous studies, the Gcn5 subcomplex acetylation within the nucleosome occurred on histone H3 with a single lysine acetylation on both histone H4 and histone H2B **(Figure 26A)**. Just as we observed with free histone H3, acetylation on nucleosomal histone H3 contained a total of 6 acetylation sites with strikingly diminished overall acetylation in the Y413A bromodomain mutant. In the Y413A HAT reaction the majority of histone H3 resolved as a mono (band 1) or di-acetylated (band 2) species, instead of progressing to a hex-acetylated state observed in the WT reaction. The effects of the bromodomain on Gcn5 catalytic HAT activity appeared to be specific to histone H3 as acetylation of H2B and H4 remained unchanged regardless of bromodomain acetyl-lysine binding function.

To determine how nucleosomal histone H3 acetylation on individual lysine residues was affected when the bromodomain was disrupted, acetyl-lysine specific antibodies were used to compare the distribution of site specific acetylation between WT and Y413 reactions **(Figure 26B)**. H3K14ac was found to be enriched in lower acetylation states catalyzed by the Y413A mutant, suggesting a loss of acetylation processivity relative to the WT subcomplex. The most striking observation was the comparison of H3K18ac abundance and distribution between the WT and Y413A HAT reactions. H3K18ac was nearly undetectable when the bromodomain acetyl-lysine

Figure 26

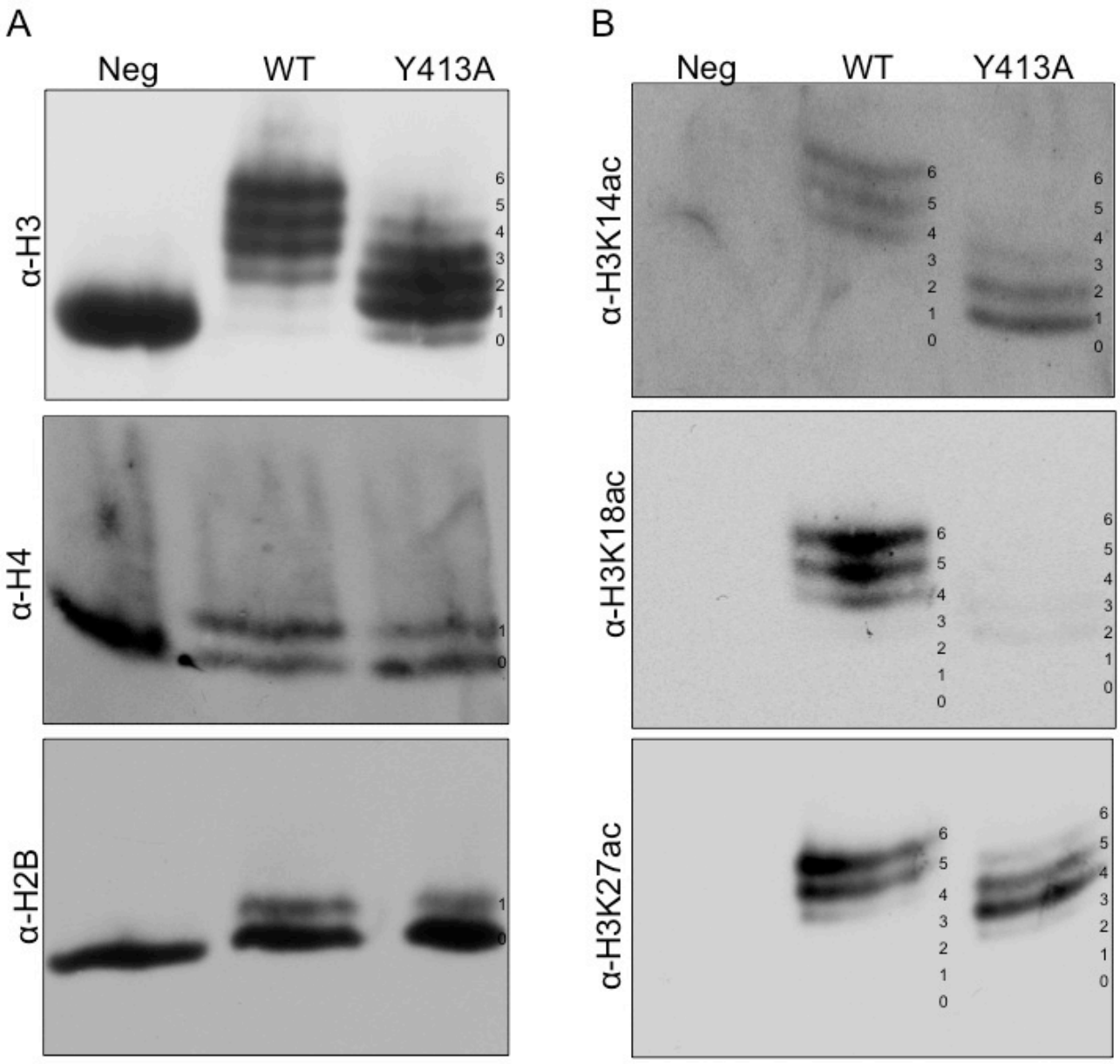


Figure 26. Gcn5 site-specific acetylation of nucleosomal histone H3 is dependent on bromodomain function. WT or Y413A ADA subcomplex HAT assays were performed with nucleosomes and resolved on acid-urea gels. Unacetylated histone H3 from a nonenzymatic reaction served as a negative control (Neg). (A) Western blots using antibodies against Gcn5 histone substrates H3, H4, and H2B. (B) Western blots using histone H3 acetyl-lysine antibodies against lysine 14 (H3K14ac), lysine 18 (H3K18ac), and lysine 27 (H3K27ac).

binding function was disrupted in the Y413A Gcn5 subcomplex indicating the acetylation of K18 is heavily dependent on a functional Gcn5 bromodomain. H3K27ac remained detectable at in both WT and Y413A reactions, however this modification was now enriched in lower acetylation states in the Y413A reaction compared with that of the WT reaction, which is consistent with the observation on free histone H3 utilizing mass spectrometry analysis.

3.3D Gcn5 acetylation of H3K18 is dependent on H3K14ac in a bromodomain-mediated manner

Because Gcn5 acetylation of H3K18 is dependent on the acetyl-lysine binding function of the bromodomain, we aimed to uncover mechanistic insight into how the bromodomain facilitates H3K18ac. We hypothesized this could be due to bromodomain-mediated crosstalk between a previously deposited lysine residue and proper acetylation of K18 acetylation. Because H3K14 and H3K23 are the first lysines acetylated by the Gcn5 subcomplex and their acetylation as independent of the Gcn5 bromodomain, we reasoned that the bromodomain may be binding H3K14ac and/or H3K23ac and thereby facilitating proper acetylation of K18ac via the Gcn5 HAT domain. To test whether H3K18ac is affected when H3K14 or H3K23ac is unable to be acetylated, we generated histone H3 point mutants where we obtained clones for H3K14R H3K23R **(Figure 27A)**. Because arginine is structurally similar to lysine and also has a positive charge, this mutation would be least detrimental on histone structure and protein-protein interactions. We generated *S. cerevisiae* H3K14R and H3K23R point mutations to test in *in vitro* HAT assays. Using these substrates we validated the specificity and reactivity of histone acetyl-lysine antibodies against H3K14ac, H3K2ac

Figure 27

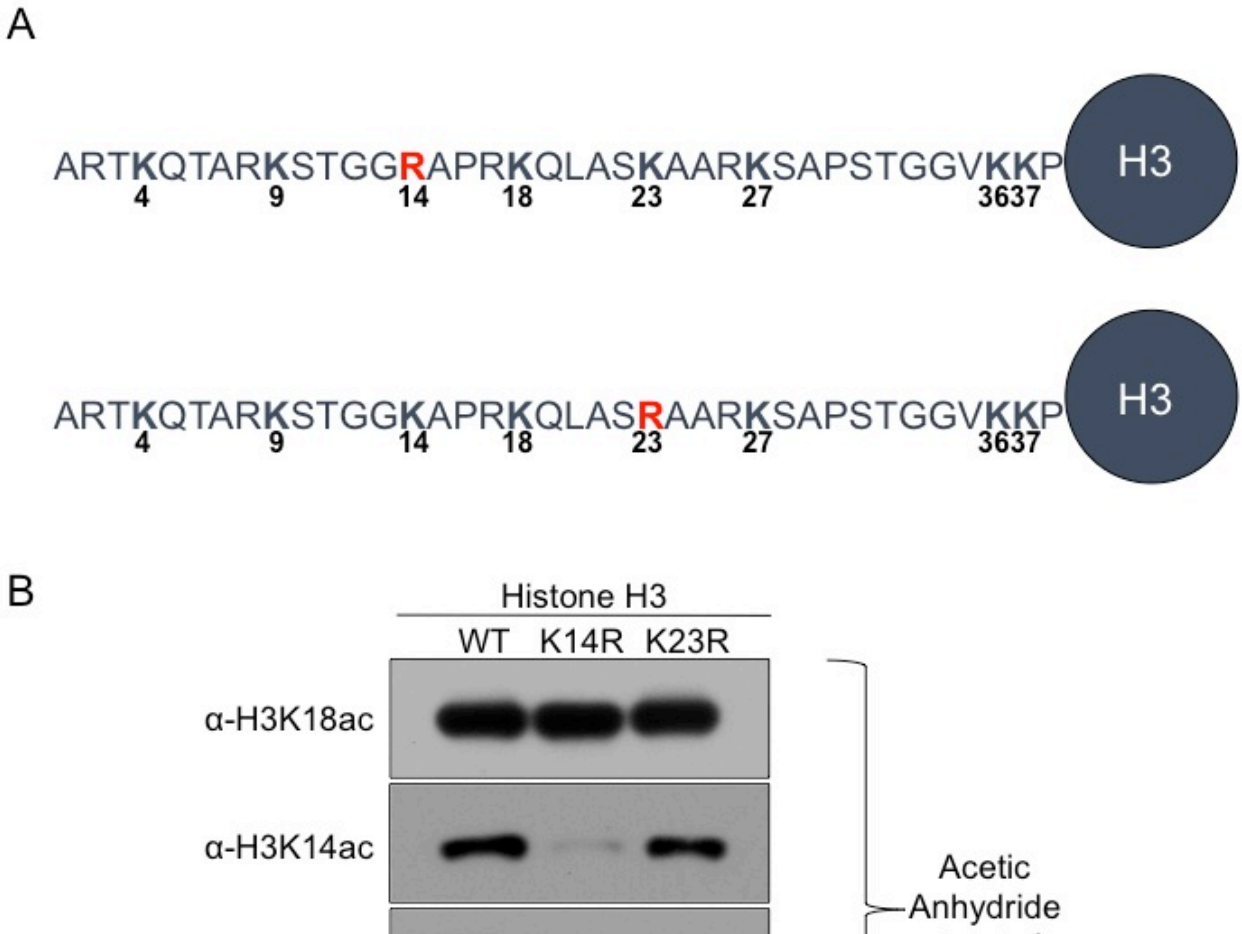


Figure 27. Generation of histone lysine to arginine point mutants and acetyl-lysine specific antibodies . (A) Histone H3K14R and H3K23R point mutations were generated. (B) WT histone H3, H3K14R, and H3K23R were chemically acetylated with acetic anhydride and western blots with acetyl-lysine specific antibodies were performed. Histone H3K23R point mutant construct was generated by Dr. Tonya M. Gilbert.

and H3K18ac. While PTM specific antibodies are very useful tools in determining site specific modifications, they have several disadvantages associated with them. Issues with cross-reactivity, broad epitope specificity, and being unable to provide a quantitative measure have encouraged the use of mass spectrometry and proteomics techniques for identifying and measuring histone PTMs (153, 154). We chemically acetylated these substrates with acetic anhydride so that every available lysine residue on these proteins was acetylated. Arginines are not acetylated in this treatment, and therefore anti-H3K14ac and anti-H3K23ac antibodies should not recognize H3K14R and H3K23R point mutants, which was verified by western blot **(Figure 27B)**.

We subjected each histone H3 substrate, WTH3, H3K14R, H3K23R to an *in vitro* HAT reaction with the WT Gcn5 subcomplex. HAT reactions were resolved on acid-urea gels to compare the pattern of overall acetylation among each substrate **(Figure 28)**. In both H3K14R and H3K23R histone mutants, only five lysines were detectable acetylated due to the loss of either H3K14 or H3K23 as a Gcn5 lysine target. The acetylation rate was evident as the progression from an unacetylated to a hyperacetylated state. We observed acetylation rates to be reduced in both histone K to R point mutants relative to WT histone H3.The H3K14R mutant was acetylated at a substantially slower rate, indicated by a greater intensity in the lowest, unacetylated histone H3 band (band 0).

To determine whether H3K14 of H3K23 acetylation might affect K18 acetylation levels, we utilized acetyl-lysine specific antibodies on HAT reactions, which were resolved on either SDS-PAGE, or acid-urea gels. Acetylation levels were then compared using antibodies that have been tested and verified against hyperacetylated

Figure 28

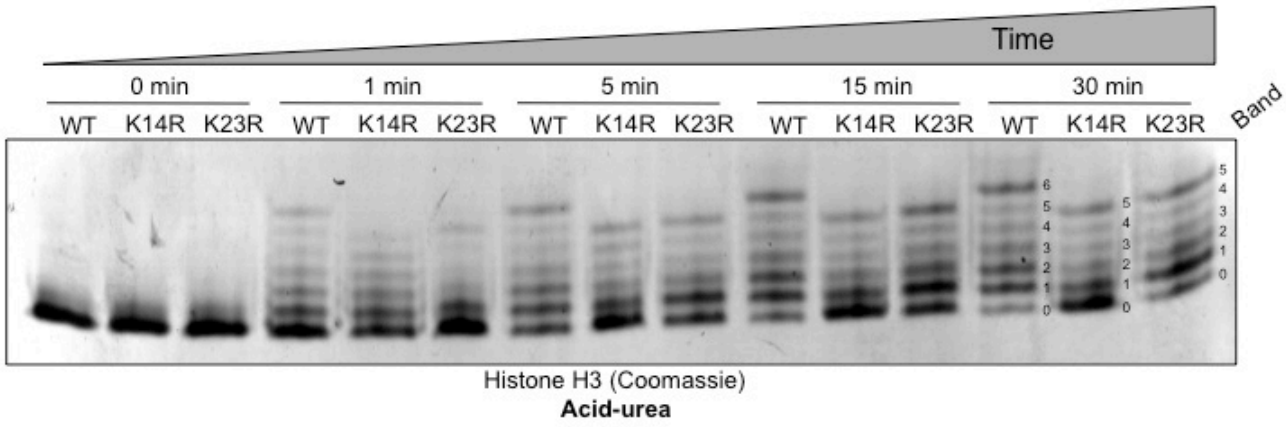


Figure 28. Histone H3 K to R mutants disrupt the rate of acetylation by the Gcn5 subcomplex. Histone H3 lysine to arginine point mutations were generated for H3K14R and H3K23R. The HAT activity of the Gcn5/Ada2/Ada3 subcomplex was compared among wildtype histone H3, H3K14R, and H3K23R over a time course resolved on an acid urea gel.

histone substrates to ensure cross reactivity was not occurring and the K to R mutation did not disrupt antibody epitope recognition. Western blots using anti-H3K18ac, anti-H3K14ac, and H3K23ac were first performed on SDS-PAGE resolved HAT reactions to determine the total acetylation level catalyzed by the Gcn5 subcomplex on WT and K to R mutant histone H3 substrates **(Figure 29A)**. Here we observed a marked decrease of K18ac on the H3K14R, whereas H3K18ac was minimally affected o the H3K23R substrate. When these reactions were resolved on acid-urea gels and subjected to the same Western blot analysis, a striking decrease of H3K18ac was again observed on the H3K14R mutant relative to WTH3 and H3K23R substrates **(Figure 29B)**. Here, H3K18ac was only observed on the H3K14R histone in the highest acetyl-H3 band (band 5). Together, these data suggest that acetylation of H3K18by the Gcn5 subcomplex is dependent upon the presence of an acetylatable lysine at H3K14, but not H3K23.

3.3E H3K18ac is dependent upon Gcn5 bromodomain acetyl lysine binding function *in vivo*

Because our *in vitro* data revealed a striking decrease in H3K18 acetylation when the Gcn5 bromodomain acetyl-lysine binding function was disrupted, we wanted to determine if this effect also occurred *in vivo* within the context of the cell. We generated *S. cerevisiae* yeast strains that contained either WT or Y413A Gcn5 **(Figure 30A)**. WT Gcn5 or Y413A Gcn5 plasmids were introduced into both ΔGcn5 and ΔGcn5ΔYng1 background strains. A ΔGcn5ΔYng1 background was used because *in vivo* lysine acetylation of histone H3 is catalyzed by both Gcn5 and the HAT NuA3-complex HAT,

Figure 29

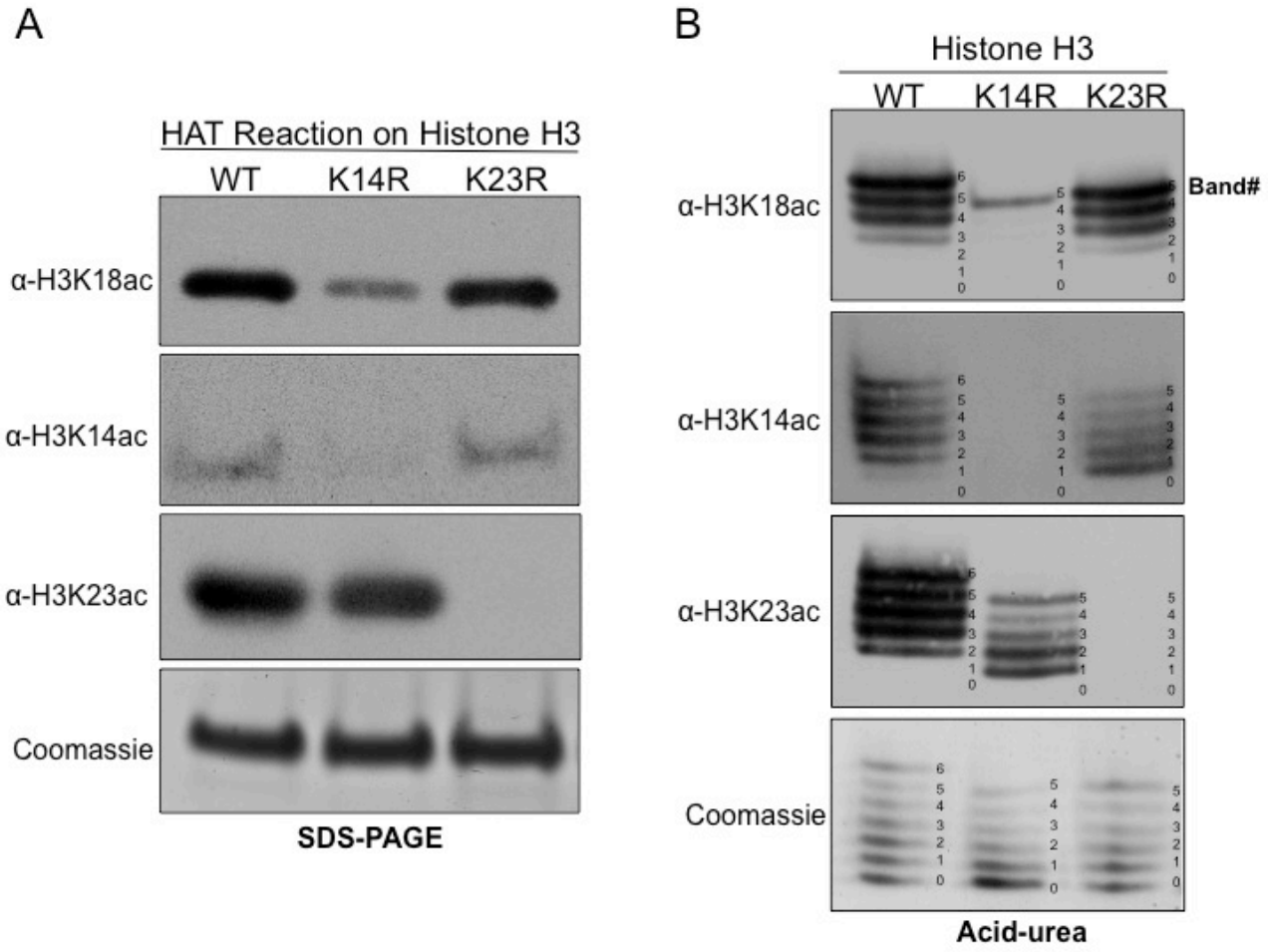


Figure 29. Histone H3 K to R mutants reveal acetylation of specific sites alters GCN5 activity (A) Histone H3 acetyl-lysine specific antibodies were used to determine relative levels of H3K18ac, H3K14ac, and H3K23ac resolved on SDS-PAGE. Antibodies were first validated on hyperacetylated wildtype H3, H3K14R, and H3K23R chemically treated with acetic anhydride (first column). Site-specific antibodies were used to compare relative levels of Gcn5/Ada2/Ada3 *in vitro* acetylation on H3K18, H3K14, and H3K23 among wildtype (WT) H3, H3K14R, and H3K23R histone substrates (second column). (B) Gcn5/Ada2/Ada3 *in vitro* HAT reactions with either wild type (WT) H3, H3K14R, or H3K23R were resolved on acid urea gels and western blots were performed using histone H3 acetyl-lysine specific antibodies against H3K18ac, H3K14ac, or H3K23ac.

Sas3 (155, 156). Yng1 is a subunit of NuA3, required for NuA3 localization to chromatin, and necessary for proper NuA3 acetylation of histone H3 (34, 37). By deleting Yng1 we posited that Sas3 would minimally acetylate histone H3, and Gcn5 acetylation can be independently assessed without any redundant HAT activity by NuA3.

Histones were isolated from each strain and resolved over SDS-PAGE gels. Histone H3 acetyl-lysine specific antibodies were used to compare global acetylation levels at individual lysine residues **(Figure 30B)**. In both the ΔGcn5 and ΔGcn5ΔYng1 background strains we observed a striking decrease in global K18ac in the Y413A Gcn5 bromodomain mutant strain. Global H3K14ac and H3K23ac levels were unaffected by the Y413A Gcn5 bromodomain mutation. These results indicate that the global acetylation of H3K18ac levels is dependent upon the Gcn5 bromodomain *in vivo*.

3.4 Discussion

3.4A General discussion

We found that ablation of the acetyl-lysine binding function of the Gcn5 bromodomain diminished the amount of total acetylation on and disrupted the order in which lysines were acetylated on free and nucleosomal histone H3. Ultimately, Gcn5 subcomplex site specific acetylation of H3K14 and H3K23 was show to be independent of bromodomain function, whereas downstream acetylation of lysines H3K9, H3K18, H3K27, and H3K36 was altered upon mutation of the Gcn5 bromodomain **(Figure 31A)**.

Figure 30

A

B

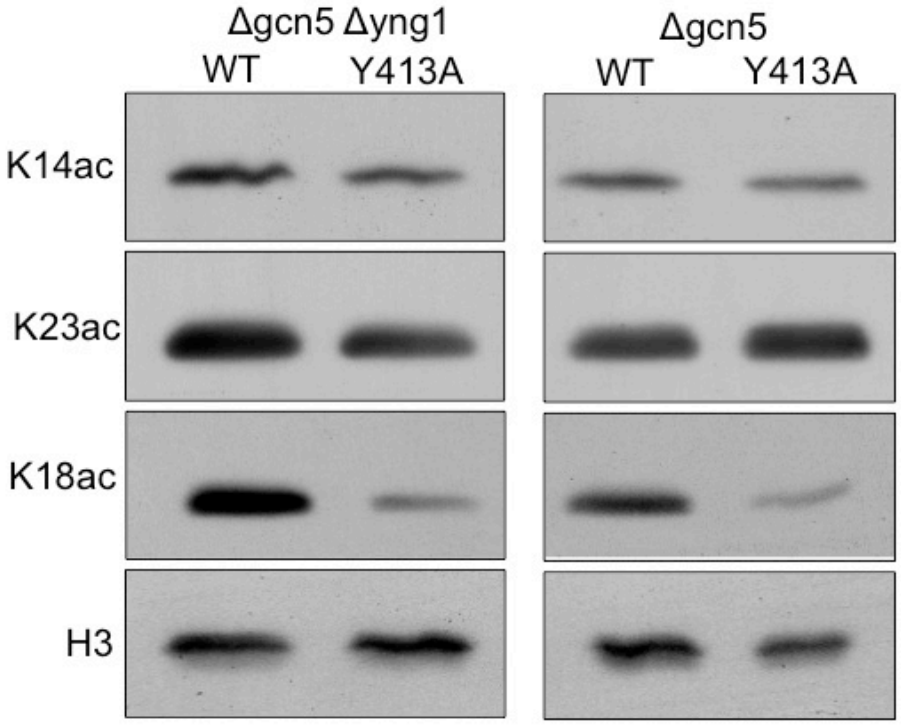


Figure 30. Y413A Gcn5 bromodomain mutation significantly decreases global H3K18 acetylation levels *in vivo*. (A) Yeast strains were generated to contain either WT Gcn5 or the Y413A Gcn5 point mutation. Gcn5 plasmids were introduced into yeast strains that had a background of either gcn5 deletion or a gcn5 and yng1 deletion. (B) Western blots using acetyl-lysine specific antibodies were used to look at global changes in site specific histone H3 acetylation levels between WT Gcn5 and Y413A Gcn5 yeast strains. Background yeast strains were obtained from the lab of Dr. Brian Strahl (UNC, NC) and generated by Ana Raman.

Previously, the effect of the Gcn5 bromodomain on catalytic HAT activity and site specificity on histone proteins has been poorly characterized. *In vivo* studies have shown that Gcn5 bromodomain point mutants, including the Y413A and the P371T/M372A used in our analysis, suggest that the bromodomain acts to regulate HAT activity at Gcn5-targeted promoters and acts to maintain proper nucleosome remodeling (32, 73, 74, 92, 145, 149, 157). The site specificity of the Gcn5 subcomplex, especially concerning that of H3K18 acetylation, was similarly altered utilizing two independent bromodomain mutants. These mutations included a non-disruptive Y413A mutant that ablated Gcn5 bromodomain acetyl-lysine binding while maintaining the structural integrity of the bromodomain. Additionally, a disruptive P371T/M372A bromodomain mutant introduced in the ZA loop has been shown to disable the bromodomain and in turn ablate acetyl-lysine binding. However, when purifying the Gcn5 subcomplex with the structurally disruptive P371T/M372A mutation we observed an uneven stoichiometry in the complex members where approximately a 2-fold increase of Ada3 co-purified with the subcomplex. We reasoned that the disruptive bromodomain mutation may have affected the structural interaction of Gcn5 with its binding partners resulting in uneven stoichiometry of the trimeric subcomplex. Given the changes we observed with both our disruptive (P371T/M372A) and nondisruptive (Y413A) Gcn5 bromodomain point mutants, additional bromodomain mutations to residues such as Gcn5 Y364 and N407, which are predicted by structural studies to be important for acetyl-lysine binding, might display a similar loss in H3K18ac (42, 92, 143, 157). Furthermore, the interaction of the Gcn5 bromodomain with acetyl-lysine residues has been suggested to underlie cooperative acetylation of Gcn5 complexes on nucleosomal substrates (145). Our

results confirm the bromodomain regulates overall histone H3 acetylation and is required for site specific acetylation events by Gcn5.

The Gcn5 bromodomain clearly altered the order of acetylation on H3K18, H3K27, and H3K36. Acetylation events on H3K14, H3K23 and possibly H3K9 are likely dominated by residues surrounding the HAT domain of Gcn5, while downstream acetylation events of H3K18, H3K27, and H3K36 may be aided by bromodomain interaction with previously deposited acetyl-lysine modifications. Our data and several previous studies have confirmed that H3K14 is the preferred lysine acetylated by Gcn5 and H3K14ac has also been shown to be a binding target of the Gcn5 bromodomain (79-81, 113). Our data showed that Gcn5-catalyzed H3K18ac was greatly diminished under conditions when the Gcn5 bromodomain was impaired in addition to HAT reactions using the H3K14R substrate when H3K14 could not be acetylated. Therefore, we propose a reader/writer model where Gcn5 catalyzes H3K14ac where the Gcn5 bromodomain in turn binds to the H3K14ac mark thus stimulating Gcn5 HAT activity on H3K18 **(Figure 31B)**. While, our study provides valuable insight into the interplay between the Gcn5 HAT and bromodomain in terms of histone H3 site specificity, several mechanistic questions still remain. Whether the Gcn5-mediated crosstalk of H3K14ac and H3K18ac occurs on the same histone H3 protein or on whether the bromodomain might engage H3K14ac on one histone tail and promote H3K18ac on a different histone H3 molecule is currently unknown. Additionally, while H3K23ac did not appear to affect K18ac levels, further testing would be useful as to how downstream acetylation events other than K18ac, such as H3K27ac and H3K36ac might be affected when H3K23 or H3K14 are unable to be acetylated as in our H3 K to R mutant HAT assays.

Figure 31

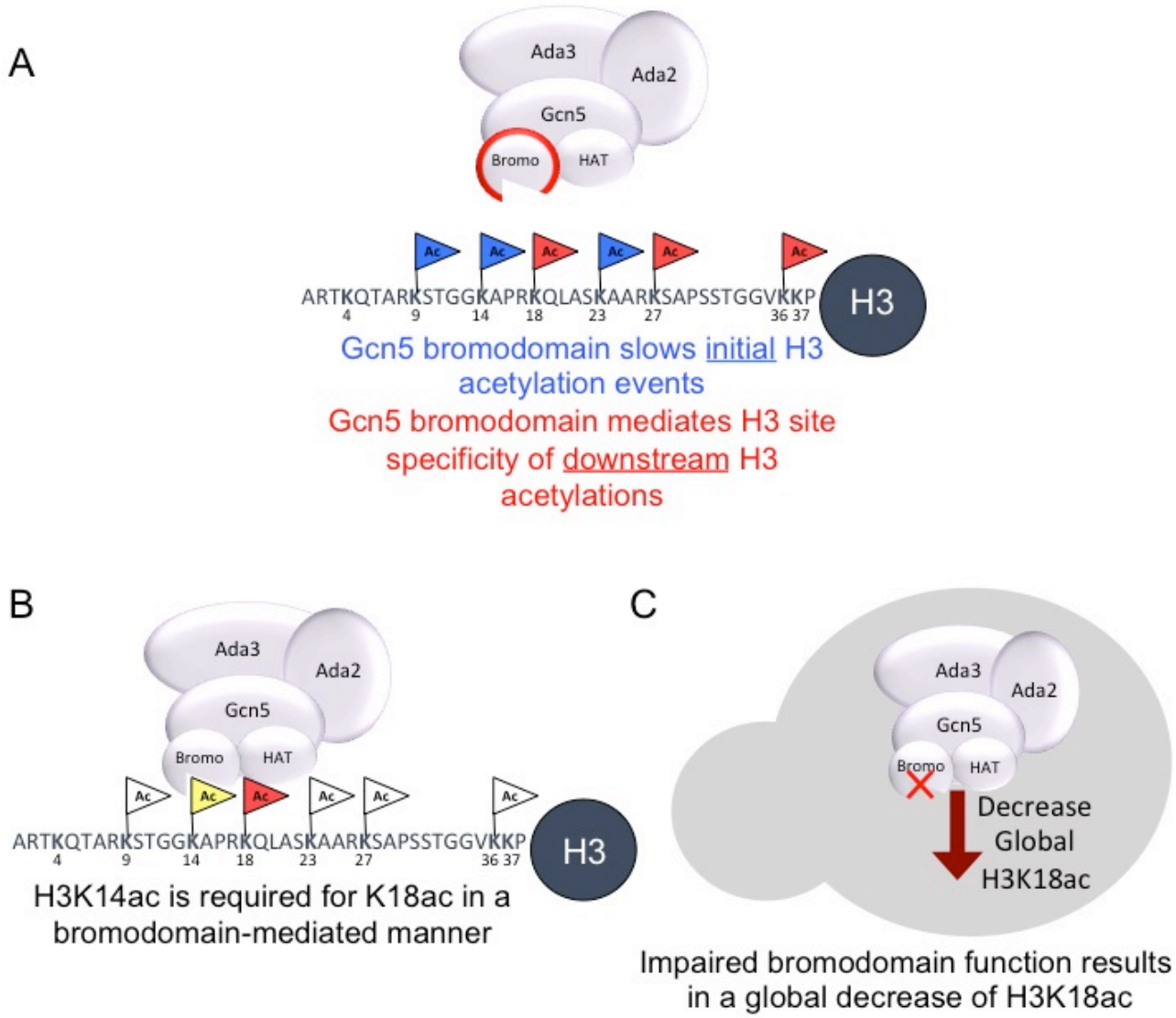


Figure 31. The Gcn5 bromodomain regulates catalytic histone H3 HAT activity in regard to site specific acetylation. Schematic showing (A) the Gcn5 bromodomain acts to slow initial histone H3 acetylation, while it regulates the site specificity of downstream acetylation events, especially on H3K18. (B) The acetylation of H3K14 is necessary for proper Gcn5 acetylation of H3K18. (C) Global histone H3K18ac is decreased when Gcn5 bromodomain acetyl-lysine binding function is ablated.

While ablation of Gcn5 bromodomain acetyl-lysine binding function was shown to have a profound affect on histone H3 acetylation, Gcn5 acetylates many non-histone substrates that are involved in several biological pathways. Gcn5 acetylation of the transcriptional co-activator PGC-1α, has been shown to prevent hepatic gluconeogenesis **(Figure 32A)** (76, 77)**.** Gcn5 HAT activity is necessary for cell cycle progression as acetylation of CDC-6 at three lysine residues ensures proper S-phase progression (158). Understanding if and how the Gcn5 bromodomain might affect catalytic HAT activity on these and other non-histone substrates could provide insight on Gcn5 function in endogenous molecular pathways.

3.4B The function and deposition of H3K18 acetylation *in vivo*

Our findings suggested interplay between the Gcn5 bromodomain reader function and its catalytic writer HAT function that showed to have the greatest affect on histone H3K18 acetylation. Crosstalk among histone PTMs such as acetylation, methylation and phosphorylation is gaining wide appreciation as a regulatory mechanism to foster or prevent interactions among chromatin effector complexes and substrates. It has been shown that histone H3 phosphorylation of threonine 11 has been shown to reduce the ability of Gc5n to acetylate H3K14 (159). The yeast histone acetyltransferase complex, NuA3, has been shown to bind H3K4 trimethylation via its PHD finger of its Yng1 subunit and then promotes the acetylation of H3K14 via its HAT subunit, Sas3 (34, 35, 37). Similarly, Gcn5-containing complexes, such as ADA and SAGA, are also able to engage H3K4me3 through the subunit Sgf29 that contains a tandem tudor domain (88, 160). However, the impact of how H3K4me3 binding affects Gcn5 HAT site specificity is described in further detail in Appendix section A.2.

Figure 32

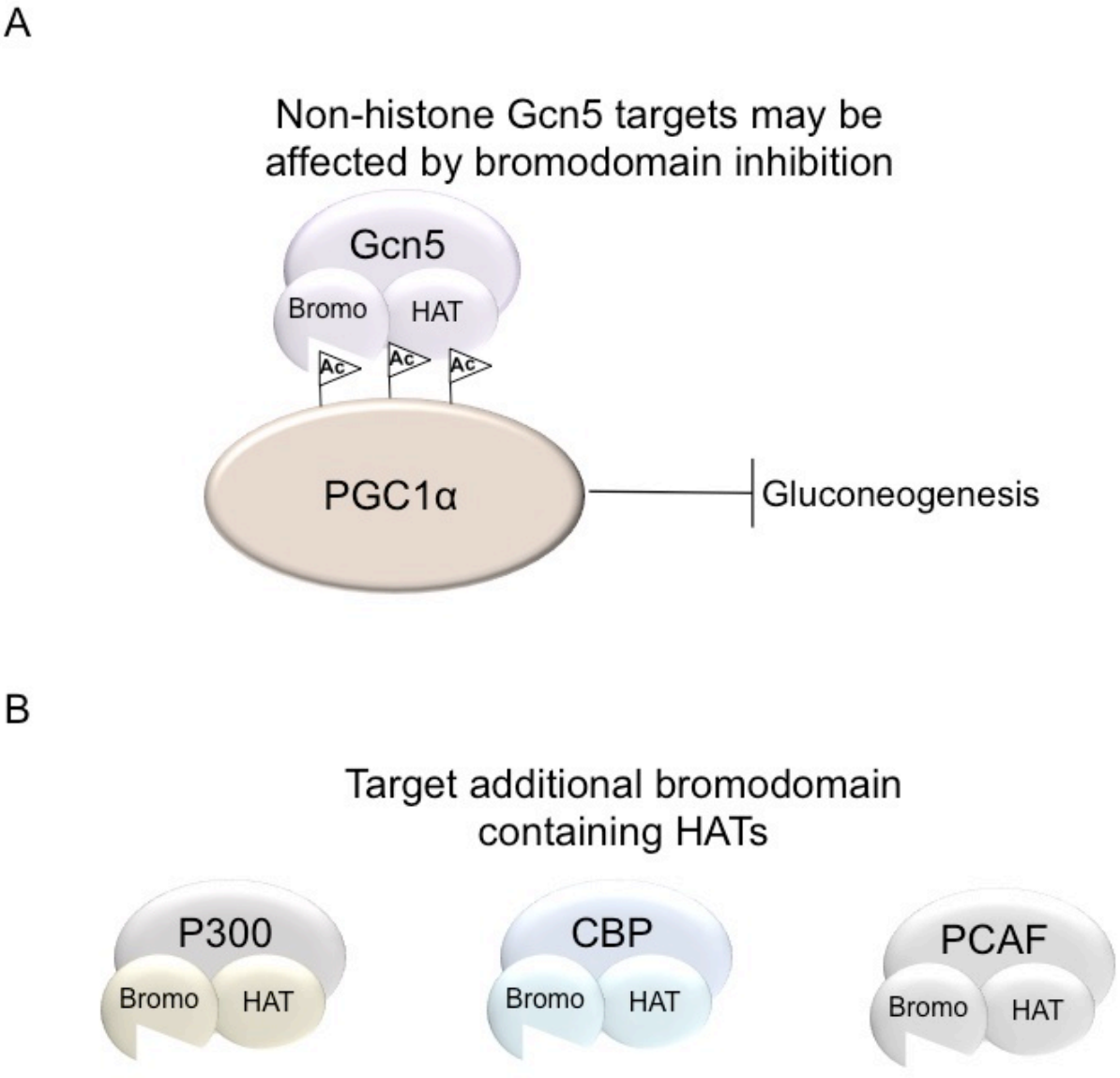


Figure 32. Future directions of research. (A) How the bromodomain of Gcn5 affects acetylation of non-histone Gcn5 targets, such as PGC1α, could have implications on cellular processes such as gluconeogenesis. (B) Studying how the bromodomains of additional HATs, such as P300, CBP, and PCAF affect catalytic HAT activity could further elucidate reader and writer interplay and potentially uncover these bromodomains as drug targets for modulating the activity of HATs.

In the context of prior *in vivo* studies using bromodomain mutations, our work suggests the existence of endogenous pathways that may target the acetyl-lysine binding capacity of the bromodomain as a means to affect and regulate catalytic HAT activity, especially that of H3K18. Relevant to our findings that K18ac acetylation levels decrease upon disruption of Gcn5 bromodomain both *in vitro* and *in vivo*, it has been previously reported that deletion of Gcn5 completely abolishes global H3K18 acetylation in yeast strains (160) **(Figure 31C)**. Levels of histone H3K14ac and H3K9ac were unaffected when Gcn5 was deleted, which could be the result of these lysine residues being redundant targets by multiple HAT enzymes such as NuA3 (160).

However, regulation of H3K18ac in the context of mammals and humans may be much more complicated than in yeast as multiple mammalian HATs have been reported to acetylate H3K18, including Gcn5, PCAF, P300, and CBP (90, 91, 161, 162). One study examined the impact of HAT deletions on histone H3 lysine specific acetylation and found that deletion of GCN5/PCAF paralogues resulted in decreased H3K9ac, while deletion of P300/CBP decreased global levels of H3K27ac and H3K18ac (91). However, additional studies report a stronger correlation of Gcn5/PCAF with H3K18ac in mammals. A recent report showed H3K18ac levels were regulated by PCAF at distinct inflammatory genes in renal cells (90). Furthermore, another study linked histone H3 lysine acetylation levels with the subunit Sgf29, which acts within the same HAT module as the Gcn5/PCAF, Ada2, and Ada3 subcomplex (160). Interestingly, when Sgf29 is knocked out in yeast or knocked down in human cell lines, the effects on global H3 acetylation levels of individual lysine residues very closely resembles the patterns we observed with our Gcn5 Y413A bromodomain mutation (160). Specifically, when Sgf29

as a whole or when either of the tandem tudor domains was deleted, H3K18ac levels were significantly decreased on a global level (160). Because the tandem tudor domain of Sgf29 and the bromodomain of Gcn5 are found within many of the same complexes, perhaps they have a dual function that contributes to complex avidity in anchoring to chromatin and affecting enzymatic HAT activity. Determining which complex is responsible for K18ac could vary in a manner specific to cell-type, pathway, or gene, which warrants greater understanding into the site specificity of mammalian HATs. Interestingly, Gcn5, PCAF, P300, and CBP all contain bromodomains in addition to their HAT domains. It would be interesting to see if bromodomain acetyl-lysine binding ablation of these additional HATs would have an impact on catalytic activity in a manner similar to that which we observed with Gcn5 **(Figure 32B)**.

3.4C The Gcn5 bromodomain as a potential therapeutic target

Because Gcn5 has been implicated in several disease states such as diabetes, spinocerebellar ataxia, and cancer, it is therefore poised as potential therapeutic target (60, 62, 76, 77, 105). However, the enzymatic HAT domain of Gcn5 participates in widespread acetylation and general transcription of nearly the entire transcribed genome and thereby inhibiting Gcn5 catalytic function would be extremely detrimental to the cell (64, 85, 100). This is made evident as deletion of the Gcn5 HAT domain resulted in embryonic lethality (64, 100). Thereby, the Gcn5 HAT domain would present itself as a poor drug target due to the very fundamental roles it plays in the cell **(Figure 33A)**. However, with the success of recent bromodomain inhibitors, such as JQ1 and I-BET that inhibit the BET bromodomains, the Gcn5 bromodomain could hold promise as a drug target that could be exploited by small molecule inhibition. Our study showed that

Gcn5 bromodomain disruption did not lead to a widespread acetylation decrease in all Gcn5 lysine targets, as would be the case with Gcn5 HAT inhibition, but instead only a single lysine, H3K18ac, was significantly decreased **(Figure 33B)**. Therefore inhibiting the Gcn5 bromodomain as opposed to the HAT domain would provide a more targeted decrease in histone H3 acetylation, and would be expected to have less detrimental affects than abolishing global widespread Gcn5 acetylation.

When considering small molecule inhibitors that might block the bromodomain, it would be likely that an inhibitor against the Gcn5 bromodomain could work to also inhibit the highly homologous bromodomain of the Gcn5 paralogue, PCAF. The bromodomains of PCAF and Gcn5 share 95% similarity, and while Gcn5 is conserved from yeast through humans, the PCAF gene generated from a duplication and divergence of the GCN5 gene (47, 89, 99). PCAF also has implications in disease and has been linked with H3K18ac in a recent study. This group found that K18ac linked PCAF with the regulation of inflammation in renal disease (90).

Figure 33

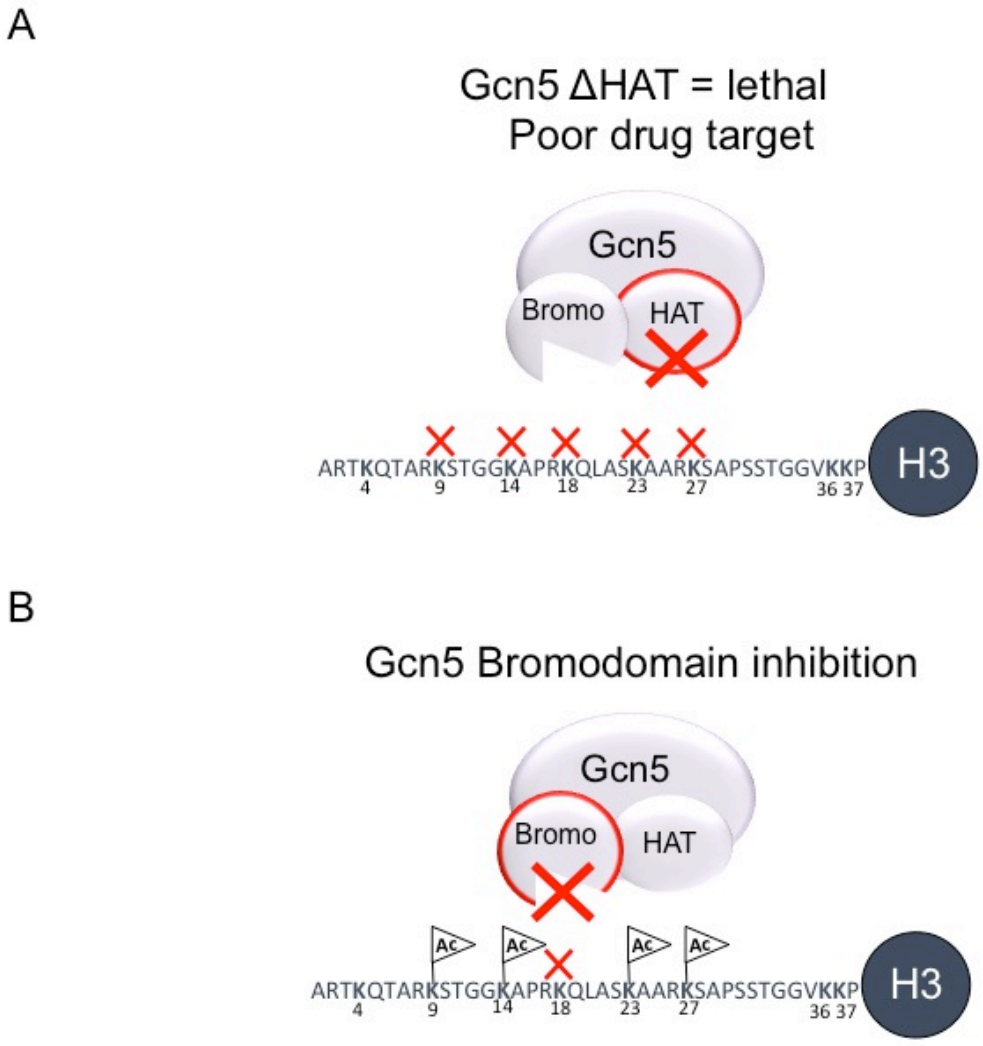


Figure 33. The Gcn5 bromodomain is a potential therapeutic target. (A) The Gcn5 HAT domain is a poor therapeutic target for small molecule inhibition because the HAT domain is involved in widespread acetylation and gene transcription. This is made evident as deletion of the Gcn5 HAT domain results in embryonic lethality. (B) Because our findings indicate that disabling the Gcn5 bromodomain results in the decrease of only a single acetylation (H3K18), this could be a more attractive Gcn5 drug target compared with the HAT domain.

Chapter 4: Tools for studying *in vitro* activity of histone modifying enzymes

4.1 Introduction

Exploiting certain eukaryotic model organisms has proved extremely useful for elucidating basic biological ramifications of epigenetic phenomena, like the fundamental roles of histone modifications and chromatin interacting proteins. For example, studying the chromatin of the single-celled prokaryote *Tetrahymena thermophila*, which has both an actively transcribed euchromatic nucleus and a heterochromatic nucleus, led to the discovery of Gcn5 as a transcriptional activator and novel histone modifications, such as H3K23 trimethylation (57, 163). Furthermore, genetic manipulation and mutation analysis in *S. cerevisiae* and *S. pombe* have provided unprecedented insight into the biological contributions of individual histone residues and subunit architecture of effector protein complexes (164, 165). However, *in vitro* characterization of histone binding protein complexes lends valuable insight into chromatin interactions, enzymatic activity, genome localization and pathway regulation. Advances in technology such as improved sensitivity in proteomics and DNA sequencing have greatly increased our understanding of epigenetic mechanisms as well.

While the past 20 years have seen hallmark studies and technological advancements in the field of epigenetics, developing biologically relevant *in vitro* assays still presents difficulties in terms of studying combinatorial PTM cross talk using higher-

ordered substrates. Epigenetic *in vitro* assays that contain both higher-ordered enzymes, such as protein complexes, and substrates, such as the nucleosome core particle, will provide a more accurate understanding of the processes occurring *in vivo* at the level of chromatin. Finally, sensitive and quantitative means of measuring the gain or loss of histone modifications has been achieved utilizing site-specific antibodies and quantitative proteomics, yet this becomes complicated as many chromatin writer and eraser enzymes target multiple sites on a single histone molecule (113, 133, 165). Therefore, continued improvement of *in vitro* assays and tools for PTM detection are necessary for further characterization of chromatin interacting enzymes and readers.

Studying how the activity and interactions of effector protein complexes are affected in the presence of a certain PTM requires specialized substrates that are specifically modified at a desired residue. Here we describe a method that employs native chemical ligation to generate recombinant histone H3 that contains customized PTMs patterns. This strategy utilizes solid phase peptide synthesis and recombinant histone expression to generate synthetic substrates that can be modified at any given residue of interest without containing heterogeneous modifications on adjacent residues. These substrates can be further reconstituted into higher ordered nucleosome core particles to be tested in biologically relevant assays.

4.2 Methods

4.2A Peptide synthesis

Peptides for native chemical ligation chemistry were synthesized using standard Fmoc-solid phase peptide chemistry on a Prelude Peptide Synthesizer (Protein

Technologies). However, N-terminal alanine (position 1) was Boc-Ala. Dawson TGR Resin (NovaBioChem) for native chemical ligation was used on a 0.1 mmole scale with 6 equivalents of amino acids (3mLs each 2mM amino acid) for a 0.6 mmole amino acid coupling. A 1:1 ratio of [HATU]:[amino acid] was used. Purified peptide was lyophilized and its mass was confirmed with an Applied Biosystems Voyager DE-STR MALDI-TOF mass spectrometer (Life Technologies). Peptide sequences are as follows:

S. cerevisiae histone H3 1-31

Boc-ARTKQTARKSTGGKAPRKQLASKAARKSAPS-resin

S. cerevisiae histone H3K4me3 1-31

Boc-ARTK(me3)QTARKSTGGKAPRKQLASKAARKSAPS-resin

4.2B Generation of Nbz C-terminal group

Resin was washed thoroughly with DCM 3 times for 5 mins. 0.5 mmole of p-nitrochloroformate (Sigma) in 10 mLs DCM was added to resin which rotated at room temp for 60 mins. Resin was thoroughly washed with DCM. 0.5M DIPEA (Sigma) in 10 mLs of DMF (turns bright yellow upon addition) and rotated at room temp for 30 mins. Resin was washed thoroughly with DMF followed by a thorough washing with DCM. Resin was cleaved with TFA:H2O:TIS (95:2.5:2.5) for three hours. Peptide was precipitated with diethyl ether, dried and stored at -80 degrees C for ligation reaction.

4.2C Cloning of truncated histone H3 for native chemical ligation

A pET3a plasmid containing *S. cerevisiae* histone H3 was obtained from Dr. Gregory Bowman, The Johns Hopkins University. Plasmid was transformed in DH5a

cells and minipreps were performed to concentrate plasmid. Nested PCR was used due to the extensive addition of restriction sites, a Factor Xa protease site and an N-terminal cysteine at position 32 of histone H3. Primers are as follows:

Forward Primer 1

5’ GAAGGTCGTTGTGGTGGTGTTAAGAAG 3’

Forward Primer 2

5’ CGCGGATCCATCGAAGGTCGTTGTGGT 3’

Reverse Primer

5’ GGAGCTCGAGCTATGATCTTTCACCTCTTAA 3’

The first round of PCR was performed with Forward Primer 1 and Reverse Primer. The final product was run, excised, and purified from agarose gel. This was used as template for PCR round two with Forward Primer 2 and the Reverse primer using the product from Round one as the template. Final product insert was gel purified and digested with BamHI and XhoI. In addition a pET28 vector obtained from the lab of Dr. Gregory Bowman and digested with BamHI and XhoI and gel purified. Ligation of PCR product insert and pET28 was confirmed by sequencing.

4.2D Expression and purification of truncated histone H3 for native chemical ligation from inclusion bodies

Native chemical ligation truncated histone H3 construct was transformed into BL21 cells and plated for fresh colonies on a Kanamycin plates. An over night culture was grown and added to LB media and at 37°C grown until OD=0.7. Bacteria were

induced with 200uM IPTG and grown over night at 20°C. Bacteria were spun down at 4°C at 8000 rpm for 10 minutes. Inclusion pellet was resuspended in Wash Buffer (50mM Tris-Cl pH = 7.8; 100 mM NaCl; 1 mM EDTA). Bacteria were subject to freeze thaw cycle and 5mM Beta-mercaptoethanol and 0.2mM PMSF were added. Cells were sonicated at 50% power and 50% duty cycle for 30 seconds and centrifuged at 4°C for 20 mins at 23,000xg. Pellet was resuspended and spun down in Triton Wash Buffer (Wash Buffer + 1% Triton) 3 times. The inclusion pellet can be frozen at -20°C.

Inclusion pellet is smeared alongside 50 mL conical and 1.5 mL DMSO is added and agitated with pellet for 30 mins. 40 mL of Extraction Buffer (7M guanidine-HCl; 20 mM Tris pH=7.8; 10 mM B-Merc) is added to the pellet and DMSO ad let to agitate at room temp for 3 hours. Suspension was spun down at 23,000xg for 20 mins and Nickel agarose beads were added to supernatant and let agitate over night at 4°C. Beads and protein were washed with Extraction Buffer over a gravity column and eluted with 200 mM imidiazole in Extraction Buffer. Elutions were dialyzed in H2O and 10 mM B-merc so that protein crashed out. Protein was spun down and aliquotted. 2 mg lots were purified over HPLC using a C8 column, and pure fractions were lyophilized.

4.2E Factor Xa cleavage and purification of truncated histone H3 product for native chemical ligation

Lyophilized aliquots were resuspended in Factor Xa Buffer (1mM CaCl2; 50 mM NaCl; 20 mM Tris; 5 mM B-merc) and 0.5 ug Factor Xa was added and allowed to react at room temperature for 30 mins. Reactions were quenched by adding 8M urea.

Reaction products were separated over HPLC and run on SDS PAGE to determine fractions containing the desired singly cleaved product.

4.2F Native chemical ligation

Purified histone H3 tail peptide with Nbz and truncated histone H3 with N-terminal cysteine at postion 32 were combined using an excess of peptide to protein. Both peptide and protein were dissolved in Ligation Buffer (0.2 M phosphate buffer pH=7; 0.1M 4-mercaptophenyl acetic acid (Sigma); 6M guanidine; 0.02 TCEP) and combined. Reaction was pH'd to 7.0 using NaOH and allowed to react overnight for 18 hours at room temp. Reaction products were separated over HPLC and fractions were run over SDS PAGE to determine ligated product.

4.2G Octamer reconstitution

Histone H3 generated from native chemical ligation and recombinant *S. cerevisiae* histone H4, H2A, and H2B were reconstituted into histone octamers as described previously (150, 152). Recombinant histones H4, H2A, and H2B were provided by the lab of Dr. Gregory Bowman (The Johns Hopkins University, Baltimore, MD).

4.3 Results

4.3A Peptide synthesis for native chemical ligation

The histone code hypothesis suggests that histone PTMs and certain combinations of PTMs recruit effector proteins and protein complexes that can elicit downstream biological events such as replication, transcription and DNA repair(27, 28).

Figure 34

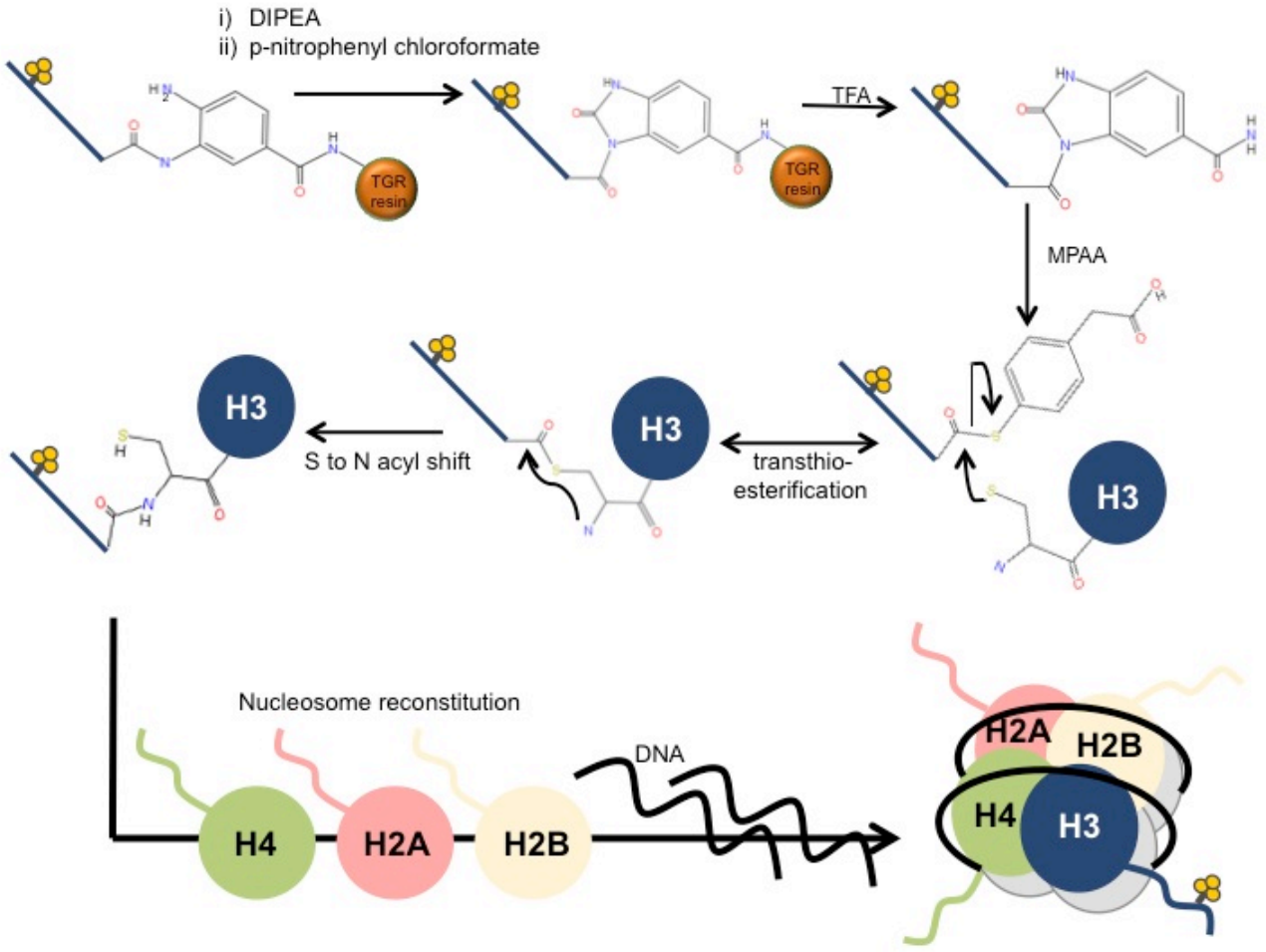


Figure 34. Stepwise schematic for designer nucleosomes. Histone H3 tail is synthesized via Fmoc solid phase synthesis using a TGR resin which results in a histone H3 tail peptide with a C-terminal Nbz group. This product is reacted with a recombinantly expressed truncated histone H3 molecule containing an N-terminal cysteine residue which reacts with the histone tail peptide via transthiosterification. After undergoing an S to N acyl shift, a scarless histone H3 molecule results. This synthesized histone H3 can be reconstituted with histone H4, H2A, and H2B into octamers which can be combined with DNA to generate recombinant nucleosomes.

Developing *in vitro* assays that test how catalytic activity of chromatin effector enzymes is affected in the presence of certain PTMs would help to elucidate molecular mechanisms that may contribute to the histone code hypothesis. However, studying epigenetic processes *in vitro* such as PTM cross talk, how the presence of one modification affects the presence or absence of subsequent modification, requires intricate histone substrates that are modified at a single site and unmodified at a secondary site of interest. Moreover, ensuring that histone substrates are properly reconstituted into the biologically relevant nucleosome core particle is critical when studying substrate specificity of histone modifying enzymes.

To generate specifically modified histone substrates we sought to use native chemical ligation to link a synthesized histone H3 tail peptide to a truncated histone core protein and further reconstitute these modified histones into nucleosomes **(Figure 34)**. This technique has been previously employed to generate designer, wild-type *Xenopus laevis* histone H3 utilizing a truncated histone H3 protein with a reactive N-terminal cysteine. We adapted this technique to generate unmodified and H3K4me3 yeast histone H3 utilizing a TGR resin that results in a C-terminal Nbz chemical group on the histone H3 tail peptide (166). This Nbz-group is susceptible to transthioesterification via the reactive cysteine on the truncated histone H3 and will result in a scarless histone H3 final product with single cysteine mutation. We can then further fold modified histone H3 into nucleosomes to use as biologically relevant substrates in *in vitro* reactions.

We chose the ligation site to occur between residue 31 and 32 of *S. cerevisiae* histone H3 as previously reported (166). This site was chosen because mutatation of threonine 32 of histone H3 to a cysteine was compatible with ligation chemistry and

Figure 35

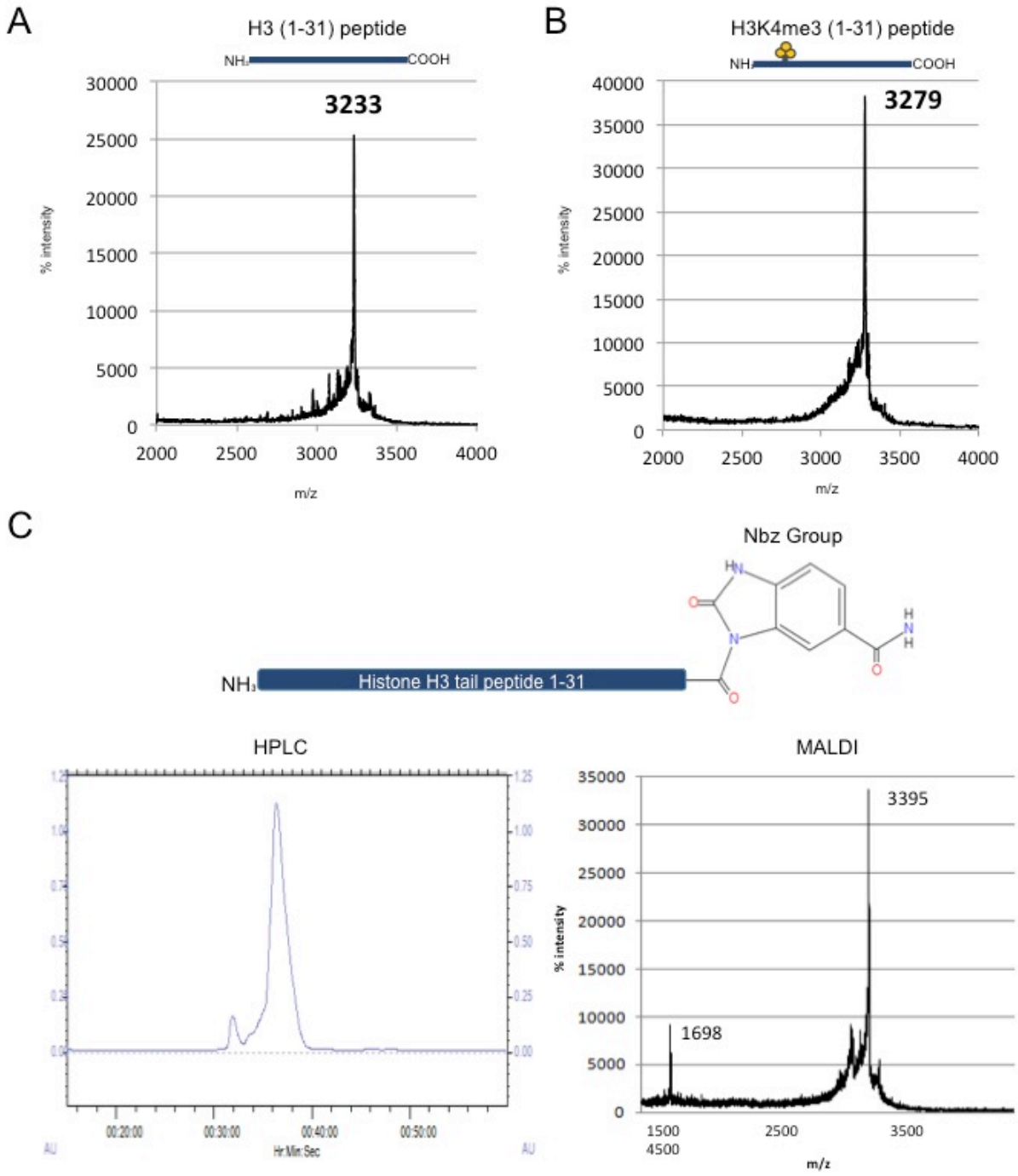


Figure 35. Histone H3 tail peptide synthesis with C-terminal Nbz group. (A) MALDI-TOF mass spectrum of unmodified histone H3 peptide residues 1-31. (B) MALDI-TOF mass spectrum of histone H3K4me3 modified tail peptide residues 1-31. (C) HPLC chromatogram and MALDI-TOF spectrum oh histone H3 unmodified tail peptide with C-terminal Nbz group.

Figure 36

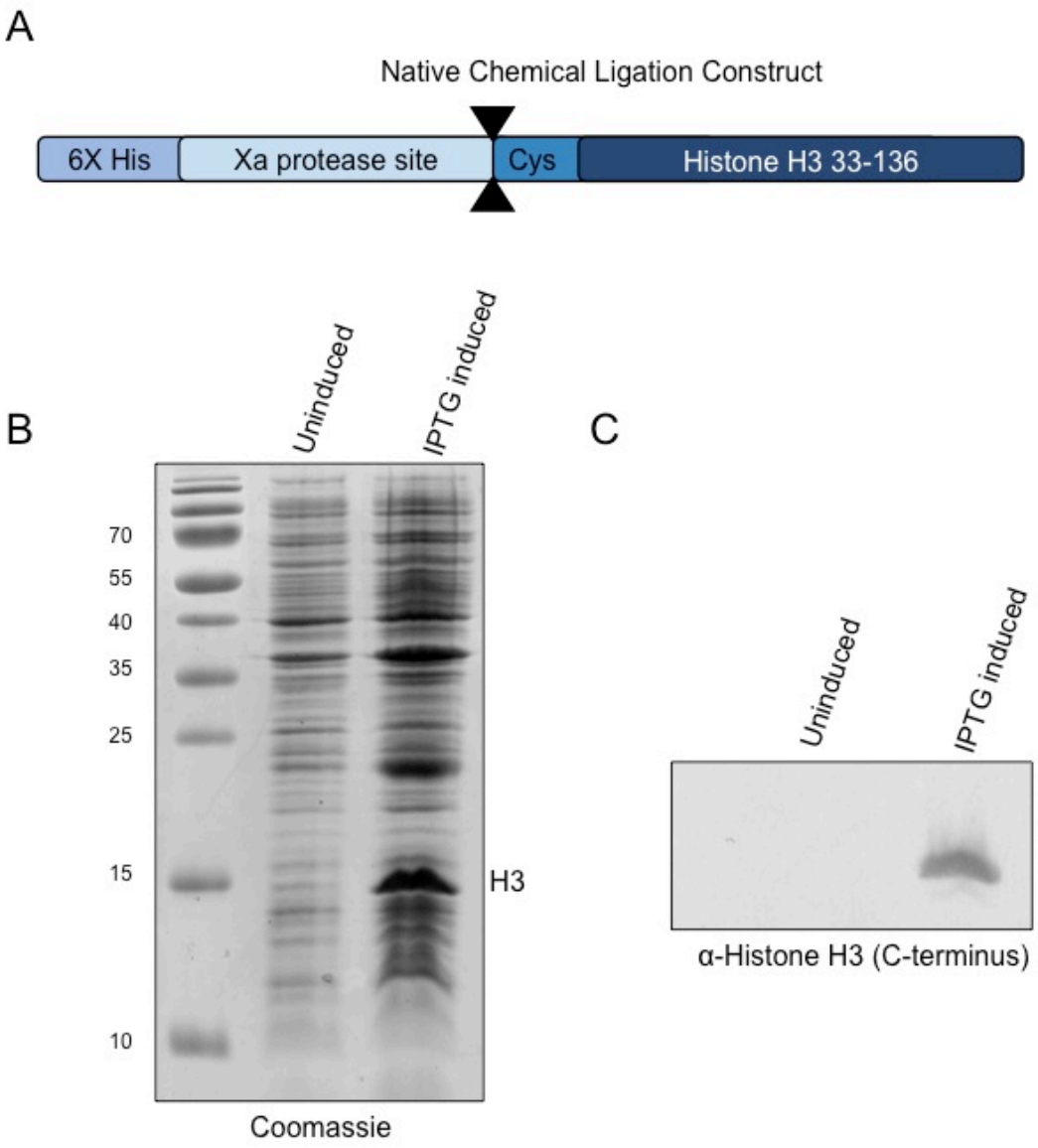


Figure 36. Recombinant expression of truncated histone H3 with N-terminal cysteine. (A) Construct of truncated histone H3 used for native chemical ligation which includes a 6X his tag, a Xa protease site, an N-terminal cysteine and the histone H3 sequence 33 – 136. (B) IPTG induction of *E. coli* transformed with the native chemical ligation truncated histone H3 construct. (C) Western blot using an antibody against the C-terminus of histone H3 to confirm expression of histone H3.

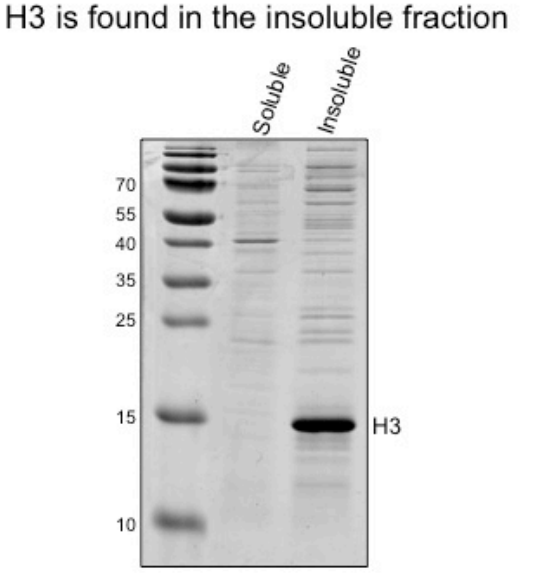
threonine 32 is not highly conserved among species. Additionally, synthesizing a 31-mer histone H3 tail peptide can be done with acceptable efficiency. Therefore, we generated both an unmodified and H3K4me3 H3 tail peptide residues 1-31 of the *S. cer* sequence using a TGR resin. Peptides were synthesized via Fmoc slid phase synthesis, purified over HPLC, and peptide synthesis was confirmed utilizing MALDI-TOF **(Figures 35A, 35B)**. Formation of the Nbz group was performed on the resin and the peptide and C-terminal Nbz group were cleaved and purified over HPLC and subjected to MALDI-TOF analysis to ensure purity and the correct size of the Nbz C-terminal histone H3 tail peptide **(Figure 35C)**.

4.3B Generation of a truncated histone H3 for native chemical ligation

A construct for a truncated histone H3 was cloned to contain *S. cerevisiae* histone H3 residues 33-136 and a cysteine in position 32 in place of the native threonine **(Figure 36A)**. Additionally an N-terminal 6X-his tag was added for purification and a Factor Xa protease cleavage site was used to remove the tag and leave a free N-terminal cysteine necessary to carry out the native chemical ligation reaction. This construct was transformed into *E. coli* and induced using IPTG and expression was confirmed using a western blot against the C-terminus of histone H3 **(Figures 36B, 36C).** The protein was further purified from insoluble inclusion bodies and bound, washed, and eluted from Nickel agarose beads to obtain a pure product **(Figures 37A, 37B)**. After purification, the N-terminal tag was cleaved using Factor Xa protease to leave an N-terminal cysteine residues necessary for the reaction with the Nbz peptide. The Factor Xa protease also promiscuously cleaves at an off-target site on the C-terminal region of histone H3 **(Figure 38A)** (166). Therefore uncleaved, singly cleaved,

Figure 37

A



B

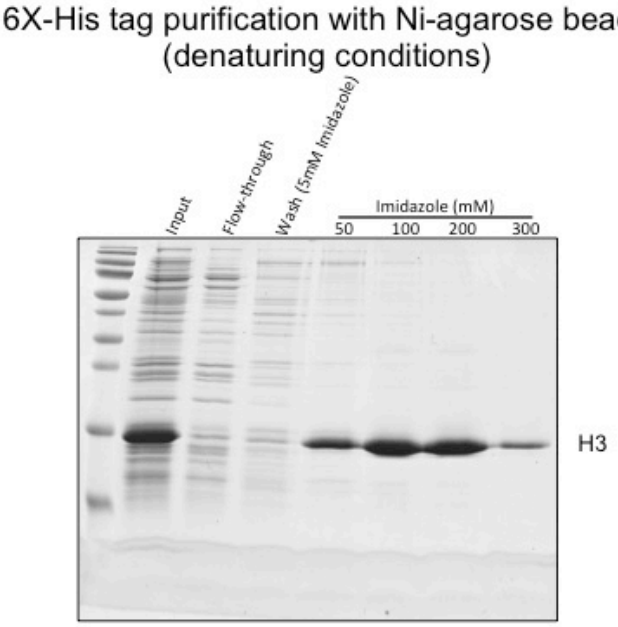


Figure 37. Purification of truncated histone H3 with N-terminal cysteine. (A) Truncated histone H3 for native chemical ligation was purified from processed inclusion bodies in *E. coli.* (B) Further purification of truncated histone H3 over nickel beads was performed. Final product is >95% pure as determined by Coomassie staining of the gel.

Figure 38

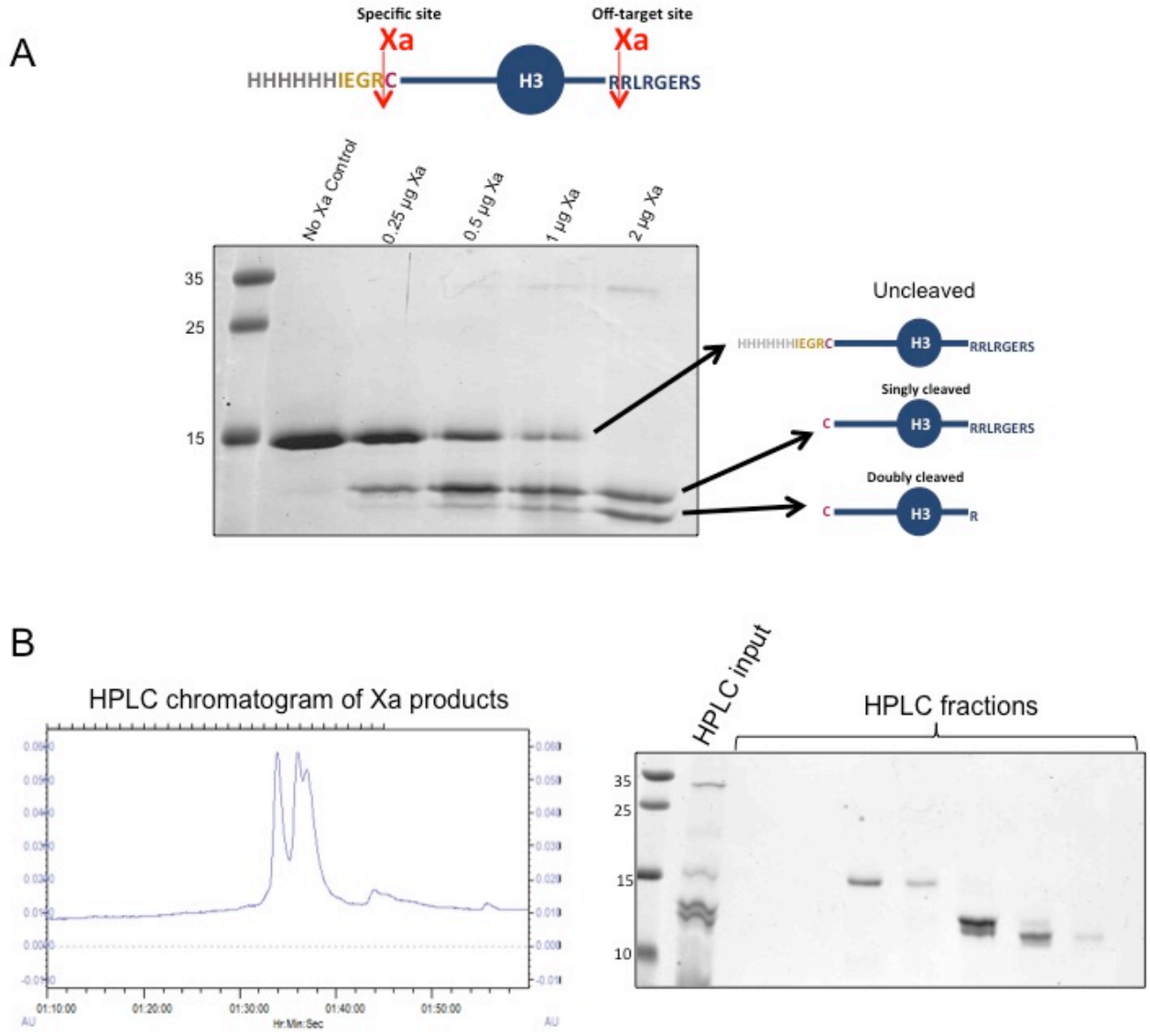


Figure 38. Xa cleavage and purification to generate N-terminal cysteine on histone H3 residues 33 – 136. (A) Xa cleavage of truncated histone H3 using increasing concentrations of Xa enzyme. A second off target cleavage site results in an undesireable, doubly cleaved product. (B) Cleaved products were separated over HPLC and run on a gel to determine which fractions (middle peak) contained the cleaved H3 product necessary for native chemical ligation.

and doubly cleaved histone H3 products were separated over HPLC and the fractions were confirmed over an SDS PAGE gel, so that the desired singly cleaved product could be isolated **(Figures 38B)**.

4.3C Histone H3 native chemical ligation and histone octamer reconstitution

The histone H3 tail peptide residues 1-31 and the truncated histone H3 protein residues 33-136 with a cysteine residue at position 32 were subjected to a small-scale native chemical ligation reaction **(Figure 39A)**. We observed a shift in size from a 12 kDa truncated protein to a 15 kDa ligated histone H3 product indicating the reaction had proceeded to a fully ligated histone H3 protein. We performed the native chemical ligation of histone H3 on a larger scale where the reaction was separated over HPLC and after fractions were run on an SDS-PAGE gel, we concluded that the first HPLC peak contained our ligated histone H3 product **(Figures 39B, 39C)**. Finally, using recombinantly expressed histone H4, H2A, and H2B obtained from the lab of Dr. Gregory Bowman (The Johns Hopkins University, Baltimore, MD), we reconstituted recombinant *S. cerevisiae* histone octamers **(Figures 40A, 40B).**

4.4 Discussion

Designing *in vitro* assays that provide a biologically relevant depiction of what is occurring *in vivo* is critical when understanding the fundamental processes of epigenetic mechanisms. Overcoming challenges associated with in *vitro* assays such as working with enzymatic subcomplexes or complexes, using nucleosomal templates as

Figure 39

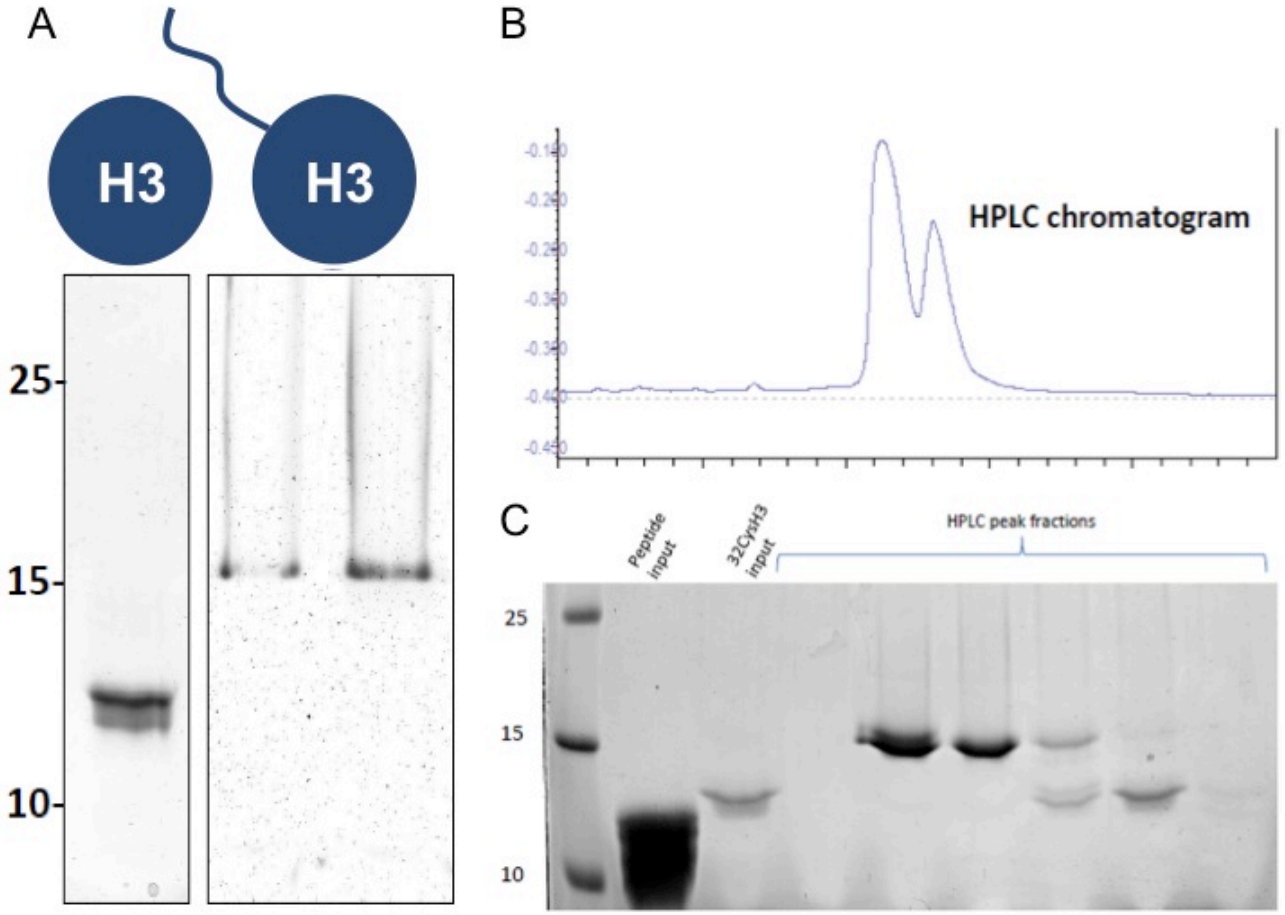


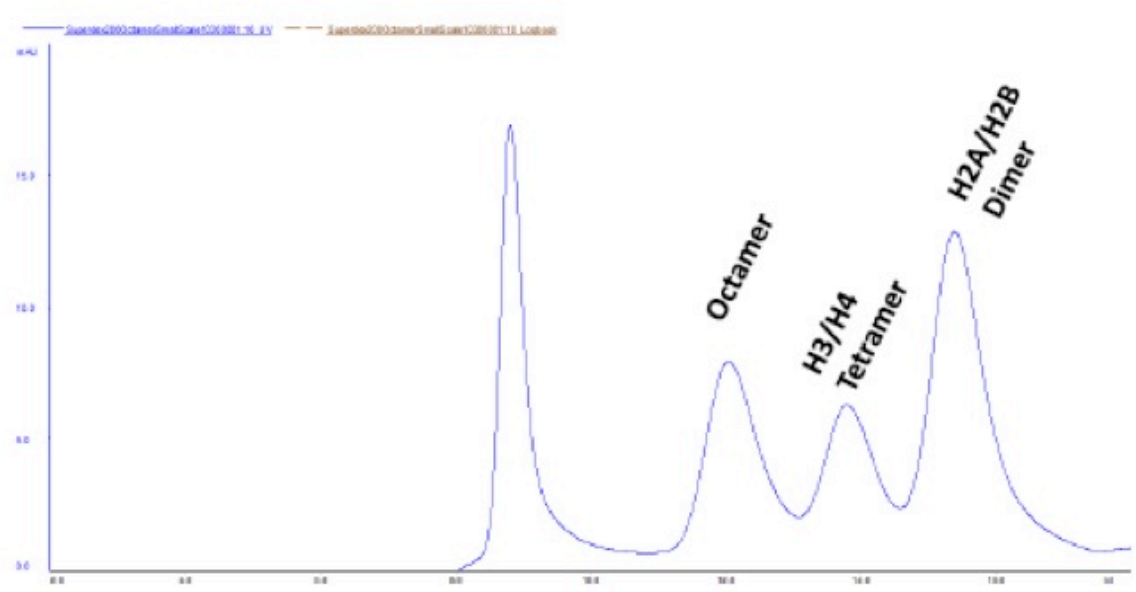
Figure 39. Native chemical ligation of histone H3. (A) Small scale reaction showing Coomassie stained gel of pre and post native chemical ligation reaction with histone tail peptide and truncated histone protein. (B) Products of large scale native chemical ligation reaction was separated over HPLC. (C) HPLC fractions were resolved on an SDS PAGE gel.

substrates, measuring enzymatic activity on substrates with multiple target sites, and studying PTM cross-talk would provide a more accurate representation of chromatin regulation. Using native chemical ligation we generated specifically modified histone substrates, which were further reconstituted into histone octamers. Because these histones were generated using solid phase peptide synthesis and recombinant protein expression we can obtain a homogenously modified histone H3 substrate (166). This provides an advantage over histone substrates generated from enzymatic reactions or purified from lysates as they can contain numerous modifications.

Because the Gcn5 subcomplex also interacts with the tandem Tudor domain containing protein Sgf29, reported to bind H3K4me3, we aimed to generate a ligated histone H3 that contains the H3K4me3 modification (88, 167). Furthermore, this technique can be used to study at acetyl-lysine and bromodomain mediated HAT activity of Gcn5 by generating histones that are differentially pre-acetylated at various lysine residues and measuring how Gcn5 HAT activity is altered upon these specific acetyl marks. How these modifications and others that have been predicted to be involved with histone H3 acetylation cross talk, such as histone H3 serine 10 phosphorylation (H3S10ph) affect catalytic HAT activity is an area of active investigation (159, 168).

Figure 40

A



B

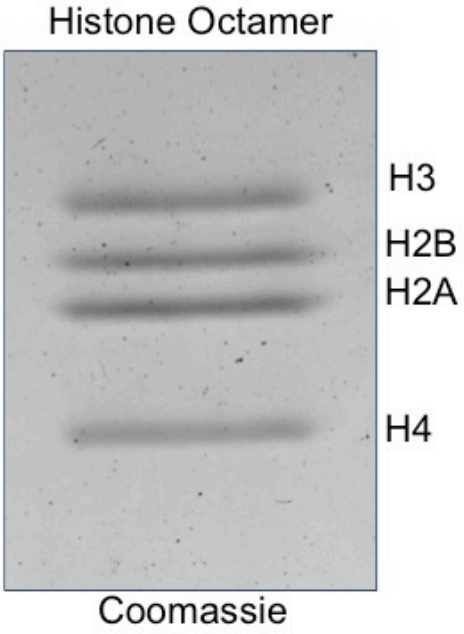


Figure 40. Recombinant histone octamer reconstitution. (A) Recombinant *S. cerevisiae* nucleosomes were reconstituted and separated over FPLC size exclusion. (B) Histone octamers were resolved on SDS-PAGE to ensure histone composition.

Appendix

A.1 Recombinant expression of Gcn5 HAT module subunits

When studying *in vitro* enzymatic activity of histone writer and eraser enzymes, it is critical to ensure biologically relevant enzymes and substrates are being utilized. This can prove difficult when studying histone-modifying enzymes as they are often found in large, multisubunit protein complexes, such as SAGA, CoREST, and Polycomb Repressive Complex or exist as very large proteins with multiple domains, such as P300 (85, 92, 169, 170). When studying enzymatic activity of chromatin proteins, taking the associated reader motifs and DNA binding domains into account is extremely critical as these could impact substrate specificity and kinetic parameters. For example, monomeric Gcn5 is unable to acetylate nucleosomes, its biological substrate, *in* vitro (78, 96, 116). However, when Gcn5 is in the context of its binding partners, Ada2 and Ada3, which contain nucleosome and DNA binding domains, the nucleosome core particle is extensively acetylated by Gcn5 (79, 96, 116).

Recombinant subcomplexes and complexes can be expressed in tandem using a polycistronic vector containing genes of the desired complex or subcomplex that can be expressed in *E. coli*. A polycistronic vector containing the genes for *S. cerevisae* Gcn5, Ada2, and an N-terminal truncated Ada3 has been developed by the laboratory of Dr. Song Tan, in which they have optimized a protocol to express and purify the intact trimeric subcomplex (117). Because this trimeric subcomplex interacts with additional proteins in complexes such as SAGA and ADA, we speculated that the N-terminal region may be necessary for interactions with other subunits, so we generated a new

polycistronic clone that contained the full length Ada3 protein **(Figure 41A)**. Furthermore, additional complex members can be recombinantly expressed individually. The protein Sgf29, which contains a tandem Tudor domain capable of binding H3K4me3, has also been shown to interact with the Gcn5 trimeric subcomplex as it is found to recruit SAGA to promoters via its interaction with H3K4me3 (88, 160). *S. cerevisiae* Sgf29 was expressed with a cleavable MBP tag to improve solubility, and we were able to obtained pure, recombinantly expressed Sgf29 **(Figure 41B)**.

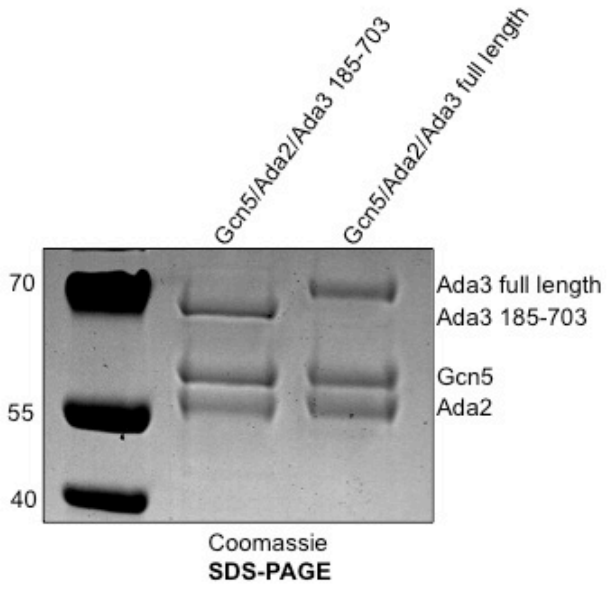
A.2 H3K4 trimethylation affects activity of the Gcn5 HAT module

Association of recombinantly expressed *S. cerevisiae* Sgf29 with the *S. cerevisiae* Gcn5 subcomplex proved to be difficult as Sgf29 would not interact with the subcomplex. However, when *S. pombe* Sgf29 and the Gcn5 subcomplex were recombinantly expressed, this resulted in a stable Gcn5/Ada2/Ada3/Sgf29 subcomplex termed the Gcn5 module. Because Sgf29 contains the H3K4me3-binding tandem tudor domain, the activity of the Gcn5 HAT module was compared on unmodified nucleosomal histone H3 and H3K4me3 nucleosomal histone H3 substrates. Overall histone H3 acetylation patterns and site specific acetylation was observed using acid-urea gel resolution and western blot analysis with general histone H3 and site specific antibodies **(Figure 42)**.

We observed that when the H3K4me3 mark was present, the rate and overall acetylation on histone H3 was enhanced by Gcn5 HAT module when observing western blots for total histone H3. Site specific antibodies revealed that H3K14ac occurred on the first acetylation state (band 1) in both the unmodified and H3K4me3 substrates,

Figure 41

A



B

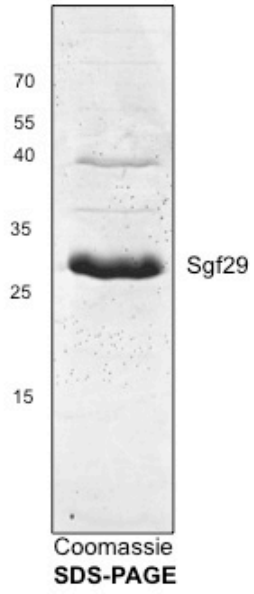


Figure 41. Recombinant expression of HAT module subunits. (A) Recombinantly expressed Gcn5/Ada2/Ada3 subcomplex from pST44-yAda3t2HISx3-yAda2x3-yGcn5x5 polycistronic vector containing yeast GCN5, ADA2, and ADA3 obtained courtesy of Dr. Song Tan (Penn State, PA), encoded a truncated Ada3 (with residues 185-703) compared to Gcn5/Ada2/Ada3 subcomplex cloned with the full-length Ada3 protein. (B) Recombinant expression of the protein Sgf29 from pET28 vector with cleavable MBP tag.

which was in agreement with our earlier findings and previous work **(Figure 42)** (111, 113). The site specificity of HAT module acetylation did not appear to be significantly altered between unmodified and H3K4me3 substrates since acetylation of specific residues (K14, K23, K18) occurred on the same acetylation state bands between both substrates. However, the rates among the specific acetylation events of H3K18 and H3K14 were observed to be more significantly altered than that of H3K23, which becomes evident at later times points (2.5 mins) by comparing the acetylation patterns between unmodified and H3K4me3 substrates **(Figure 42)**. These results are in agreement with a previous study that reports a decrease in H3K14ac and H3K18ac levels *in vivo* when Sgf29 was deleted (160). This work was performed in collaboration with Alison E. Ringel and the lab of Dr. Cynthia Wolberger.

A.3 TAP-tag purification of yeast HAT complexes and HAT assays

While recombinant expression of certain protein complexes can increase purity and yield, problems with solubility may arise especially because some effector complexes, such as SAGA, contain over 15 protein subunits (88). Additionally, proteins within these complexes may be modified by PTMs *in vivo* that could affect enzymatic activity, stability, protein architecture, and substrate specificity, which would otherwise be lost when complexes are recombinantly expressed in *E. coli.* Therefore, by purifying endogenous complexes from eukaryotic organisms, such as yeast, we can obtain effector complexes in their entirety and with endogenous modifications. We used recombinant expression from a polycistronic vector and TAP-tag pulldowns of endogenous HAT complexes to test *in vitro* activity of these enzymes in the context of their binding partners

Figure 42

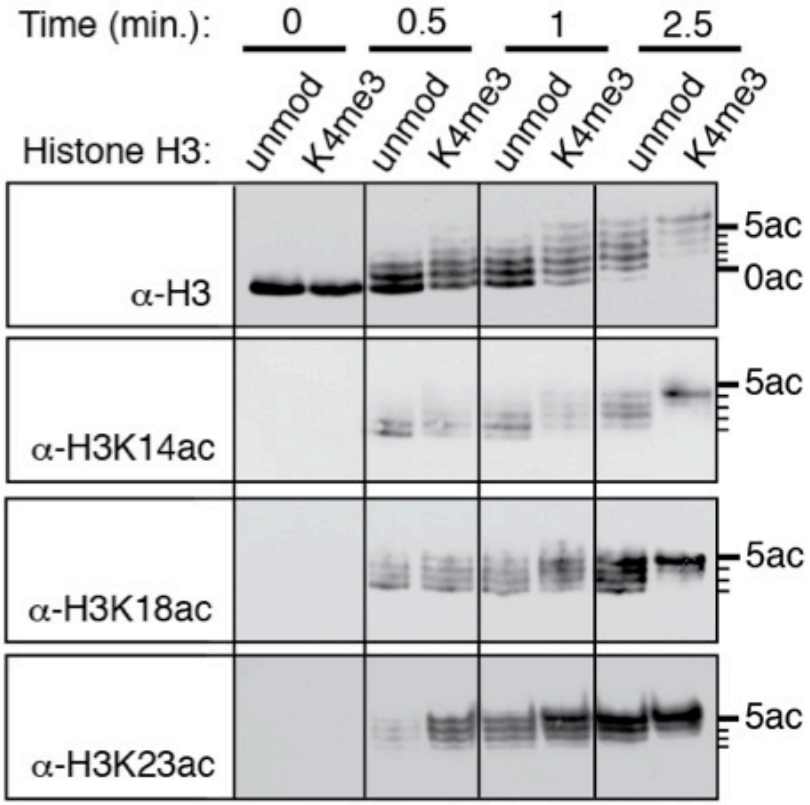


Figure 42. The presence of H3K4me3 increases overall acetylation by the HAT module on nucleosomes. HAT reactions comparing the activity of the *S. pombe* HAT module (Gcn5, Ada2, Ada3, and Sgf29) on either unmodified nucleosomes or H3K4me3 modified nucleosomes. Western blot of a HAT reaction resolved on an acid urea gel using antibodies for total H3, H3K14ac, H3K18ac, and H3K23ac. In collaboration with Alison E. Ringel and the Lab of Dr. Cynthia Wolberger (The Johns Hopkins University School of Medicine).

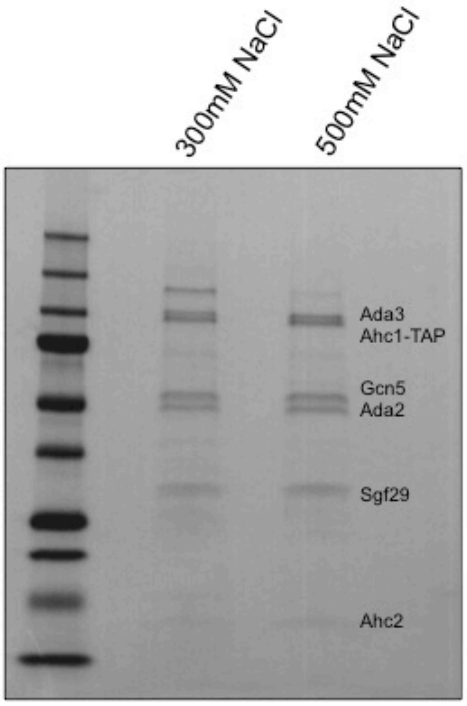
We have successfully purified the ADA complex, consisting of the proteins Gcn5, Ada2, Ada3, Sgf29, Ahc1, and Ahc2 performed in the lab of Dr. Alan J. Tackett (University of Arkansas for Medical Sciences, Little Rock, AR). Unlike Gcn5, Ada2, Ada3 and Sgf29, all of which are found in additional complexes, Ahc1 and Ahc2 are distinct to the ADA complex (88). Therefore, to ensure we were not pulling down multiple complexes, we tagged Ahc1 with a TAP-tag in which we purified the six-membered ADA complex from *S. cerevisiae* **(Figure 43A)**. We observed a very strong interaction among these proteins as all complex members remained intact after washing with up to 500mM salt. This endogenous ADA complex was shown to be active in an *in vitro* HAT assays where it acetylates histone H3 **(Figure 43B)**. In addition to the endogenously purified ADA complex, two subsets of the NuA3 complex, NuA3a and NuA3b, were purified by Dr. Tonya M. Gilbert and HAT assays were performed with recombinant histone H3 and resolved over an acid-urea gel **(Figure 44)** (34)**.** Acid-urea gel analysis revealed that the ADA complex acetylated up to 6 sites on histone H3.

A.4 Cryo EM of the six-member ADA complex

While TAP-tag purification provides full-membered, endogenously modified complexes it cannot yield protein amounts sufficient for crystallography. However, advances in Cryo-EM, which require a much lesser amount of protein, may lend structural insight into these large multisubunit protein complexes. We obtained preliminary Cryo-EM structural data on the intact endogenous ADA complex, which revealed three unique averages of the complex structure **(Figures 45A, 45B)** (in collaboration with Johnathan Chittuluru and Dr. Francisco Asturias (Scripps, San Diego, CA).

Figure 43

A



B

ADA Complex		+			+
Gcn5 Subcomplex	+			+	
Histone H3	+	+		+	+
Acetyl CoA				+	+
Positive Control			+		

Figure 43. Purification and assay development with the ADA complex. (A) The six membered ADA complex was purified from *S. cerevisiae* using a TAP tag on the Ahc-1 subunit. Complex was washed with 300mM and 500mM salt. (B) Enzymatic assay assessing HAT activity of the recombinant Gcn5 subcomplex and the endogenous ADA complex on histone H3. A western blot using an antibody against histone H3 acetylation was used to determine activity.

A.5 Quantitative mass spectrometry for measuring histone H3K4 methylation

In addition to measuring *in vitro* acetylation on lysine residues, quantitative mass spectrometry was also used to measure the effectiveness of an inhibitor against the lysine demethylase, LSD1. LSD1 is an actively pursued drug target as it is involved in many disease states and is known to demethylate H3K4me2 (63, 171). This method, termed MassSQUIRM (mass spectrometric quantitation using isotopic reductive methylation), which exploits the reactive methylation of amine groups using deutero-formaldehyde to chemically methylate all lysine residues resulting in a dimethylated state (172, 173). We developed an assay to test the effectiveness of a new LSD1 inhibitor (12d or bizine). Using massSQUIRM we were able to show that this inhibitor prevented LSD1 from demethylating H3K4me2 to an unmodified H3K4 state (63) **(Figure 46).**

A.6 Methods

NuPAGE SDS PAGE 12% gels (Invitrogen) were used to resolve histone H3. Acid-urea gels were assembled and run as previously described (120). Gels were washed with nano-pure water and stained with SimplyBlue Safe Stain (Invitrogen).

The following methods were done in collaboration with Alison E. Ringel, Lab of Dr. Cynthia Wolberger, and used for HAT module reactions on unmodified and H3K4me3 nucleosomal substrates. Nucleosomes were acetylated in buffer containing 20 mM HEPES, pH 7.6, 50 mM NaCl, 1 mM DTT, 50 μM acetyl-CoA, 20

Figure 44

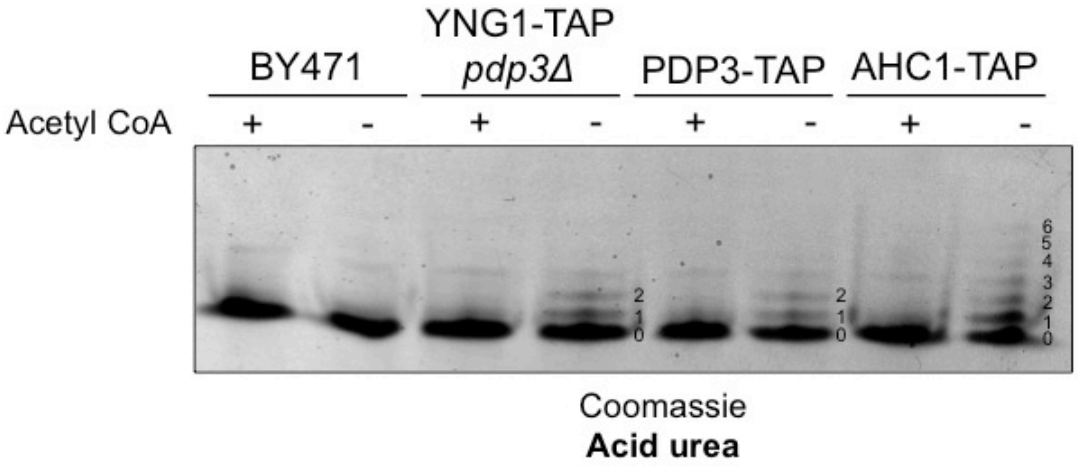


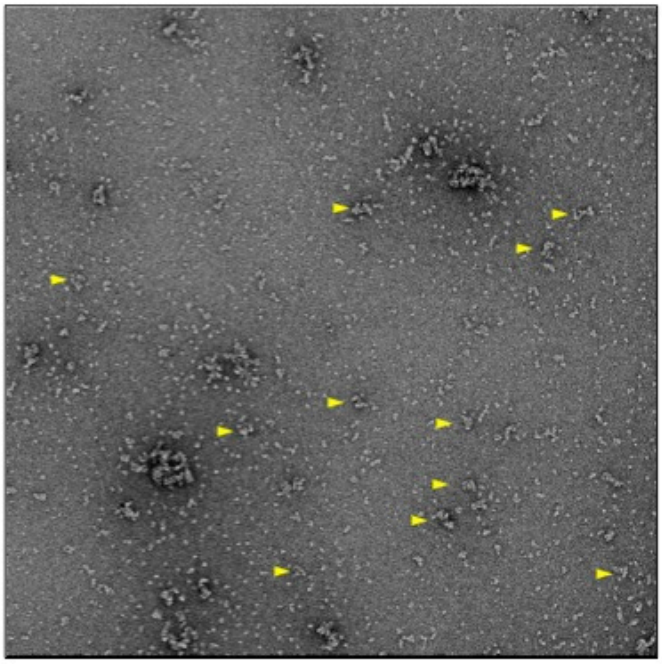
Figure 44. *In vitro* histone H3 acetylation by endogenous purified yeast HAT complexes. *In vitro* HAT assays were performed using endogenous HAT complexes purified from yeast. Assays were performed using either of the complexes NuA3a (YNG1-TAP), NuA3b (PDP3-TAP), or ADA (AHC1-TAP) using recombinant *S. cerevisiae* histone H3 and acetyl CoA. Reactions were resolved on an acid urea gel and stained with Coomassie to reveal the number of acetylation sites generated by each complex. TAP tag pulldowns were performed by Dr. Tonya M. Gilbert.

μg/mL BSA and 0.2 μM or 1 μM *Xenopus* nucleosome core particle (NCP). Reactions were incubated in buffer at 30°C for five minutes, initiated by the addition of 50 nM HAT module, quenched at different time points by flash-freezing in liquid nitrogen, and then lyophilized for analysis by acid-urea gel electrophoresis. Acid-urea gels were assembled and run as previously described (120). Histones were visualized with either SYPRO Ruby protein stain (LifeTechnologies) or by western blotting. For western blotting, proteins were transferred to PVDF membrane as previously described (120). Membranes were blocked overnight in 5% nonfat milk at 4°C and washed in TBS. Primary antibodies were diluted in 1% nonfat milk in TBS supplemented with 0.1% Tween (TBST) as follows: anti-H3 (Abcam ab1791, 1/25,000), anti-H3K14ac (07-353, EMD Millipore, 1/5000), anti-H3K18ac (EMD Millipore 07-354, 1/7500), anti-H3K23ac (EMD Millipore 07-355, 1/5000), or anti-H3K4me3 (Abcam ab8580, 1/5000). Each primary antibody was applied for 1 hour at room temperature followed by washing in TBST. Goat anti-rabbit IgG-horseradish peroxidase secondary antibody (Amersham Biosciences) was diluted to 1/5000 in 1% nonfat milk and TBST and applied for 1 hour at room temperature and washed in TBST. Blots were developed using Pierce ECL Western Blotting Substrate (ThermoScientific) and exposed using film.

Purifications of endogenously TAP-tagged *S. cerevisiae* NuA3a (*YNG1;pdp3*Δ), NuA3b (*PDP3*), and ADA (Ahc1) were performed to maintain complex integrity as previously described (34). Complexes were resuspended in HAT buffer (20 mM Tris pH 7.5, 50 mM NaCl, 5% glycerol) and mixed with 1 μg of recombinantly expressed *S. cerevisiae* histone H3 and 30 μM acetyl CoA in a total volume of 60 μL. Acetyl CoA was

Figure 45

A



B

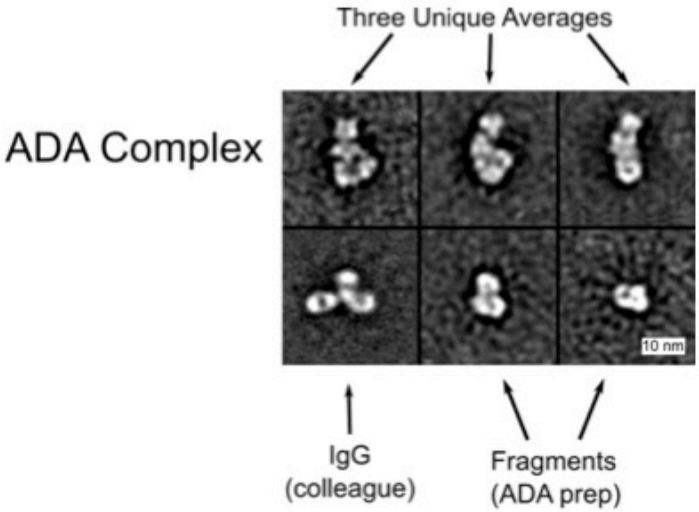


Figure 45. Cryo electron microscopy of the ADA complex. (A) Cryo EM image of the endogenously purified ADA complex (yellow arrows). (B) Image comparing different averages of the ADA complex compared to IgG antibody and fragments of the ADA complex. These preliminary results were performed in collaboration with Jonathan Chittuluru and Dr. Francisco Asturias (Scripps, San Diego, CA).

omitted from the negative control reactions. All reactions were incubated for 30 min at 30 °C and frozen in liquid nitrogen. TAP-tag purifications were performed by Dr. Tonya M. Gilbert.

MassSQUIRM inhibition experiments were performed in triplicate as described previously12. The reaction mixtures containing 13.3 μM H3K4me2-biotin peptide (1 ARTKme2QTA RKS TGG KAP RKQ LYKbio), 50 mM HEPES (pH 7.5), and 50 μM phenelzine or 12d, were incubated at 25 °C for 5 min, prior to initiation with 215 nM GST-LSD1. The demethylase reactions were run at 25 °C for 30 min and then analyzed as reported previously (63).

Figure 46

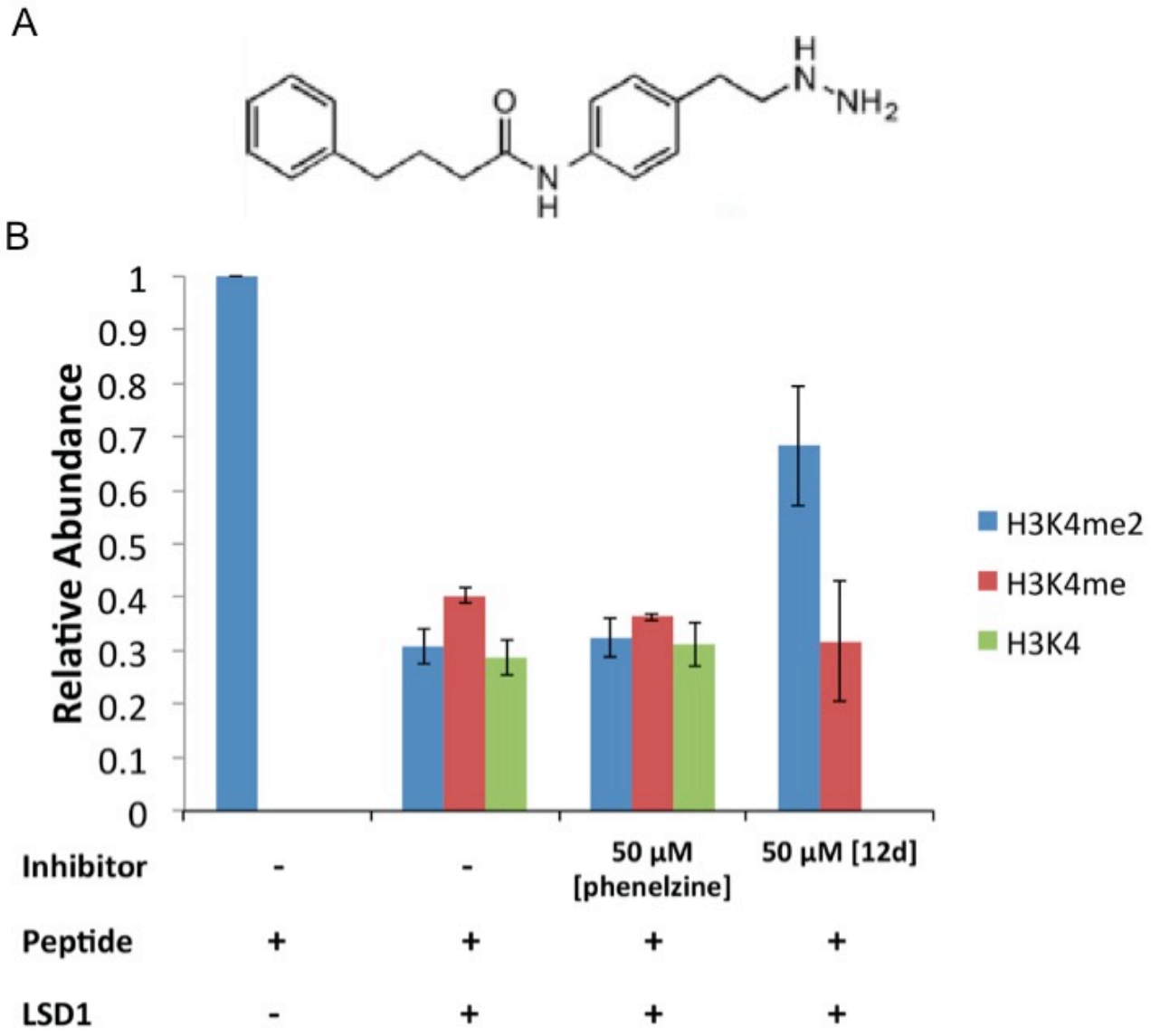


Figure 46. Quantitative mass spectrometry for measuring histone demethylation. (A) Structure of bizine inhibitor for LSD1 (B) Quantitation of H3K4me states in an enzymatic assay testing the inhibition of phenelzine and 12d on LSD1 as determined by MassSQUIRM. These data were performed in collaboration with the lab of Dr. Alan Tackett and with Dr. Philip Cole as previously published Prusevich, P., Kalin, J. H., Ming, S. A., Basso, M., Givens, J., Li, X., Hu, J., Taylor, M. S., Cieniewicz, A. M., Hsiao, P. Y., Huang, R., Roberson, H., Adejola, N., Avery, L. B., Casero, R. A.,Jr, Taverna, S. D., Qian, J., Tackett, A. J., Ratan, R. R., McDonald, O. G., Feinberg, A. P., and Cole, P. A. (2014) *A selective phenelzine analogue inhibitor of histone demethylase LSD1.* ACS Chem. Biol. 9, 1284-1293 .

References

1. Holliday, R. and Pugh, J. E. (1975) *DNA modification mechanisms and gene activity during development.* Science 187, 226-232

2. Riggs, A. D. (1975) *X inactivation, differentiation, and DNA methylation.* Cytogenet. Cell Genet. 14, 9-25

3. Eissenberg, J. C. (2014) *Epigenetics: Modifying the genetic blueprint.* Mo. Med. 111, 428-433

4. Alberts, B. (1994) *Molecular Biology of the Cell*, pp. 139-194

5. Berger, S. L. (2007) *The complex language of chromatin regulation during transcription.* Nature 447, 407-412

6. Rivera, C. M. and Ren, B. (2013) *Mapping human epigenomes.* Cell 155, 39-55

7. Karlic, H., Herrmann, H., Varga, F., Thaler, R., Reitermaier, R., Spitzer, S., Ghanim, V., Blatt, K., Sperr, W. R., Valent, P., and Pfeilstocker, M. (2014) *The role of epigenetics in the regulation of apoptosis in myelodysplastic syndromes and acute myeloid leukemia.* Crit. Rev. Oncol. Hematol. 90, 1-16

8. Kanherkar, R. R., Bhatia-Dey, N., and Csoka, A. B. (2014) *Epigenetics across the human lifespan.* Front. Cell. Dev. Biol. 2, 49

9. Egger, G., Liang, G., Aparicio, A., and Jones, P. A. (2004) *Epigenetics in human disease and prospects for epigenetic therapy.* Nature 429, 457-463

10. Kornberg, R. D. and Lorch, Y. (1999) *Twenty-five years of the nucleosome, fundamental particle of the eukaryote chromosome.* Cell 98, 285-294

11. Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997) *Crystal structure of the nucleosome core particle at 2.8 A resolution.* Nature 389, 251-260

12. Oudet, P., Gross-Bellard, M., and Chambon, P. (1975) *Electron microscopic and biochemical evidence that chromatin structure is a repeating unit.* Cell 4, 281-300

13. Roth, S. Y. and Allis, C. D. (1992) *Chromatin condensation: Does histone H1 dephosphorylation play a role?* Trends Biochem. Sci. 17, 93-98

14. Felsenfeld, G. (1978) *Chromatin.* Nature 271, 115-122

15. Rill, R. L., Shaw, B. R., and Van Holde, K. E. (1978) *Isolation and characterization of chromatin subunits.* Methods Cell Biol. 18, 69-103

16. Turner, B. M. (2000) *Histone acetylation and an epigenetic code.* Bioessays 22, 836-845

17. Cheung, P., Allis, C. D., and Sassone-Corsi, P. (2000) *Signaling to chromatin through histone modifications.* Cell 103, 263-271

18. Mizzen, C. A. and Allis, C. D. (1998) *Linking histone acetylation to transcriptional regulation.* Cell Mol. Life Sci. 54, 6-20

19. De Koning, L., Corpet, A., Haber, J. E., and Almouzni, G. (2007) *Histone chaperones: An escort network regulating histone traffic.* Nat. Struct. Mol. Biol. 14, 997-1007

20. Ekwall, K. (2007) *Epigenetic control of centromere behavior.* Annu. Rev. Genet. 41, 63-81

21. Brien, G. L. and Bracken, A. P. (2009) *Transcriptomics: Unravelling the biology of transcription factors and chromatin remodelers during development and differentiation.* Semin. Cell Dev. Biol. 20, 835-841

22. Clapier, C. R. and Cairns, B. R. (2009) *The biology of chromatin remodeling complexes.* Annu. Rev. Biochem. 78, 273-304

23. Lalonde, M. E., Cheng, X., and Cote, J. (2014) *Histone target selection within chromatin: An exemplary case of teamwork.* Genes Dev. 28, 1029-1041

24. Fischle, W., Wang, Y., and Allis, C. D. (2003) *Histone and chromatin cross-talk.* Curr. Opin. Cell Biol. 15, 172-183

25. Strahl, B. D. and Allis, C. D. (2000) *The language of covalent histone modifications.* Nature 403, 41-45

26. Kouzarides, T. (2007) *Chromatin modifications and their function.* Cell 128, 693-705

27. Lee, J. S., Smith, E., and Shilatifard, A. (2010) *The language of histone crosstalk.* Cell 142, 682-685

28. Jenuwein, T. and Allis, C. D. (2001) *Translating the histone code.* Science 293, 1074-1080

29. Peserico, A. and Simone, C. (2011) *Physical and functional HAT/HDAC interplay regulates protein acetylation balance.* J. Biomed. Biotechnol. 2011, 371832

30. Kouzarides, T. (2007) *SnapShot: Histone-modifying enzymes.* Cell 131, 822

31. Vu, L. P., Luciani, L., and Nimer, S. D. (2013) *Histone-modifying enzymes: Their role in the pathogenesis of acute leukemia and their therapeutic potential.* Int. J. Hematol. 97, 198-209

32. Filippakopoulos, P. and Knapp, S. (2012) *The bromodomain interaction module.* FEBS Lett. 586, 2692-2704

33. Taverna, S. D., Li, H., Ruthenburg, A. J., Allis, C. D., and Patel, D. J. (2007) *How chromatin-binding modules interpret histone modifications: Lessons from professional pocket pickers.* Nat. Struct. Mol. Biol. 14, 1025-1040

34. Gilbert, T. M., McDaniel, S. L., Byrum, S. D., Cades, J. A., Dancy, B. C., Wade, H., Tackett, A. J., Strahl, B. D., and Taverna, S. D. (2014) *A PWWP domain-containing protein targets the NuA3 acetyltransferase complex via histone H3 lysine 36 trimethylation to coordinate transcriptional elongation at coding regions.* Mol. Cell. Proteomics 13, 2883-2895

35. Martin, D. G., Baetz, K., Shi, X., Walter, K. L., MacDonald, V. E., Wlodarski, M. J., Gozani, O., Hieter, P., and Howe, L. (2006) *The Yng1p plant homeodomain finger is a*

methyl-histone binding module that recognizes lysine 4-methylated histone H3. Mol. Cell. Biol. 26, 7871-7879

36. Ruthenburg, A. J., Li, H., Milne, T. A., Dewell, S., McGinty, R. K., Yuen, M., Ueberheide, B., Dou, Y., Muir, T. W., Patel, D. J., and Allis, C. D. (2011) *Recognition of a mononucleosomal histone modification pattern by BPTF via multivalent interactions.* Cell 145, 692-706

37. Taverna, S. D., Ilin, S., Rogers, R. S., Tanny, J. C., Lavender, H., Li, H., Baker, L., Boyle, J., Blair, L. P., Chait, B. T., Patel, D. J., Aitchison, J. D., Tackett, A. J., and Allis, C. D. (2006) *Yng1 PHD finger binding to H3 trimethylated at K4 promotes NuA3 HAT activity at K14 of H3 and transcription at a subset of targeted ORFs.* Mol. Cell 24, 785-796

38. Wysocka, J., Swigut, T., Xiao, H., Milne, T. A., Kwon, S. Y., Landry, J., Kauer, M., Tackett, A. J., Chait, B. T., Badenhorst, P., Wu, C., and Allis, C. D. (2006) *A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling.* Nature 442, 86-90

39. Baker, L. A., Allis, C. D., and Wang, G. G. (2008) *PHD fingers in human diseases: Disorders arising from misinterpreting epigenetic marks.* Mutat. Res. 647, 3-12

40. Hudson, B. P., Martinez-Yamout, M. A., Dyson, H. J., and Wright, P. E. (2000) *Solution structure and acetyl-lysine binding activity of the GCN5 bromodomain.* J. Mol. Biol. 304, 355-370

41. Yang, X. J. (2004) *Lysine acetylation and the bromodomain: A new partnership for signaling.* Bioessays 26, 1076-1087

42. Owen, D. J., Ornaghi, P., Yang, J. C., Lowe, N., Evans, P. R., Ballario, P., Neuhaus, D., Filetici, P., and Travers, A. A. (2000) *The structural basis for the recognition of acetylated histone H4 by the bromodomain of histone acetyltransferase gcn5p.* EMBO J. 19, 6141-6149

43. Ruthenburg, A. J., Li, H., Patel, D. J., and Allis, C. D. (2007) *Multivalent engagement of chromatin modifications by linked binding modules.* Nat. Rev. Mol. Cell Biol. 8, 983-994

44. Schaft, D., Roguev, A., Kotovic, K. M., Shevchenko, A., Sarov, M., Shevchenko, A., Neugebauer, K. M., and Stewart, A. F. (2003) *The histone 3 lysine 36 methyltransferase, SET2, is involved in transcriptional elongation.* Nucleic Acids Res. 31, 2475-2482

45. Ruthenburg, A. J., Allis, C. D., and Wysocka, J. (2007) *Methylation of lysine 4 on histone H3: Intricacy of writing and reading a single epigenetic mark.* Mol. Cell 25, 15-30

46. Shilatifard, A. (2008) *Molecular implementation and physiological roles for histone H3 lysine 4 (H3K4) methylation.* Curr. Opin. Cell Biol. 20, 341-348

47. Spedale, G., Timmers, H. T., and Pijnappel, W. W. (2012) *ATAC-king the complexity of SAGA during evolution.* Genes Dev. 26, 527-541

48. Csordas, A. (1990) *On the biological role of histone acetylation.* Biochem. J. 265, 23-38

49. Kuo, M. H., Brownell, J. E., Sobel, R. E., Ranalli, T. A., Cook, R. G., Edmondson, D. G., Roth, S. Y., and Allis, C. D. (1996) *Transcription-linked acetylation by Gcn5p of histones H3 and H4 at specific lysines.* Nature 383, 269-272

50. Marmorstein, R. and Zhou, M. M. (2014) *Writers and readers of histone acetylation: Structure, mechanism, and inhibition.* Cold Spring Harb Perspect. Biol. 6, a018762

51. Vidali, G., Ferrari, N., and Pfeffer, U. (1988) *Histone acetylation: A step in gene activation.* Adv. Exp. Med. Biol. 231, 583-596

52. ALLFREY, V. G., FAULKNER, R., and MIRSKY, A. E. (1964) *Acetylation and methylation of histones and their possible role in the regulation of rna synthesis.* Proc. Natl. Acad. Sci. U. S. A. 51, 786-794

53. Verdin, E. and Ott, M. (2014) *50 years of protein acetylation: From gene regulation to epigenetics, metabolism and beyond.* Nat. Rev. Mol. Cell Biol.

54. PHILLIPS, D. M. (1963) *The presence of acetyl groups of histones.* Biochem. J. 87, 258-263

55. Hebbes, T. R., Thorne, A. W., and Crane-Robinson, C. (1988) *A direct link between core histone acetylation and transcriptionally active chromatin.* EMBO J. 7, 1395-1402

56. Turner, B. M. and Fellows, G. (1989) *Specific antibodies reveal ordered and cell-cycle-related use of histone-H4 acetylation sites in mammalian cells.* Eur. J. Biochem. 179, 131-139

57. Brownell, J. E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D. G., Roth, S. Y., and Allis, C. D. (1996) *Tetrahymena histone acetyltransferase A: A homolog to yeast Gcn5p linking histone acetylation to gene activation.* Cell 84, 843-851

58. Choudhary, C., Kumar, C., Gnad, F., Nielsen, M. L., Rehman, M., Walther, T. C., Olsen, J. V., and Mann, M. (2009) *Lysine acetylation targets protein complexes and co-regulates major cellular functions.* Science 325, 834-840

59. Carlson, S. and Glass, K. C. (2014) *The MOZ histone acetyltransferase in epigenetic signaling and disease.* J. Cell. Physiol. 229, 1571-1574

60. Chen, Y. C., Gatchel, J. R., Lewis, R. W., Mao, C. A., Grant, P. A., Zoghbi, H. Y., and Dent, S. Y. (2012) *Gcn5 loss-of-function accelerates cerebellar and retinal degeneration in a SCA7 mouse model.* Hum. Mol. Genet. 21, 394-405

61. Dekker, F. J. and Haisma, H. J. (2009) *Histone acetyl transferases as emerging drug targets.* Drug Discov. Today 14, 942-948

62. Palhan, V. B., Chen, S., Peng, G. H., Tjernberg, A., Gamper, A. M., Fan, Y., Chait, B. T., La Spada, A. R., and Roeder, R. G. (2005) *Polyglutamine-expanded ataxin-7 inhibits STAGA histone acetyltransferase activity to produce retinal degeneration.* Proc. Natl. Acad. Sci. U. S. A. 102, 8472-8477

63. Prusevich, P., Kalin, J. H., Ming, S. A., Basso, M., Givens, J., Li, X., Hu, J., Taylor, M. S., Cieniewicz, A. M., Hsiao, P. Y., Huang, R., Roberson, H., Adejola, N., Avery, L. B., Casero, R. A.,Jr, Taverna, S. D., Qian, J., Tackett, A. J., Ratan, R. R., McDonald, O. G., Feinberg, A. P., and Cole, P. A. (2014) *A selective phenelzine analogue inhibitor of histone demethylase LSD1.* ACS Chem. Biol. 9, 1284-1293

64. Wang, L. and Dent, S. Y. (2014) *Functions of SAGA in development and disease.* Epigenomics 6, 329-339

65. Heerboth, S., Lapinska, K., Snyder, N., Leary, M., Rollinson, S., and Sarkar, S. (2014) *Use of epigenetic drugs in disease: An overview.* Genet. Epigenet 6, 9-19

66. Mann, B. S., Johnson, J. R., Cohen, M. H., Justice, R., and Pazdur, R. (2007) *FDA approval summary: Vorinostat for treatment of advanced primary cutaneous T-cell lymphoma.* Oncologist 12, 1247-1252

67. Mann, B. S., Johnson, J. R., He, K., Sridhara, R., Abraham, S., Booth, B. P., Verbois, L., Morse, D. E., Jee, J. M., Pope, S., Harapanhalli, R. S., Dagher, R., Farrell, A., Justice, R., and Pazdur, R. (2007) *Vorinostat for treatment of cutaneous manifestations of advanced primary cutaneous T-cell lymphoma.* Clin. Cancer Res. 13, 2318-2322

68. Filippakopoulos, P. and Knapp, S. (2014) *Targeting bromodomains: Epigenetic readers of lysine acetylation.* Nat. Rev. Drug Discov. 13, 337-356

69. Sanchez, R., Meslamani, J., and Zhou, M. M. (2014) *The bromodomain: From epigenome reader to druggable target.* Biochim. Biophys. Acta 1839, 676-685

70. Filippakopoulos, P., Qi, J., Picaud, S., Shen, Y., Smith, W. B., Fedorov, O., Morse, E. M., Keates, T., Hickman, T. T., Felletar, I., Philpott, M., Munro, S., McKeown, M. R., Wang, Y., Christie, A. L., West, N., Cameron, M. J., Schwartz, B., Heightman, T. D., La Thangue, N., French, C. A., Wiest, O., Kung, A. L., Knapp, S., and Bradner, J. E. (2010) *Selective inhibition of BET bromodomains.* Nature 468, 1067-1073

71. Taverna, S. D. and Cole, P. A. (2010) *Drug discovery: Reader's block.* Nature 468, 1050-1051

72. Nicodeme, E., Jeffrey, K. L., Schaefer, U., Beinke, S., Dewell, S., Chung, C. W., Chandwani, R., Marazzi, I., Wilson, P., Coste, H., White, J., Kirilovsky, J., Rice, C. M., Lora, J. M., Prinjha, R. K., Lee, K., and Tarakhovsky, A. (2010) *Suppression of inflammation by a synthetic histone mimic.* Nature 468, 1119-1123

73. Syntichaki, P., Topalidou, I., and Thireos, G. (2000) *The Gcn5 bromodomain co-ordinates nucleosome remodelling.* Nature 404, 414-417

74. Dhalluin, C., Carlson, J. E., Zeng, L., He, C., Aggarwal, A. K., and Zhou, M. M. (1999) *Structure and ligand of a histone acetyltransferase bromodomain.* Nature 399, 491-496

75. Lee, J., Yun, N., Kim, C., Song, M. Y., Park, K. S., and Oh, Y. J. (2014) *Acetylation of cyclin-dependent kinase 5 is mediated by GCN5.* Biochem. Biophys. Res. Commun. 447, 121-127

76. Dominy, J. E.,Jr, Lee, Y., Jedrychowski, M. P., Chim, H., Jurczak, M. J., Camporez, J. P., Ruan, H. B., Feldman, J., Pierce, K., Mostoslavsky, R., Denu, J. M., Clish, C. B., Yang, X., Shulman, G. I., Gygi, S. P., and Puigserver, P. (2012) *The deacetylase Sirt6 activates the acetyltransferase GCN5 and suppresses hepatic gluconeogenesis.* Mol. Cell 48, 900-913

77. Kelly, T. J., Lerin, C., Haas, W., Gygi, S. P., and Puigserver, P. (2009) *GCN5-mediated transcriptional control of the metabolic coactivator PGC-1beta through lysine acetylation.* J. Biol. Chem. 284, 19945-19952

78. Candau, R., Zhou, J. X., Allis, C. D., and Berger, S. L. (1997) *Histone acetyltransferase activity and interaction with ADA2 are critical for GCN5 function in vivo.* EMBO J. 16, 555-565

79. Grant, P. A., Duggan, L., Cote, J., Roberts, S. M., Brownell, J. E., Candau, R., Ohba, R., Owen-Hughes, T., Allis, C. D., Winston, F., Berger, S. L., and Workman, J. L. (1997) *Yeast Gcn5 functions in two multisubunit complexes to acetylate nucleosomal histones: Characterization of an ada complex and the SAGA (spt/ada) complex.* Genes Dev. 11, 1640-1650

80. Grant, P. A., Eberharter, A., John, S., Cook, R. G., Turner, B. M., and Workman, J. L. (1999) *Expanded lysine acetylation specificity of Gcn5 in native complexes.* J. Biol. Chem. 274, 5895-5900

81. Jiang, J., Lu, J., Lu, D., Liang, Z., Li, L., Ouyang, S., Kong, X., Jiang, H., Shen, B., and Luo, C. (2012) *Investigation of the acetylation mechanism by GCN5 histone acetyltransferase.* PLoS One 7, e36660

82. Poux, A. N., Cebrat, M., Kim, C. M., Cole, P. A., and Marmorstein, R. (2002) *Structure of the GCN5 histone acetyltransferase bound to a bisubstrate inhibitor.* Proc. Natl. Acad. Sci. U. S. A. 99, 14065-14070

83. Rojas, J. R., Trievel, R. C., Zhou, J., Mo, Y., Li, X., Berger, S. L., Allis, C. D., and Marmorstein, R. (1999) *Structure of tetrahymena GCN5 bound to coenzyme A and a histone H3 peptide.* Nature 401, 93-98

84. Tanner, K. G., Langer, M. R., Kim, Y., and Denu, J. M. (2000) *Kinetic mechanism of the histone acetyltransferase GCN5 from yeast.* J. Biol. Chem. 275, 22048-22055

85. Bonnet, J., Wang, C. Y., Baptista, T., Vincent, S. D., Hsiao, W. C., Stierle, M., Kao, C. F., Tora, L., and Devys, D. (2014) *The SAGA coactivator complex acts on the whole transcribed genome and is required for RNA polymerase II transcription.* Genes Dev. 28, 1999-2012

86. Pray-Grant, M. G., Schieltz, D., McMahon, S. J., Wood, J. M., Kennedy, E. L., Cook, R. G., Workman, J. L., Yates, J. R.,3rd, and Grant, P. A. (2002) *The novel SLIK histone*

acetyltransferase complex functions in the yeast retrograde response pathway. Mol. Cell. Biol. 22, 8774-8786

87. Eberharter, A., Sterner, D. E., Schieltz, D., Hassan, A., Yates, J. R.,3rd, Berger, S. L., and Workman, J. L. (1999) *The ADA complex is a distinct histone acetyltransferase complex in saccharomyces cerevisiae.* Mol. Cell. Biol. 19, 6621-6631

88. Lee, K. K., Sardiu, M. E., Swanson, S. K., Gilmore, J. M., Torok, M., Grant, P. A., Florens, L., Workman, J. L., and Washburn, M. P. (2011) *Combinatorial depletion analysis to assemble the network architecture of the SAGA and ADA chromatin remodeling complexes.* Mol. Syst. Biol. 7, 503

89. Wang, Y. L., Faiola, F., Xu, M., Pan, S., and Martinez, E. (2008) *Human ATAC is a GCN5/PCAF-containing acetylase complex with a novel NC2-like histone fold module that interacts with the TATA-binding protein.* J. Biol. Chem. 283, 33808-33815

90. Huang, J., Wan, D., Li, J., Chen, H., Huang, K., and Zheng, L. (2014) *Histone acetyltransferase PCAF regulates inflammatory molecules in the development of renal injury.* Epigenetics, 0

91. Jin, Q., Yu, L. R., Wang, L., Zhang, Z., Kasper, L. H., Lee, J. E., Wang, C., Brindle, P. K., Dent, S. Y., and Ge, K. (2011) *Distinct roles of GCN5/PCAF-mediated H3K9ac and CBP/p300-mediated H3K18/27ac in nuclear receptor transactivation.* EMBO J. 30, 249-262

92. Zeng, L., Zhang, Q., Gerona-Navarro, G., Moshkina, N., and Zhou, M. M. (2008) *Structural basis of site-specific histone recognition by the bromodomains of human coactivators PCAF and CBP/p300.* Structure 16, 643-652

93. Sendra, R., Tse, C., and Hansen, J. C. (2000) *The yeast histone acetyltransferase A2 complex, but not free Gcn5p, binds stably to nucleosomal arrays.* J. Biol. Chem. 275, 24928-24934

94. Balasubramanian, R., Pray-Grant, M. G., Selleck, W., Grant, P. A., and Tan, S. (2002) *Role of the Ada2 and Ada3 transcriptional coactivators in histone acetylation.* J. Biol. Chem. 277, 7989-7995

95. Hoke, S. M., Genereaux, J., Liang, G., and Brandl, C. J. (2008) *A conserved central region of yeast Ada2 regulates the histone acetyltransferase activity of Gcn5 and interacts with phospholipids.* J. Mol. Biol. 384, 743-755

96. Horiuchi, J., Silverman, N., Marcus, G. A., and Guarente, L. (1995) *ADA3, a putative transcriptional adaptor, consists of two separable domains and interacts with ADA2 and GCN5 in a trimeric complex.* Mol. Cell. Biol. 15, 1203-1209

97. Syntichaki, P. and Thireos, G. (1998) *The Gcn5.ada complex potentiates the histone acetyltransferase activity of Gcn5.* J. Biol. Chem. 273, 24414-24419

98. Nagy, Z. and Tora, L. (2007) *Distinct GCN5/PCAF-containing complexes function as co-activators and are involved in transcription factor and global histone acetylation.* Oncogene 26, 5341-5357

99. Suganuma, T., Gutierrez, J. L., Li, B., Florens, L., Swanson, S. K., Washburn, M. P., Abmayr, S. M., and Workman, J. L. (2008) *ATAC is a double histone acetyltransferase complex that stimulates nucleosome sliding.* Nat. Struct. Mol. Biol. 15, 364-372

100. Bu, P., Evrard, Y. A., Lozano, G., and Dent, S. Y. (2007) *Loss of Gcn5 acetyltransferase activity leads to neural tube closure defects and exencephaly in mouse embryos.* Mol. Cell. Biol. 27, 3405-3416

101. Lin, W., Zhang, Z., Chen, C. H., Behringer, R. R., and Dent, S. Y. (2008) *Proper Gcn5 histone acetyltransferase expression is required for normal anteroposterior patterning of the mouse skeleton.* Dev. Growth Differ. 50, 321-330

102. Lin, W., Srajer, G., Evrard, Y. A., Phan, H. M., Furuta, Y., and Dent, S. Y. (2007) *Developmental potential of Gcn5(-/-) embryonic stem cells in vivo and in vitro.* Dev. Dyn. 236, 1547-1557

103. Caron, C., Boyault, C., and Khochbin, S. (2005) *Regulatory cross-talk between lysine acetylation and ubiquitination: Role in the control of protein stability.* Bioessays 27, 408-415

104. Linares, L. K., Kiernan, R., Triboulet, R., Chable-Bessia, C., Latreille, D., Cuvier, O., Lacroix, M., Le Cam, L., Coux, O., and Benkirane, M. (2007) *Intrinsic ubiquitination activity of PCAF controls the stability of the oncoprotein Hdm2.* Nat. Cell Biol. 9, 331-338

105. Chen, L., Wei, T., Si, X., Wang, Q., Li, Y., Leng, Y., Deng, A., Chen, J., Wang, G., Zhu, S., and Kang, J. (2013) *Lysine acetyltransferase GCN5 potentiates the growth of non-small cell lung cancer via promotion of E2F1, cyclin D1, and cyclin E1 expression.* J. Biol. Chem. 288, 14510-14521

106. Helmlinger, D., Hardy, S., Eberlin, A., Devys, D., and Tora, L. (2006) *Both normal and polyglutamine- expanded ataxin-7 are components of TFTC-type GCN5 histone acetyltransferase- containing complexes.* Biochem. Soc. Symp. (73), 155-163

107. McCullough, S. D., Xu, X., Dent, S. Y., Bekiranov, S., Roeder, R. G., and Grant, P. A. (2012) *Reelin is a target of polyglutamine expanded ataxin-7 in human spinocerebellar ataxia type 7 (SCA7) astrocytes.* Proc. Natl. Acad. Sci. U. S. A. 109, 21319-21324

108. McMahon, S. J., Pray-Grant, M. G., Schieltz, D., Yates, J. R.,3rd, and Grant, P. A. (2005) *Polyglutamine-expanded spinocerebellar ataxia-7 protein disrupts normal SAGA and SLIK histone acetyltransferase activity.* Proc. Natl. Acad. Sci. U. S. A. 102, 8478-8482

109. Kikuchi, H., Takami, Y., and Nakayama, T. (2005) *GCN5: A supervisor in all-inclusive control of vertebrate cell cycle progression through transcription regulation of various cell cycle-related genes.* Gene 347, 83-97

110. Liu, X., Tesfai, J., Evrard, Y. A., Dent, S. Y., and Martinez, E. (2003) *C-myc transformation domain recruits the human STAGA complex and requires TRRAP and GCN5 acetylase activity for transcription activation.* J. Biol. Chem. 278, 20405-20412

111. Cieniewicz, A. M., Moreland, L., Ringel, A. E., Mackintosh, S. G., Raman, A., Gilbert, T. M., Wolberger, C., Tackett, A. J., and Taverna, S. D. (2014) *The bromodomain of Gcn5 regulates site specificity of lysine acetylation on histone H3.* Mol. Cell. Proteomics 13, 2896-2910

112. Grant, P. A., Eberharter, A., John, S., Cook, R. G., Turner, B. M., and Workman, J. L. (1999) *Expanded lysine acetylation specificity of Gcn5 in native complexes.* J. Biol. Chem. 274, 5895-5900

113. Kuo, Y. M. and Andrews, A. J. (2013) *Quantitating the specificity and selectivity of Gcn5-mediated acetylation of histone H3.* PLoS One 8, e54896

114. Suka, N., Suka, Y., Carmen, A. A., Wu, J., and Grunstein, M. (2001) *Highly specific antibodies determine histone acetylation site usage in yeast heterochromatin and euchromatin.* Mol. Cell 8, 473-479

115. Eberharter, A., Sterner, D. E., Schieltz, D., Hassan, A., Yates, J. R.,3rd, Berger, S. L., and Workman, J. L. (1999) *The ADA complex is a distinct histone acetyltransferase complex in saccharomyces cerevisiae.* Mol. Cell. Biol. 19, 6621-6631

116. Balasubramanian, R., Pray-Grant, M. G., Selleck, W., Grant, P. A., and Tan, S. (2002) *Role of the Ada2 and Ada3 transcriptional coactivators in histone acetylation.* J. Biol. Chem. 277, 7989-7995

117. Barrios, A., Selleck, W., Hnatkovich, B., Kramer, R., Sermwittayawong, D., and Tan, S. (2007) *Expression and purification of recombinant yeast Ada2/Ada3/Gcn5 and piccolo NuA4 histone acetyltransferase complexes.* Methods 41, 271-277

118. Syntichaki, P. and Thireos, G. (1998) *The Gcn5.ada complex potentiates the histone acetyltransferase activity of Gcn5.* J. Biol. Chem. 273, 24414-24419

119. Papazyan, R. and Taverna, S. D. (2013) *Separation and purification of multiply acetylated proteins using cation-exchange chromatography.* Methods Mol. Biol. 981, 103-113

120. Shechter, D., Dormann, H. L., Allis, C. D., and Hake, S. B. (2007) *Extraction, purification and analysis of histones.* Nat. Protoc. 2, 1445-1457

121. Tackett, A. J., Dilworth, D. J., Davey, M. J., O'Donnell, M., Aitchison, J. D., Rout, M. P., and Chait, B. T. (2005) *Proteomic and genomic characterization of chromatin complexes at a boundary.* J. Cell Biol. 169, 35-47

122. Gradolatto, A., Smart, S. K., Byrum, S., Blair, L. P., Rogers, R. S., Kolar, E. A., Lavender, H., Larson, S. K., Aitchison, J. D., Taverna, S. D., and Tackett, A. J. (2009) *A noncanonical bromodomain in the AAA ATPase protein Yta7 directs chromosomal positioning and barrier chromatin activity.* Mol. Cell. Biol. 29, 4604-4611

123. Byrum, S. D., Taverna, S. D., and Tackett, A. J. (2013) *Purification of a specific native genomic locus for proteomic analysis.* Nucleic Acids Res. 41, e195

124. Byrum, S. D., Larson, S. K., Avaritt, N. L., Moreland, L. E., Mackintosh, S. G., Cheung, W. L., and Tackett, A. J. (2013) *Quantitative proteomics identifies activation of hallmark pathways of cancer in patient melanoma.* J. Proteomics Bioinform 6, 43-50

125. Byrum, S. D., Raman, A., Taverna, S. D., and Tackett, A. J. (2012) *ChAP-MS: A method for identification of proteins and histone posttranslational modifications at a single genomic locus.* Cell. Rep. 2, 198-205

126. Nesvizhskii, A. I., Keller, A., Kolker, E., and Aebersold, R. (2003) *A statistical model for identifying proteins by tandem mass spectrometry.* Anal. Chem. 75, 4646-4658

127. Vizcaino, J. A., Deutsch, E. W., Wang, R., Csordas, A., Reisinger, F., Rios, D., Dianes, J. A., Sun, Z., Farrah, T., Bandeira, N., Binz, P. A., Xenarios, I., Eisenacher, M., Mayer, G., Gatto, L., Campos, A., Chalkley, R. J., Kraus, H. J., Albar, J. P., Martinez-Bartolome, S., Apweiler, R., Omenn, G. S., Martens, L., Jones, A. R., and Hermjakob, H. (2014) *ProteomeXchange provides globally coordinated proteomics data submission and dissemination.* Nat. Biotechnol. 32, 223-226

128. Cote, R. G., Griss, J., Dianes, J. A., Wang, R., Wright, J. C., van den Toorn, H. W., van Breukelen, B., Heck, A. J., Hulstaert, N., Martens, L., Reisinger, F., Csordas, A., Ovelleiro, D., Perez-Rivevol, Y., Barsnes, H., Hermjakob, H., and Vizcaino, J. A. (2012) *The PRoteomics IDEntification (PRIDE) converter 2 framework: An improved suite of tools to facilitate data submission to the PRIDE database and the ProteomeXchange consortium.* Mol. Cell. Proteomics 11, 1682-1689

129. Vizcaino, J. A., Cote, R. G., Csordas, A., Dianes, J. A., Fabregat, A., Foster, J. M., Griss, J., Alpi, E., Birim, M., Contell, J., O'Kelly, G., Schoenegger, A., Ovelleiro, D., Perez-Riverol, Y., Reisinger, F., Rios, D., Wang, R., and Hermjakob, H. (2013) *The PRoteomics IDEntifications (PRIDE) database and associated tools: Status in 2013.* Nucleic Acids Res. 41, D1063-9

130. Wang, R., Fabregat, A., Rios, D., Ovelleiro, D., Foster, J. M., Cote, R. G., Griss, J., Csordas, A., Perez-Riverol, Y., Reisinger, F., Hermjakob, H., Martens, L., and Vizcaino, J. A. (2012) *PRIDE inspector: A tool to visualize and validate MS proteomics data.* Nat. Biotechnol. 30, 135-137

131. Panyim, S. and Chalkley, R. (1969) *High resolution acrylamide gel electrophoresis of histones.* Arch. Biochem. Biophys. 130, 337-346

132. Arnold, K. M., Lee, S., and Denu, J. M. (2011) *Processing mechanism and substrate selectivity of the core NuA4 histone acetyltransferase complex.* Biochemistry 50, 727-737

133. Henry, R. A., Kuo, Y. M., and Andrews, A. J. (2013) *Differences in specificity and selectivity between CBP and p300 acetylation of histone H3 and H3/H4.* Biochemistry 52, 5746-5759

134. Hersman, E., Nelson, D. M., Griffith, W. P., Jelinek, C., and Cotter, R. J. (2012) *Analysis of histone modifications from tryptic peptides of deuteroacetylated isoforms.* Int. J. Mass. Spectrom. 312, 5-16

135. Fiedler, K. L., Bheda, P., Dai, J., Boeke, J. D., Wolberger, C., and Cotter, R. J. (2013) *A quantitative analysis of histone methylation and acetylation isoforms from their deuteroacetylated derivatives: Application to a series of knockout mutants.* J. Mass Spectrom. 48, 608-615

136. Ito, T., Umehara, T., Sasaki, K., Nakamura, Y., Nishino, N., Terada, T., Shirouzu, M., Padmanabhan, B., Yokoyama, S., Ito, A., and Yoshida, M. (2011) *Real-time imaging of histone H4K12-specific acetylation determines the modes of action of histone deacetylase and bromodomain inhibitors.* Chem. Biol. 18, 495-507

137. Su, X., Zhang, L., Lucas, D. M., Davis, M. E., Knapp, A. R., Green-Church, K. B., Marcucci, G., Parthun, M. R., Byrd, J. C., and Freitas, M. A. (2007) *Histone H4 acetylation dynamics determined by stable isotope labeling with amino acids in cell culture and mass spectrometry.* Anal. Biochem. 363, 22-34

138. Dilworth, D. J., Tackett, A. J., Rogers, R. S., Yi, E. C., Christmas, R. H., Smith, J. J., Siegel, A. F., Chait, B. T., Wozniak, R. W., and Aitchison, J. D. (2005) *The mobile nucleoporin Nup2p and chromatin-bound Prp20p function in endogenous NPC-mediated transcriptional control.* J. Cell Biol. 171, 955-965

139. Dhalluin, C., Carlson, J. E., Zeng, L., He, C., Aggarwal, A. K., and Zhou, M. M. (1999) *Structure and ligand of a histone acetyltransferase bromodomain.* Nature 399, 491-496

140. Sterner, D. E., Grant, P. A., Roberts, S. M., Duggan, L. J., Belotserkovskaya, R., Pacella, L. A., Winston, F., Workman, J. L., and Berger, S. L. (1999) *Functional*

organization of the yeast SAGA complex: Distinct components involved in structural integrity, nucleosome acetylation, and TATA-binding protein interaction. Mol. Cell. Biol. 19, 86-98

141. Syntichaki, P., Topalidou, I., and Thireos, G. (2000) *The Gcn5 bromodomain co-ordinates nucleosome remodelling.* Nature 404, 414-417

142. Hassan, A. H., Awad, S., Al-Natour, Z., Othman, S., Mustafa, F., and Rizvi, T. A. (2007) *Selective recognition of acetylated histones by bromodomains in transcriptional co-activators.* Biochem. J. 402, 125-133

143. Mujtaba, S., He, Y., Zeng, L., Farooq, A., Carlson, J. E., Ott, M., Verdin, E., and Zhou, M. M. (2002) *Structural basis of lysine-acetylated HIV-1 tat recognition by PCAF bromodomain.* Mol. Cell 9, 575-586

144. Zhang, Q., Chakravarty, S., Ghersi, D., Zeng, L., Plotnikov, A. N., Sanchez, R., and Zhou, M. M. (2010) *Biochemical profiling of histone binding selectivity of the yeast bromodomain family.* PLoS One 5, e8903

145. Li, S. and Shogren-Knaak, M. A. (2009) *The Gcn5 bromodomain of the SAGA complex facilitates cooperative and cross-tail acetylation of nucleosomes.* J. Biol. Chem. 284, 9411-9417

146. Deng, Z., Chen, C. J., Chamberlin, M., Lu, F., Blobel, G. A., Speicher, D., Cirillo, L. A., Zaret, K. S., and Lieberman, P. M. (2003) *The CBP bromodomain and nucleosome*

targeting are required for zta-directed nucleosome acetylation and transcription activation. Mol. Cell. Biol. 23, 2633-2644

147. Hassan, A. H., Prochasson, P., Neely, K. E., Galasinski, S. C., Chandy, M., Carrozza, M. J., and Workman, J. L. (2002) *Function and selectivity of bromodomains in anchoring chromatin-modifying complexes to promoter nucleosomes.* Cell 111, 369-379

148. Marcus, G. A., Silverman, N., Berger, S. L., Horiuchi, J., and Guarente, L. (1994) *Functional similarity and physical association between GCN5 and ADA2: Putative transcriptional adaptors.* EMBO J. 13, 4807-4815

149. Pizzitutti, F., Giansanti, A., Ballario, P., Ornaghi, P., Torreri, P., Ciccotti, G., and Filetici, P. (2006) *The role of loop ZA and Pro371 in the function of yeast Gcn5p bromodomain revealed through molecular dynamics and experiment.* J. Mol. Recognit. 19, 1-9

150. Luger, K., Rechsteiner, T. J., and Richmond, T. J. (1999) *Expression and purification of recombinant histones and nucleosome reconstitution.* Methods Mol. Biol. 119, 1-16

151. Lowary, P. T. and Widom, J. (1998) *New DNA sequence rules for high affinity binding to histone octamer and sequence-directed nucleosome positioning.* J. Mol. Biol. 276, 19-42

152. Dyer, P. N., Edayathumangalam, R. S., White, C. L., Bao, Y., Chakravarthy, S., Muthurajan, U. M., and Luger, K. (2004) *Reconstitution of nucleosome core particles from recombinant histones and DNA.* Methods Enzymol. 375, 23-44

153. Fuchs, S. M. and Strahl, B. D. (2011) *Antibody recognition of histone post-translational modifications: Emerging issues and future prospects.* Epigenomics 3, 247-249

154. Nishikori, S., Hattori, T., Fuchs, S. M., Yasui, N., Wojcik, J., Koide, A., Strahl, B. D., and Koide, S. (2012) *Broad ranges of affinity and specificity of anti-histone antibodies revealed by a quantitative peptide immunoprecipitation assay.* J. Mol. Biol. 424, 391-399

155. Lafon, A., Petty, E., and Pillus, L. (2012) *Functional antagonism between Sas3 and Gcn5 acetyltransferases and ISWI chromatin remodelers.* PLoS Genet. 8, e1002994

156. Rosaleny, L. E., Ruiz-Garcia, A. B., Garcia-Martinez, J., Perez-Ortin, J. E., and Tordera, V. (2007) *The Sas3p and Gcn5p histone acetyltransferases are recruited to similar genes.* Genome Biol. 8, R119

157. Mujtaba, S., Zeng, L., and Zhou, M. M. (2007) *Structure and acetyl-lysine recognition of the bromodomain.* Oncogene 26, 5521-5527

158. Paolinelli, R., Mendoza-Maldonado, R., Cereseto, A., and Giacca, M. (2009) *Acetylation by GCN5 regulates CDC6 phosphorylation in the S phase of the cell cycle.* Nat. Struct. Mol. Biol. 16, 412-420

159. Liokatis, S., Stutzer, A., Elsasser, S. J., Theillet, F. X., Klingberg, R., van Rossum, B., Schwarzer, D., Allis, C. D., Fischle, W., and Selenko, P. (2012) *Phosphorylation of histone H3 Ser10 establishes a hierarchy for subsequent intramolecular modification events.* Nat. Struct. Mol. Biol. 19, 819-823

160. Bian, C., Xu, C., Ruan, J., Lee, K. K., Burke, T. L., Tempel, W., Barsyte, D., Li, J., Wu, M., Zhou, B. O., Fleharty, B. E., Paulson, A., Allali-Hassani, A., Zhou, J. Q., Mer, G., Grant, P. A., Workman, J. L., Zang, J., and Min, J. (2011) *Sgf29 binds histone H3K4me2/3 and is required for SAGA complex recruitment and histone H3 acetylation.* EMBO J. 30, 2829-2842

161. Horwitz, G. A., Zhang, K., McBrian, M. A., Grunstein, M., Kurdistani, S. K., and Berk, A. J. (2008) *Adenovirus small e1a alters global patterns of histone modification.* Science 321, 1084-1085

162. Liu, Y., Xing, Z. B., Zhang, J. H., and Fang, Y. (2013) *Akt kinase targets the association of CBP with histone H3 to regulate the acetylation of lysine K18.* FEBS Lett. 587, 847-853

163. Papazyan, R., Voronina, E., Chapman, J. R., Luperchio, T. R., Gilbert, T. M., Meier, E., Mackintosh, S. G., Shabanowitz, J., Tackett, A. J., Reddy, K. L., Coyne, R. S., Hunt, D. F., Liu, Y., and Taverna, S. D. (2014) *Methylation of histone H3K23 blocks DNA damage in pericentric heterochromatin during meiosis.* Elife 3, e02996

164. Grunstein, M. and Gasser, S. M. (2013) *Epigenetics in saccharomyces cerevisiae.* Cold Spring Harb Perspect. Biol. 5, 10.1101/cshperspect.a017491

165. Suka, N., Suka, Y., Carmen, A. A., Wu, J., and Grunstein, M. (2001) *Highly specific antibodies determine histone acetylation site usage in yeast heterochromatin and euchromatin.* Mol. Cell 8, 473-479

166. Shogren-Knaak, M. A. and Peterson, C. L. (2004) *Creating designer histones by native chemical ligation.* Methods Enzymol. 375, 62-76

167. Bian, C., Xu, C., Ruan, J., Lee, K. K., Burke, T. L., Tempel, W., Barsyte, D., Li, J., Wu, M., Zhou, B. O., Fleharty, B. E., Paulson, A., Allali-Hassani, A., Zhou, J. Q., Mer, G., Grant, P. A., Workman, J. L., Zang, J., and Min, J. (2011) *Sgf29 binds histone H3K4me2/3 and is required for SAGA complex recruitment and histone H3 acetylation.* EMBO J. 30, 2829-2842

168. Lo, W. S., Trievel, R. C., Rojas, J. R., Duggan, L., Hsu, J. Y., Allis, C. D., Marmorstein, R., and Berger, S. L. (2000) *Phosphorylation of serine 10 in histone H3 is functionally linked in vitro and in vivo to Gcn5-mediated acetylation at lysine 14.* Mol. Cell 5, 917-926

169. Kelly, R. D. and Cowley, S. M. (2013) *The physiological roles of histone deacetylase (HDAC) 1 and 2: Complex co-stars with multiple leading parts.* Biochem. Soc. Trans. 41, 741-749

170. Gil, J. and O'Loghlen, A. (2014) *PRC1 complex diversity: Where is it taking us?* Trends Cell Biol. 24, 632-641

171. Rudolph, T., Beuch, S., and Reuter, G. (2013) *Lysine-specific histone demethylase LSD1 and the dynamic control of chromatin.* Biol. Chem. 394, 1019-1028

172. Blair, L. P., Avaritt, N. L., and Tackett, A. J. (2012) *Application of MassSQUIRM for quantitative measurements of lysine demethylase activity.* J. Vis. Exp. (61). pii: 3604. doi, 10.3791/3604

173. Blair, L. P., Avaritt, N. L., Huang, R., Cole, P. A., Taverna, S. D., and Tackett, A. J. (2011) *MassSQUIRM: An assay for quantitative measurement of lysine demethylase activity.* Epigenetics 6, 490-499

Curriculum Vitae

ANNE M. CIENIEWICZ

103 East Mount Royal Avenue#901, Baltimore, MD 21202 | 610-334-9796 |
amcieniewicz@gmail.com

PROFESSIONAL SUMMARY

A Pharmacology Ph.D. candidate at The Johns Hopkins University School of Medicine with a solid professional and scientific track record. Earned a B.S. in Pharmaceutical Product Development at West Chester University of Pennsylvania, while gaining pharmaceutical industry experience through an internship at Cephalon, Inc. (now acquired by Teva). Performed cell-based assays while working with the West Chester University Gap Junction Research Team resulting in two first-author publications. Awarded a prestigious grant from the National Science Foundation to independently fund thesis research at Johns Hopkins University in the study of epigenetic drug targets, which culminated in an impactful, peer-reviewed publication. Extensive scientific skillsets in biochemistry, proteomics, molecular biology, *in vitro* and *in vivo* systems, and tissue collection. Possess valuable leadership experience and effective team collaboration serving as the Co-President for the Hopkins Biotech Network, while successfully leading a cross-matrix team of biomedical doctoral candidates, MBA students, postdoctoral associates and alumni. Concurrently mentored young scientists in routine laboratory techniques and tutored underrepresented youth at a Baltimore inner-city high school. Volunteered as a grant writer for Charm City Clinic, a non-profit health clinic in Baltimore, and worked closely with a collaborative team of students and doctors to strategically secure funding for the next several years.

EDUCATION

- Ph.D. Pharmacology and Molecular Sciences — August 2010 - present
  The Johns Hopkins University School of Medicine

- Independent Coursework — Academic Year 2013 and 2014
  The Johns Hopkins University Carey Business School

- B.S., Pharmaceutical Product Development — August 2006 – December 2009
  West Chester University of Pennsylvania

RESEARCH EXPERIENCE AND RELEVANT SKILLSETS

- Graduate Research Scientist August 2010 – present
  Lab of Dr. Sean Taverna
  Pharmacology and Molecular Sciences and the Center for Epigenetics
  The Johns Hopkins University School of Medicine

  Summary: Thesis work focused on how epigenomic pathways affect gene expression with a particular emphasis on the enzymatic activity of epigenetic protein complexes, which are currently being targeted for drug discovery in many disease settings. This work has been published in a highly reputable, peer-reviewed journal as well as allowed me to attend and present at several international conferences.

  Skills: Biochemistry; Proteomics; Molecular Biology
  - *In vitro* enzymatic assay development and optimization
  - Extensive peptide, protein and protein complex purification using AKTA FPLC system and HPLC
  - PCR, site-directed mutagenesis, molecular cloning and recombinant protein expression in bacteria and yeast
  - Western blot and ELISA analysis
  - Peptide chemistry and synthesis used to perform native chemical ligation
  - Mass spectrometry: trypsin digestion, deuterium labeling, MALDI-TOF instrumentation, and mass spectra analysis
  - SDS-PAGE and Acid-Urea gel electrophoresis

- Undergraduate Research Assistant May 2007 – December 2009
  Lab of Dr. Richard Woodruff
  Gap Junction Research Team
  West Chester University of Pennsylvania Department of Biology

  Summary: Studied cell-to-cell communication and the passage of large protein molecules in neighboring germ and epithelial cells. Successfully advanced this project from insects to mammals resulting in two first author, peer-reviewed publications as an undergraduate student.

  Skills: Cell-based Assays; *In-vivo* Biology; Animal Dissection; Tissue Collection
  - Live cell imaging using fluorescence and confocal microscopy
  - Ovary dissection and tissue collection of insects, fish, amphibians, and mice
  - Cell culture experience with WB (rat liver epithelium) and HeLa cell lines
  - Microinjection of oocytes and epithelial cells

- Fluorescent labeling of proteins

- Summer Intern Researcher June 2009 – August 2009
  Drug Safety and Disposition Department
  Cephalon, Inc. (Currently Teva)

  Summary: Designed and performed *in vitro* assays to identify metabolites generated by cytochrome P450 enzymes acting on various drug compounds. Determined pharmacokinetics on rates of formation using LC-MS/MS and learned to meet deadlines in a team-oriented environment.

  Skills:
  - *In vitro* pharmacokinetics assays
  - LC-MS/MS analysis of drug metabolites

PEER-REVIEWED PUBLICATIONS

- Ringel, A.E., **Cieniewicz, A.M**., Taverna, S.D., Wolberger, C. (2015) Recognition of H3K4 trimethylation by Sgf29 promotes processive acetylation by the SAGA HAT module. *SUBMITTED*

- **Cieniewicz, A.M.**, Moreland, L., Ringel, A.E., Mackintosh, S. G., Raman A., Gilbert T.M., Wolberger C., Tackett, A.J. and Taverna, S.D. (2014) The bromodomain of Gcn5 regulates site-specificity of lysine acetylation of histone H3. Molecular and Cellular Proteomics PMID 25106422.

- Prusevich, P., Kalin, J.H., Ming, S.H., Basso, M., Givens, J., Li, X., Hu, J., Taylor, M. S., **Cieniewicz, A.M.**, Hsiao, P.Y., Huang R., Roberson, H., Adejola, N., Avery, L.B., Casero, R.A., Taverna, S.D., Qian, J., Tackett, A.J., Ratan, R.R., McDonald, O.G., Feinberg, A.P., Cole, P.A. (2014) A selective Phenelzine analog inhibitor of Histone Demethylase LSD1. ACS Chemical Biology 9(6):1284-93.

- **Cieniewicz, A.M.**, Woodruff, R.I. (2010) Passage through vertebrate gap junctions of 17/18 kDa molecules is primarily dependent upon molecular configuration. Tissue and Cell 42: 47-52.

- **Cieniewicz, A.M.**, Woodruff, R.I. (2008) Importance of molecular configuration in gap junctional permeability. Journal of Insect Physiology 54: 1293 – 300.

HONORS AND AWARDS

- National Science Foundation September 2012 – present
  Graduate Research Fellowship
  The National Science Foundation
  Received a $126,000 grant to fund thesis research focused on the characterization of an epigenetic drug target.

- Student Fellowship October 2014
  Discovery on Target Conference
  Financial support to attend an international conference and present research to biotechnology and pharmaceutical professionals and representatives.

- Published Abstract and Research Presentation November 2013
  Abcam: Chromatin Structure and Function, Grand Cayman Islands
  The bromodomain of Gcn5 regulates site specificity and processivity of lysine acetylation on histone H3. Cieniewicz, A.M., Moreland, L., Ringel, A.E., Wolberger C., Tackett, A.J. and Taverna, S.D.
  Attended and presented at an international scientific conference sponsored by Abcam, Inc., in the Grand Cayman Islands.

- West Chester University Annual Poster Session May 2009
  1st Place Undergraduate Award
  West Chester University of Pennsylvania
  Presented with distinction work on the cell-to-cell transfer of protein molecules through gap junctions during a university-wide conference.

- Centocor Scholarship Academic Year 2008
  West Chester University of Pennsylvania
  Received scholarship to pursue undergraduate education in Pharmaceutical Product Development.

LEADERSHIP AND COMMUNITY OUTREACH

- Co-President June 2014 – present
  Hopkins Biotech Network (HBN)
  HBN connects the Johns Hopkins community with the biotechnology and pharmaceutical industries, with a network of over 2000 representatives from the science and business sectors. Developed and implemented a plan to increase the HBN budget and expand HBN membership. Together, these strategies resulted in the recruitment of faculty sponsors and board members and increased funds that were used to:
    - Design and launch a new website
    - Generate a new organization logo
    - Organize and implement a bio-entrepreneurial training initiative
    - Assemble a committee for corporate outreach
    - Extend the HBN brand across Johns Hopkins campuses to unite scientists, business students, and biotechnology professionals

- Director of Industry Relations August 2013 – June 2014
  Hopkins Biotech Network (HBN)
  Responsible for collaboratively working with a team and utilizing effective communication to establish partnerships with local biotech and pharmaceutical companies. Successfully implemented funding guidelines and expectations of corporate sponsors. Organized and hosted 3 career events that were attended by hundreds of Johns Hopkins students.

- Grants Team Member June 2013 – August 2014
  Charm City Clinic
  Volunteered for non-profit health clinic in East Baltimore run by medical, graduate and undergraduate students. Responsibilities included identifying funding opportunities, hosting and organizing fundraisers, determining clinic eligibility, and filling out grant applications.

- Incentive Mentoring Program April 2011 – June 2014
  Dunbar High School, Baltimore, MD
  Served as a mentor for underrepresented high school students. Collaborated with a group of volunteers to coach, tutor, and guide students in planning for future educational and career options. Over 90% of students graduate high school and were accepted in a 2 or 4 year university.

- Healthcare Case Manager June 2013 – August 2014
  Charm City Clinic

Worked with a nonprofit health clinic in the City of Baltimore and consulted with clients to provide them with a better understanding of healthcare options and ensuring they are receiving proper benefits.

- Center for Talented Youth Mentor Summer 2012 and Summer 2013
  The Johns Hopkins University
  Supervised and trained two junior scientists in learning laboratory techniques, organizing a summer research project, and preparing a poster presentation. These individuals are now undergraduate researchers performing cutting-edge research at premier US universities.