ANALYSIS OF HISTONE LYSINE METHYLATION USING MASS SPECTROMETRY

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To My Parents, Leanne and Russ

Thank you for your support

through everything.

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LIST OF ABBREVIATIONS

ac	acetyl
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BCA	bicinchronic assay
Benz	Benzonase
BME	2-beta mercaptoethanol
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CAM	2-chloroacetamide
CBP	calmodulin binding protein
ChIP-chip	chromatin immunoprecipiation – chip
Chy	chymotrypsin
CID	collision induced dissociation
COMPASS	complex associated with Set1
CTD	carboxy terminal domain
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescense
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FDR	false discovery rate

FSC	fused silica
HAT	histone acetyltransferase
HDAC	histone deacetyltransferase
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
ID	inner diameter
IGEPAL	octylphenoxypolyethoxyethanol
K	lysine
KOH	potassium hydroxide
LTQ	linear trap quadrupole
me	monomethyl
me2	dimethyl
me3	trimethyl
MgOAC	magnesium acetate
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MudPIT	multi-dimensional protein identifiation
n/a	not applicable
NaCl	sodium chloride
NDE	no dynamic exclusion
OD	optical density
ORF	open reading frame
P	proline

PA	propionic anhydride
Pgk1	phosphoglycerate kinase 1
PRO	propionylated
RNA	ribonucleic acid
RNAPII	RNA Polymerase II
RP	reverse phase
rpm	rotations per minute
RT	retention time
RTR1	regulator of transcription 1
S	serine
SCX	strong cation exchange
SDS PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SWI-SNF	switch-sucrose non fermentable
T	threonine
TAP	tandem affinity purification
TCA	trichloroacetic acid
TCEP	tris(2-carboxyethyl)phosphine
TEV	tobacco etch virus
Tris	tris(hydroxmethyl)aminomethane
TTS	transcription termination site
ub	ubiquitination
UTX	untreated
WR	working reagent

WT	wildtype
Y	tyrosine
YPD	yeast peptone dextrose

INTRODUCTION

I. Chromatin

Eukaryotic DNA is compacted in the nucleus by wrapping around histone proteins. The combination of DNA and histones is referred to as chromatin (Li and Reinberg 2011). There are 4 core histones (histone H2A, histone H2B, histone H3, histone H4) and 1 linker histone (histone H1), plus variants of the core histones in different organisms. The histone octamer consists of two dimers of histone H2A – histone H2B and one tetramer of histone H3 – histone H4 as shown in Figure 1 (reviewed in De Koning, Corpet et al. 2007).

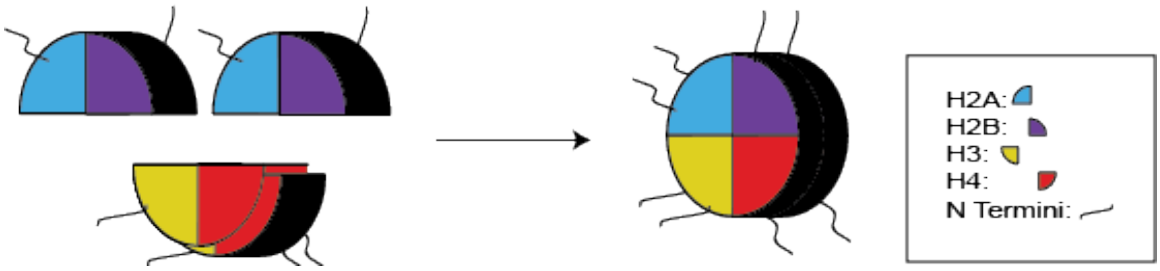

Figure 1. Core histone octamer assembly. Two dimers of histone H2A – histone H2B and one tetramer of histone H3 – histone H4 join together to form the histone octamer. The N-termini of the histones are highly charged and unstructured. The specific core histones and the N-terminal tails are illustrated as indicated in the figure legend to the right.

It is known that 147 base pairs of DNA wrap around each fully formed histone octamer forming a unit called the nucleosome. The octamer forms a highly structured globular core that has approximately 14 contact points with DNA allowing a tight interaction between the octamer and DNA as determined by X-ray crystallography (Luger, Mader et al. 1997). Interestingly the N-termini of

the core histones are highly charged and unstructured thereby making addition domains for protein-protein interaction that extend from the globular core (illustrated in Figure 1). The main function of histones is to condense and protect the DNA and allow for compaction of the DNA in the nucleus. Histone H1 interacts with the DNA between nucleosomes (Ushinsky, Bussey et al. 1997; Patterton, Landel et al. 1998) and promotes the compaction of DNA into the 30 nm fiber also known as “heterochromatin” (reviewed in Woodcock and Ghosh 2010). The uncompacted areas of chromatin that are transcriptionally active are depleted in histone H1 and are referred to as “euchromatin” (reviewed in Woodcock and Ghosh 2010).

In budding yeast *Saccharomyces cerevisiae* (which will be referred to henceforth as yeast) there are 2 genes that encode each core histone. The genes encoding the core histones are: *HTA1* and *HTA2* (histone H2A), *HTB1* and *HTB2* (histone H2B), *HHT1* and *HHT2* (histone H3), and *HHF1* and *HHF2* (histone H4). The two genes for histone H2A have extremely high sequence identity and the same is true for the two genes for histone H2B (as shown by the amino acid sequences in Figure 2). The two genes for histone H3 are identical in sequence and the same is true for histone H4 (as shown by the amino acid sequences in Figure 3).

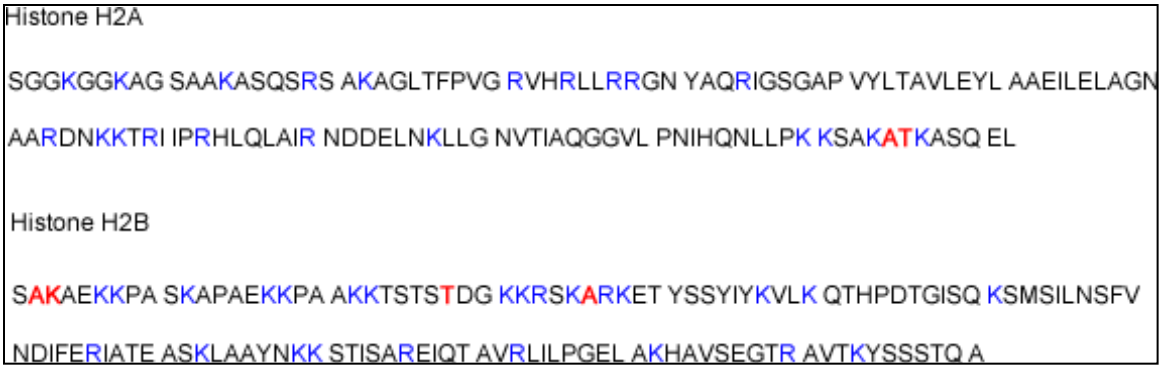

Figure 2. Amino acid sequences of histone H2A and histone H2B. Basic residues are in blue. Residues 124 and 125 (AT) in histone H2A are reversed in the protein product from *HTA2*. Residues 2 and 3 (AK) in histone H2B are changed to SA, and residues 27 (T) and 35 (A) are both changed to valine in the protein product form *HTB2.* Both versions of histone H2A are 13,989 Daltons (pI = 11.43). Histone H2B from *HTB1* is 14,252 Daltons (pI = 10.92), while histone H2B from *HTB2* is 14,237 Daltons (pI = 10.89). Sequences, molecular weights, and isoelectric points were obtained from www.yeastgenome.org.

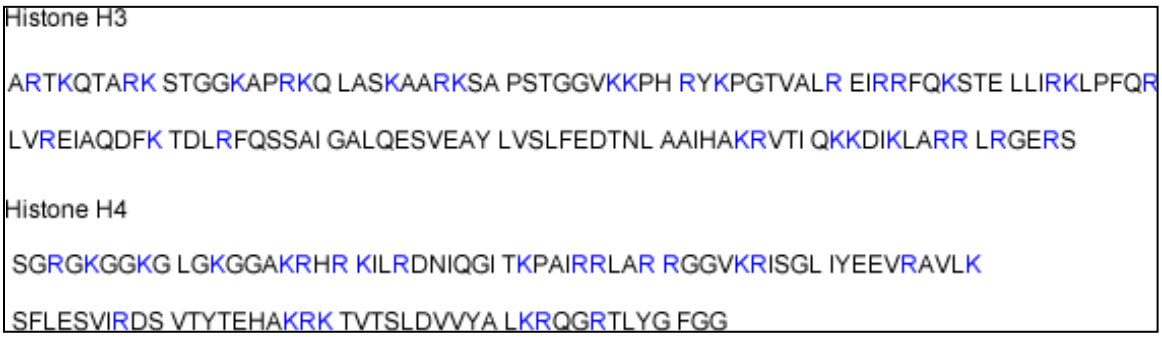

Figure 3. Amino acid sequences of histone H3 and histone H4. Basic residues are in blue. Both copies of the genes encoding histone H3 and histone H4 are identical. Histone H3 is 15,356 Daltons (pI = 12.0) and histone H4 is 11,368 Daltons (pI = 11.95). Sequences, molecular weights, and isoelectric points were obtained from www.yeastgenome.org.

The histone octamer has to be assembled, disassembled, and reassembled throughout the cell cycle especially during processing that require

access to the DNA such as DNA replication and RNA transcription. Proteins called “histone chaperones” have been identified that facilitate the assembly and disassembly of the histone octamer (Avvakumov, Nourani et al. 2011). These histone chaperones can basically work alone (i.e. Nap1 (Mosammaparast, Ewart et al. 2002)), work as a complex (i.e. FACT (Belotserkovskaya, Oh et al. 2003)), or work within an enzymatic complex (i.e. Arp4 which is a subunit of the SWR1 complex (Harata, Oma et al. 1999)). Specific karyopherins (or importins) are also needed to transport the histones from the cytoplasm into the nucleus (reviewed in Keck and Pemberton 2011). The main karyopherin involved in the import of histone H2A and histone H2B is Kap114 (Mosammaparast, Jackson et al. 2001). Kap121 and Kap123 are the main karyopherins for histone H3 and histone H4 (Mosammaparast, Guo et al. 2002).

Asf1 and Nap1 are two of the most well characterized histone chaperones. Asf1 is thought to be the main histone chaperone that interacts with histone H3 – histone H4 (Bao and Shen 2006). Nap1 is thought to be the main histone chaperone that interacts with H2A-H2B (Mosammaparast, Ewart et al. 2002). Histone chaperones and karyopherins interact with nuclear localization signals in the N-termini of the histones to import the histones into the nucleus (reviewed in Keck and Pemberton 2011).

It has also been well established that histones and chromatin structure are also important for regulation of gene expression. This regulation of gene expression is managed through covalent modifications specifically on the histone N-termini and somewhat throughout the globular portion of the histones (Li,

Carey et al. 2007). These histone modifications may also regulate the higher order structure of chromatin including dynamic assembly and disassembly of heterochromatin (Jenuwein and Allis 2001).

II. Histone Modifications

Proteins, in general, are known to have various types of modifications at certain amino acid residues post-translation. This discussion focuses on histone modifications in yeast. The most widely characterized histone modifications are acetylation and methylation. Other known modifications also include ubiquitination, sumoylation, deimination, phosphorylation, ADP ribosylation, and proline isomerization (reviewed in Kouzarides 2007). Histone lysine residues can be acetylated (Gershey, Vidali et al. 1968), methylated (mono-, di-, or tri-) (Murray 1964), ubiquitinated (Goldknopf, Taylor et al. 1975; West and Bonner 1980), or sumoylated (Nathan, Ingvarsdottir et al. 2006). Histone arginine residues can be methylated (mono- or di-) (Byvoet, Shepherd et al. 1972) or deiminated to citrulline (Cuthbert, Daujat et al. 2004). Histone serines can be phosphorylated (Ahn, Cheung et al. 2005; Cheung, Turner et al. 2005), while ADP ribosylation can occur at glutamate (Ogata, Ueda et al. 1980). Finally, cis-proline can be isomerized to trans-proline (Nelson, Santos-Rosa et al. 2006). The most well characterized histone modifications in yeast are shown in Figures 4 and 5.

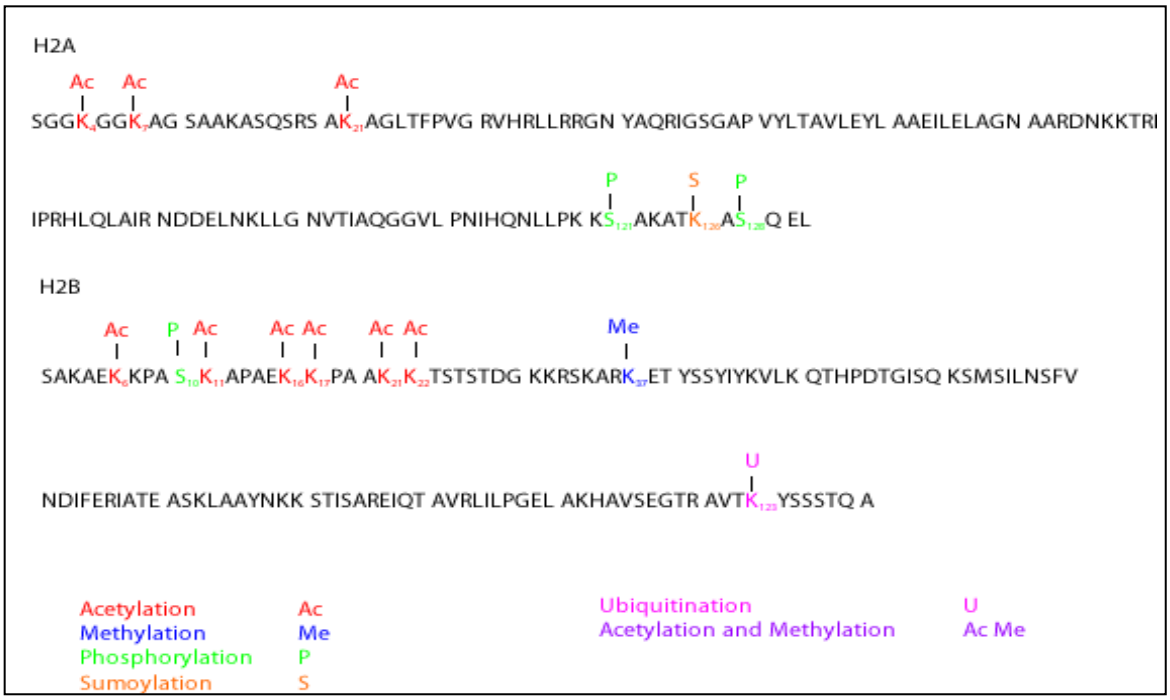

Figure 4. Most well characterized histone modifications for histone H2A and histone H2B in yeast. Color-coding for each type of modification is listed in the legend below the sequences.

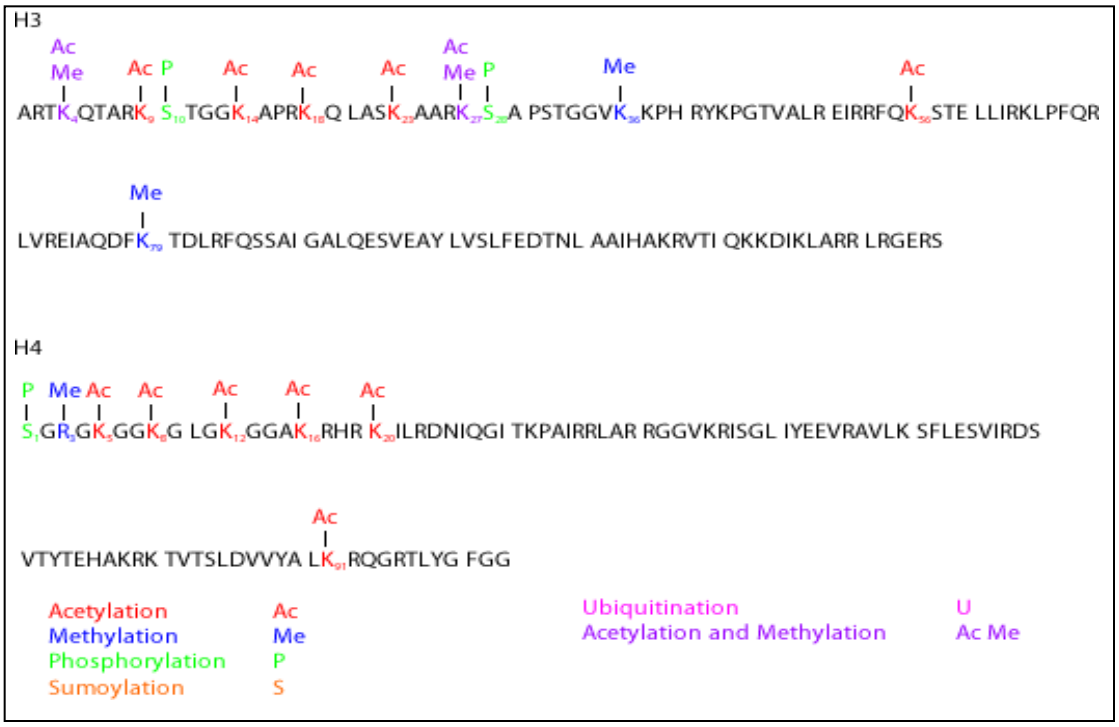

Figure 5. Most well characterized histone modifications for histone H3 and histone H4 in yeast. Color-coding for each type of modification is listed in the legend below the sequences.

In 2001 Jenuwein and Allis proposed the “histone code” hypothesis, which suggests that combinations of the different histone PTMs form a pattern of inheritance in addition to the genome (Jenuwein and Allis 2001). This hypothesis further suggests that different modifications would interact with different proteins and modifications could be interdependent (Jenuwein and Allis 2001). Individual histone modifications and combinations of histone modifications have been shown to be important in the regulation of transcription (Li, Carey et al. 2007).

To further refine the “histone code” hypothesis, the idea of “readers”, “erasers”, and “writer” was suggested. Enzymes that add a modification on histones are referred to as “writers”, while the enzmes that remove the

modifications are called “erasers” (Ruthenburg, Allis et al. 2007). Generally, histones are acetylated by histone acetyltransferases (HATs) and deacetylated by histone deacetylases (HDACs). Histones are methylated by lysine and arginine methyltransferases and demethylated by lysine and arginine demethylases (reviewed in Li, Carey et al. 2007). Thus, HATS and methyltransferases are “writers”, and the HDACS and demethylases are “erasers”.

The most widely studied HAT in yeast is Gcn5, which is part of the SAGA complex (Brown, Lechner et al. 2000). The SAGA complex is responsible for the acetylation of histone H3. HATs require acetyl-CoA as a cofactor to acetylate lysine residues as shown in Figure 6 (Takahashi, McCaffery et al. 2006). Other HATs in yeast include Esa1, Sas3, and Hat1. Esa1 is part of the NuA4 histone acetyltransferase complex and acetylates the N-terminus of histone H4 (Allard, Utley et al. 1999). Sas3 is part of the NuA3 histone acetyltransferase complex and acetylates histone H3 (John, Howe et al. 2000). Finally, Hat1 forms a complex with Hat2 and acetylates histone H4 (Parthun, Widom et al. 1996).

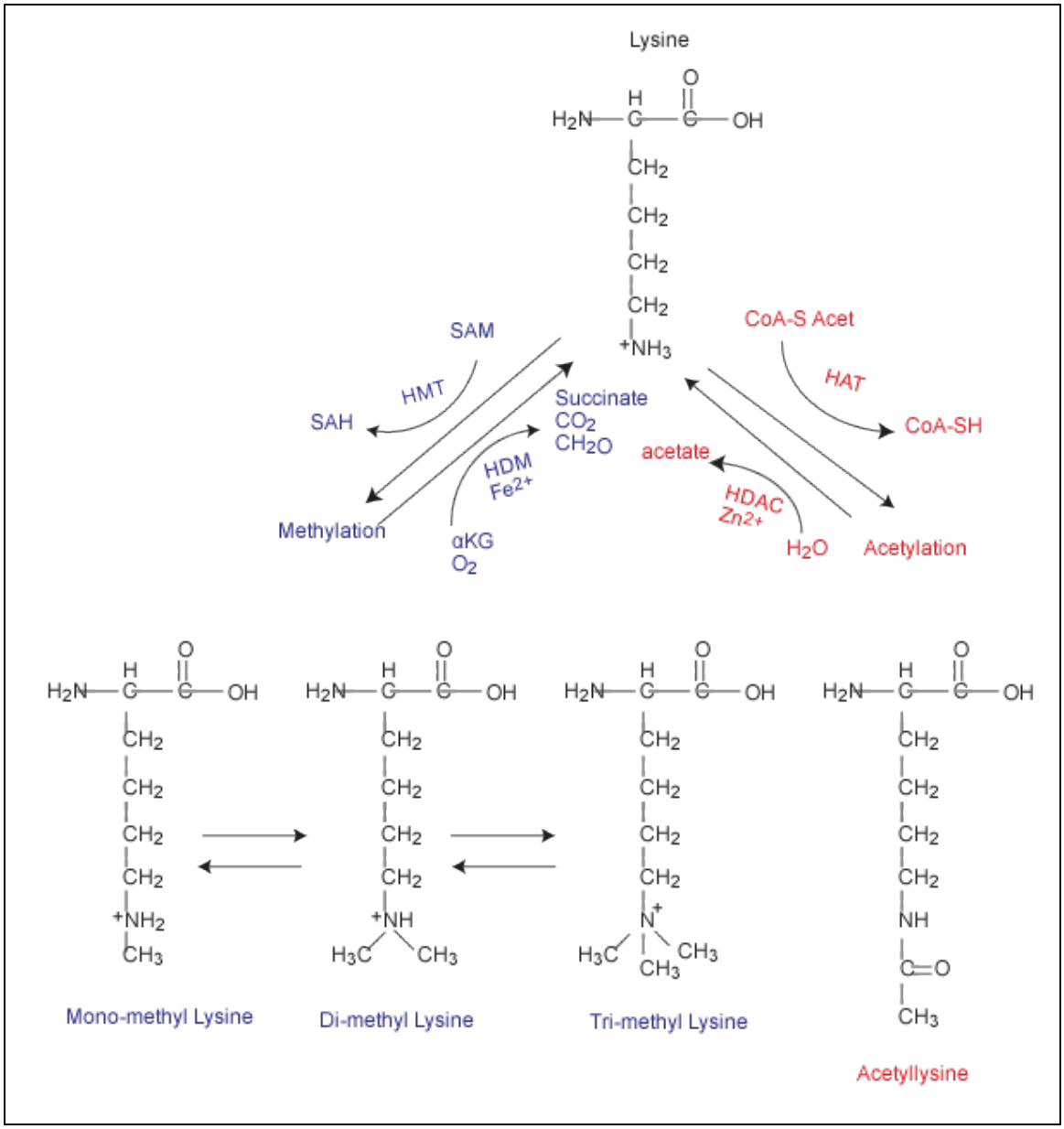

Figure 6. Mechanism of lysine methylation and acetylation. HMT = histone methyltransferase, HDM = histone demethylase, HAT = histone acetyltransferase, HDAC = histone deacetylase. Lysine methylation is processive and occurs mono-, to di-, to tri-methyl. Methylation does not change the charge on lysine, while acetylation neutralizes the charge on lysines at physiological pH.

There are four classes of HDACs, classes I, II, III, and IV. HDAC classes I, II, and IV are similar, while class III contains the sirtuin proteins, which are

involved in gene silencing and utilize an NAD^+ dependent mechanism. The class I, II, and IV HDACs use a zinc dependent mechanism to remove the acetyl group from the lysine residue as shown in Figure 6 (Hernick and Fierke 2005). Yeast have three class I HDACs (Rpd3, Hos2, and Hos1) and two class II HDACs (Hda1 and Hos3) (Ekwall 2005).

There have been three lysine methyltransferases identified in yeast: Set1 (H3K4), Set2 (H3K36), and Dot1 (H3K79) (Krogan, Dover et al. 2002; Strahl, Grant et al. 2002; van Leeuwen, Gafken et al. 2002). Both Set1 and Set2 are discussed in the next section. Dot1 is different from Set1 and Set2 in that it does not contain a catalytic SET domain and is involved in telomeric silencing (Ng, Feng et al. 2002). It does contain an AdoMet-binding domain, which means that it uses S-adenosyl methionine (SAM) as a substrate for lysine methylation like Set1 and Set2 (Ng, Feng et al. 2002).

So far, three lysine demethylases have been identified that play a role in the regulation of histone methylation in yeast. Jhd1 was the first to be identified, and it demethylates H3K36 (Tsukada, Fang et al. 2006). Rph1 specifically demethylates H3K36 tri- and di-methyl modification states (Klose, Gardner et al. 2007). Jhd2 demethylates H3K4 (Huang, Chandrasekharan et al. 2010). Figure 6 shows that these demethylases, all members of the Jumonji C (JmjC)-domain containing demethylase family; use α-ketoglutarate, iron, and oxygen to remove the methyl group from lysine (Klose, Kallin et al. 2006). The demethylase responsible for H3K79 has not yet been identified (Krogan, Dover et al. 2002).

Along with the “writers” and “erasers” there is another class of enzymes called the “readers” that is important for this discussion. “Readers” are the enzymes that preferentially bind to the modifications that “writers” place on the histones (Ruthenburg, Allis et al. 2007). These “readers” have domains that bind preferentially to either acetyl-lysine, methyl-lysine, or other modification specific forms of histones.

Proteins with a chromodomain bind to methyl-lysine (Jacobs, Taverna et al. 2001). An example of a yeast chromodomain containing protein is Eaf3, which is part of the NuA4 histone acetyltransferase complex and Rpd3 histone deacetylase complex (Reid, Moqtaderi et al. 2004; Joshi and Struhl 2005). The chromodomain of Eaf3 binds specifically to methylated H3K36, and helps to direct deacetylation in active gene coding regions (Carrozza, Li et al. 2005; Joshi and Struhl 2005).

Proteins with a bromodomain bind to acetyl-lysine (Mujtaba, Zeng et al. 2007). The acetyltransferase Gcn5 contains a bromodomain that allows the SAGA complex to bind already acetylated nucleosomes and acetylate nearby nuclesomes (Li and Shogren-Knaak 2009).

Another class of “readers” contain a plant homeodomain (PHD) finger, which binds to either methylated or unmethylated lysines (Wysocka, Swigut et al. 2006; Lan, Collins et al. 2007). An example of a PHD finger containing protein in yeast is Yng1, which is part of the NuA3 histone acetyltransferase complex and binds to methylated H3K4 (Martin, Baetz et al. 2006). The H3K4 demethylase

Jhd2 also contains a PHD finger, but it has been shown that this PHD finger does not bind to methylated H3K4 (Huang, Chandrasekharan et al. 2010).

III. The Role of Histone Modifications in Transcription

RNA Polymerase II (RNAPII) is known to be the key enzyme for transcription of mRNAs, snRNAs, and microRNAs. Histones' tight interaction with DNA provides a problem for the passage of RNA Pol II during transcription. The histones have to be removed from the DNA before RNA Pol II can transcribe the DNA. The histones then have to be put back to once again reassemble the chromatin and protect the DNA.

In yeast, a study using high-resolution microarrays showed that over gene promoters, there is an average 200 base pair nucleosome free region (Yuan, Liu et al. 2005). Sequence specific transcriptional activators can bind to or promote formation of this nucleosome-free region at the promoter and recruit general transcription factors, chromatin remodeling complexes and histone modifiers. This includes SWI-SNF, a chromatin-remodeling complex that uses ATP to disrupt the interaction between the histone octamer and DNA. HATs are also recruited by interactions with transcription factors and acetylate lysine residues on the histones (Brown, Howe et al. 2001). This acetylation neutralizes the charge on the lysine residues and is thought to decrease the interaction between the octamer and DNA and/or recruit other transcriptional activators through interactions with bromodomains. Acetylation of H3 and H4 (Figure 5) has been

shown to peak at active promoters and correlates with transcription (Figure 7) (Pokholok, Harbison et al. 2005).

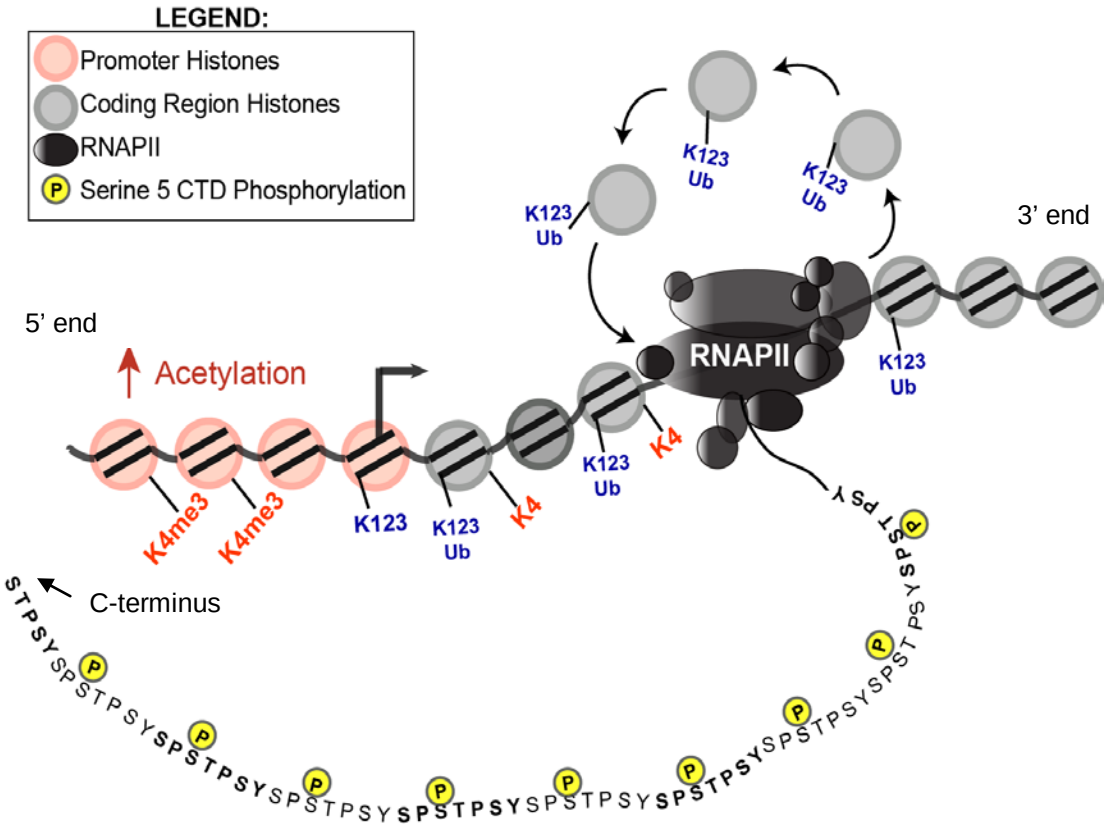

Figure 7. Model of the coupling of histone modification and transcription. Acetylation is increased at active gene promoters along with H3K4me3. H2BK123ub is present at the beginning of active genes. Serine 5 phosphorylation of the CTD of RNAPII is highest at the beginning of the gene and facilitates recruitment of histone methyltransferases to carry out co-transcriptional histone H3K4 methylation. (K4me3 = lysine 4 trimethylation, K123ub = lysine 123 ubiquitination, CTD = C-terminal domain, RNAPII = RNA Polymerase II.)

The largest subunit of RNAPII is Rpb1, which has a C-terminal domain (CTD) consisiting of 27 repeats of the amino acid sequence YSPTSPS. Phosphorylation of the CTD at serines at position 2, 5, and 7 in the repeat has

been shown to correlate with transcription (reviewed in Buratowski, 2009). Prior to the transcription initiation complex forming at the promoter, the CTD is not phosphorylated. Once RNAPII releases from the promoter, serine 5 phosporylation peaks. As transcription progresses, serine 5 phosphorylation begins to decline and serine 2 phosphorylation increases (Komarnitsky, Cho et al. 2000). It is important to note that serine 5 phosphorylation is not completely removed during early transcription, and the CTD can be doubly phosphorylated at serines 2 and 5 (Phatnani and Greenleaf 2006).

The double phosphorylation of the CTD at serines 2 and 5 is important for recruitment of the histone methyltransferase Set2. Set2 is known to methylate H3K36 (Kizer, Phatnani et al. 2005). It also binds the RNA Pol II CTD only when serines 2 and 5 are both phosphorylated, which has been validated *in vitro* by NMR and occurs *in vivo* during transcription elongation (Figure 8) (Vojnic, Simon et al. 2006). Set2 is able to methylate H3K36, resulting in a specific methylation mark that is enriched in the coding region of transcriptionally active genes (Kizer, Phatnani et al. 2005; Pokholok, Harbison et al. 2005; Strahl, Grant et al. 2002).

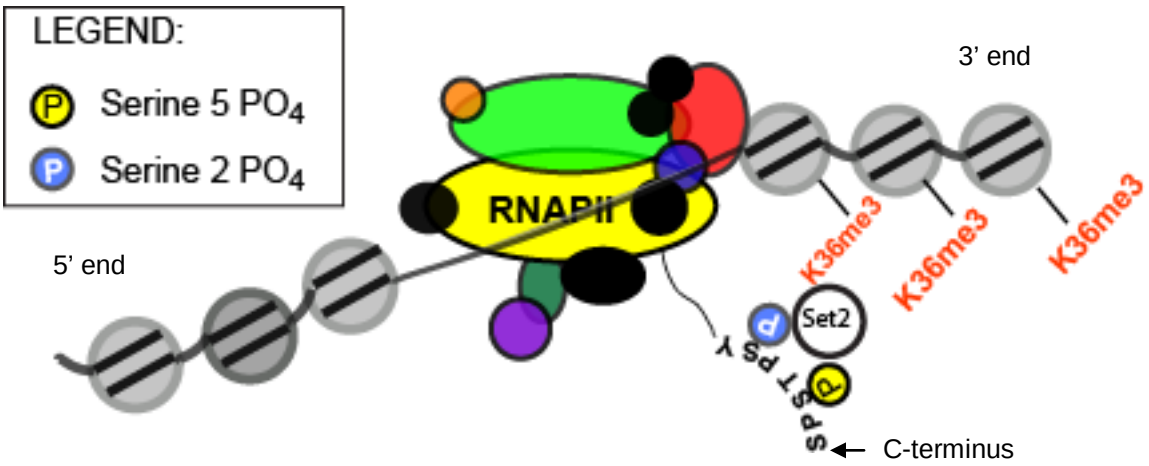

Figure 8. Set2 binds to the S2,S5-P double phosphorylated CTD of RNAPII during transcription. Set2 then methylates H3K36, a mark that is only present in transcriptionally active genes.

H3K4 can be acetylated, monomethylated, dimethylated, or trimethylated (Strahl, Ohba et al. 1999; Bernstein, Humphrey et al. 2002; Guillemette, Drogaris et al. 2011). It has been shown by ChIP-chip that H3K4me3 peaks at the transcription start site (TSS), H3K4me2 peaks in the middle of the open reading frame (ORF), and H3K4me peaks at the 3' end of the ORF (reviewed in Li, Carey et al. 2007; Pokholok, Harbison et al. 2005). The methyltransferase responsible for methylation of H3K4 is a protein called Set1 that is part of a multi-protein complex called COMPASS (complex associated with Set1) (Krogan, Dover et al. 2002). COMPASS has been shown to bind to RNAPII when the CTD has been phosphorylated at serine 5 and when another protein complex called the Paf1 complex is present (Gerber and Shilatifard 2003).

IV. MudPIT

Multidimensional Protein Identification (MudPIT) utilizes the separation abilities of high performance liquid chromatography followed by peptide analyses by mass spectrometry (Florens and Washburn 2006). This allows for more complete identification of complex mixtures than using gel separation. Samples are typically denatured in 8 M Urea, reduced with TCEP, alkylated with chloroacetamide and incubated with trypsin, which cleaves the peptide backbone C-terminal to lysine and arginine residues. The samples are then loaded into a column that is packed with strong cation exchange (SCX) resin followed by reverse phase (RP) resin. Charged peptides have a high affinity for the SCX resin and can be eluted onto the RP resin with increasing concentrations of salt (specifically ammonium acetate). The RP resin separates peptides based on their hydrophobicity (Florens and Washburn 2006).

An organic gradient of increasing acetonitrile is then run through the column to elute the peptides off the RP resin. When the peptide fragments reach the tip of the column, they are ionized by nanospray ionization. The type of mass spectrometer used to analyze the peptide fragments can vary. A typical mass spectrometer used for MudPIT is a linear ion trap instrument such as a Linear Trap Quadrupole (LTQ) (Thermo).

A linear ion trap mass spectrometer can use a peptide fragmentation method called low energy collision induced dissociation (CID). This occurs by colliding the peptide fragments with an inert gas like helium. This low energy CID generates a spectrum predominately made up of b and y ions, which are the ions

generated after the amide bond breaks. The difference between b and y ions is whether the charge is on the N-terminal end (b-ion) or the C-terminal end (y-ion) (Zhang 2004; Paizs and Suhai 2005).

The mass spectrometer selects the most abundant ions from the initial MS scan (the number can vary and is manually selected) and fragments them via CID, which is called MS/MS or MS^2. This allows for the more abundant peptides to be analyzed further (Florens and Washburn 2006). A property called dynamic exclusion can be used to limit the amount of times that a peptide is selected for fragmentation. The higher the dynamic exclusion, the more sampling of the peptides occurs, while the opposite is also true. Without dynamic exclusion, only the most abundant peptides are analyzed, and the lower abundant peptides that co-elute with the high abundance peptides are undersampled or not sampled. But with the dynamic exclusion set too high, the number of spectral counts for the more abundant peptides decreases without significantly increasing the number of proteins identified (Zhang, Wen et al. 2009). This means there is a fine line with the selection of dynamic exclusion time settings that should be optimized from experiment to experiment.

The mass spectrometer generates MS and MS/MS spectra of the detected ions from each point in the analysis. These spectra can then be searched using a database search algorithm like SEQUEST®, which will compare the precursor mass and the experimental MS/MS fragment spectra obtained against the calculated mass and theoretical MS/MS spectra from a selected peptide in a protein database, in our case the entire yeast protein database (Eng,

McCormack et al. 1994). This allows for an unbiased comparison of all the spectra to best determine the identity of the peptides and match them back to the correct protein. This approach is refered to as bottom up proteomics (reviewed in Guerrera and Kleiner 2005).

Histones present a problem for the standard approach using mass spectrometry. Histones are highly basic proteins, as shown in Figures 2 and 3. When digested with trypsin, the tryptic peptides are often highly charged and rather small. These peptides are then difficult for a mass spectrometer to detect and analyse. Another enzyme that could be used for digestion of the histones is the endoproteinase ArgC, also known as Clostripain, which cleaves the peptide backbone C-terminal to arginine residues (Gilles, Imhoff et al. 1979). However, ArgC does not have a high digestion efficiency like trypsin and would result in highly charged histone peptides containing multiple lysine residues. Techniques have emerged that block the lysine residues and neutralize their charge in purified histones, allowing for trypsin to mimic an ArgC digestion. Propionylation of lysine residues is one of these blocking techniques used to increase identification of important histone peptides (Garcia, Mollah et al. 2007). However, these techniques have not yet been coupled with MudPIT analysis, which is one of the major goals of my thesis work.

V. Rtr1 and Its Link With Histones

As mentioned above, the phosphorylation state of the CTD of RNAPII plays an important role in transcription. The transition from serine 5

phosphorylation to serine 2 phosphorylation is integral to the regulation of transcription. A protein by the name of Rtr1 has been shown to be a serine 5 phosphatase that acts on the CTD of RNAPII as shown in Figure 9 (Mosley, Pattenden et al. 2009).

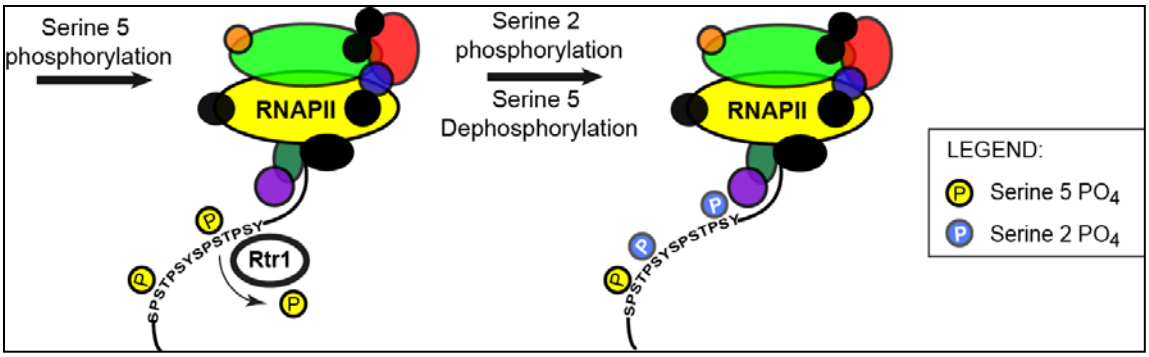

Figure 9. The role of Rtr1 in transcription. Rtr1 is known to be a serine 5 phosphatase that regulates the transition from serine 5 phosphorylation to serine 2 phosphorylation.

When *RTR1* is deleted serine 5 phosphorylation increases throughout the coding region of the gene. Along with this accumulation of serine 5 phosphorylation, RNAPII transcription decreases with the deletion of *RTR1* (Mosley, Pattenden et al. 2009). Termination defects have also been shown to occur with the deletion of *RTR1 (*Mosley, Pattenden et al. 2009).

As shown above in Figure 8, Set2, the methylase responsible for H3K36me3 binds only to the doubly phosphorylated CTD of RNAPII. Therefore, Rtr1 could play a role in the binding of Set2 to the CTD. When Rtr1 is functioning normally, it removes the serine 5 phosphorylation during transcription elongation. This decreases the amount of serine 5 phosphorylation present in the ORF of the

gene being transcribed and the amount of doubly phosphorylated CTD for Set2 to bind. Therefore we hypothesize that Set2 dissociation from the CTD is Rtr1-dependent. To test this hypothesis, the localization of H3K36me3 in wildtype and *rtr1Δ* strains was analyzed using chromatin immunoprecipitation (ChIP) followed by high-resolution microarray analyses (Figure 10).

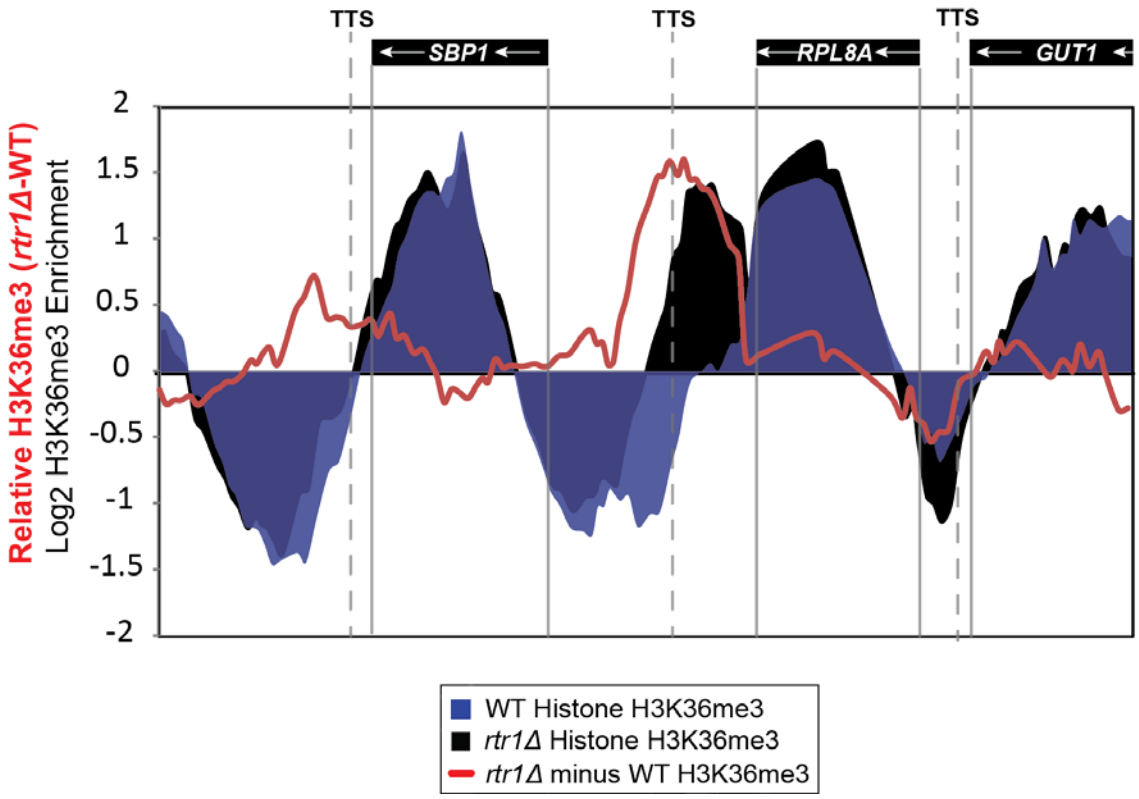

Figure 10. ChIP-microarray data from wild-type and *rtr1Δ* strains to analyze the occupancy of H3K36me3 across the yeast genome. A specific gene region is shown containing *RPL8A,* a highly transcribed ribosomal gene, and the other two genes are not as highly transcribed. A high resolution microarray was used with a probe length of 50 nucleotides. This means that approximately 3 probes were present per nucleosome, which spans 147 nucleotides of DNA.

The black peaks in Figure 10 show the relative abundance of H3K36me3 in *RTR1* deletion cells, while the blue peaks show H3K36me3 in a wildtype (WT)

strain. In the WT strain, H3K36me3 peaks in the ORF of the active gene and decreases prior to the transcription termination site (indicated by TTS in Figure 10). The *rtr1Δ* mutant strain data shows that H3K36me3 shifts past the termination transcription site. This fits with the transcription defects already observed in *rtr1Δ* strains, since RNAPII does not dissociate at the TTS when *RTR1* is deleted (data not shown). Without Rtr1 present to remove serine 5 phosphorylation, Set2 may still be bound to the CTD of RNAPII resulting in the extension of H3K36me3 past the TTS. Serine 5 phosphorylation can also be removed by the phosphatase Ssu72, which is part of the cleavage/polyadenlyation factor in yeast (Krishnamurthy, He et al. 2004). The cleavage/polyadenylation factor is localized towards the 3' end of the gene. When RNAPII reaches the 3' end of the gene with serine 5 phosphorylation still present, Ssu72 may still be able to remove this phosphorylation. Figure 10 shows that H3K36me3 does eventually drop off after the TTS in *RTR1* deletion cells. Ssu72 may be able to return serine 5 of the CTD to the unphosphorylated state, though this has not yet been tested.

To address the role of histone modifications during RNAPII elongation, we wanted to design a novel approach to histone modification analysis by mass spectrometry. Towards this goal, we combined various chemical modification approaches such as propionylation with MudPIT analysis. Once this approach was established, we began to investigate the role of Rtr1 in the regulation of cotranscriptional histone modifications through MudPIT analyis of histones

isolated from a *RTR1* deletion background. From this analysis, our goal was to determine if there was a change in the global histone modification patterns.

MATERIALS AND METHODS

I. Pre Purification

C-terminally TAP tagged histone H4 strains from the genes *HHF1* and *HHF2* were obtained from a glycerol stock stored at -80°C and were streaked onto YPD plates and grown at 30°C for two days. Cells from these plates were inoculated into two separate flasks of 30 mL YPD and grown at 30°C with shaking overnight. Cells were harvested at 4°C.

The cell pellets were resuspended in TAP lysis buffer (40 mM Hepes-KOH, pH 7.5; 10% glycerol; 350 mM NaCl; 0.1% Tween-20; 1X yeast protease inhibitor cocktail (Sigma); and 0.5 mM DTT). The resuspended cells were transferred to microcentrifuge tubes and ~200 µL acid washed glass beads were added. Cells were lysed on a disruptor genie for 20 minutes and then centrifuged at 14,000 rpm 4°C 10 minutes. The supernatant was transferred to fresh microcentrifuge tubes and used for further analyses.

Bovine Serum Albumin (BSA) was used to create a standard curve for the Bicinchronic Acid assay (BCA) using 0, 5, 25, 50, 100, and 250 µg of BSA. TAP lysis buffer was added to the various concentrations of BSA to bring the total volume to 25 µL. The prepared lysates were diluted 1:1, 1:10, and 1:100 with TAP lysis buffer. BCA working reagent (WR) was created by mixing BCA solution A and solution B 50:1. Each sample dilution and BSA sample had 200 µL of the BCA WR added. After addition of the WR each sample was vortexed and incubated at 37°C for 30 minutes. The absorbances of the samples were

measured at OD (optical density) 550 nm using a spectrophotometer. A standard curve for BSA was created in Excel and concentrations of the whole cell lysates were determined relative to the standard curve.

The concentrations determined from the BCA assay were used to load 1, 5, and 25 µg of each sample. Samples were mixed with 10 µL 2X Laemmli loading dye with BME as a reducing agent and brought up to 20 µL with TAP lysis buffer. Samples were boiled at 100°C for 10 minutes and centrifuged at 14,000 rpm 30 seconds. Samples were loaded on a 15% SDS gel alongside 5 µL Precision Plus Protein Dual Color Standard molecular weight marker (BioRad). The gel was electrophoresed at 200 volts for 1 hour.

Proteins were transferred overnight from gel to a nitrocelullose membrane at 30 volts in a wet transfer setup. The nitrocellulose membrane was removed after transfer was complete and blocked in 5% milk for 45 minutes. The nitrocellulose membrane was incubated with primary antibody (anti-CBP 1:1000) for 35 minutes then washed three times 10 minutes in ~50 mL TBS. The nitrocellulose membrane was then incubated with secondary antibody (anti-rabbit horseradish peroxidase (HRP) coupled 1:5000) for 30 minutes and then washed three times 10 minutes in ~50 mL TBS. ECL Plus (GE Healthcare) was used to develop the membrane according to the manufacturer's directions. The membrane was visualized using a Fuji digital imager with the blue laser and LBP filter.

II. Tandem Affinity Purification (TAP)

This TAP purification was based on the original TAP purification protocol (Rigaut, Shevchenko et al. 1999). Histone H4 TAP tagged cells were grown overnight at 30°C and harvested at 4°C. Cells were resuspended in TAP lysis buffer and the resulting slurry was frozen using liquid nitrogen. The frozen cells were lysed in a Waring blender with dry ice and then allowed to thaw at room temperature. The thawed lysate was treated with 100 units DNase I and 0.3 mg heparin for 10 minutes at room temperature to solublize the chromatin (Mosley, Florens et al. 2009). The lysate was then incubated with 200 µL IgG Sepharose resin overnight at 4°C with rotation.

The next day, the lysate was transferred to a Bio-Rad Econoprep column and drained by gravity flow. The column was washed with TAP lysis buffer three times. The beads were then resuspended in 1 mL TEV cleavage buffer (10 mM Tris, ph 8; 150 mM NaCl; 0.1% IGEPAL, 0.5 mM EDTA, 10% glycerol, 1X protease inhibitors (Sigma), and 1 mM DTT) to which 10 µL TEV protease was added. TEV protease cleavage was performed at 30°C for 1 hour with shaking.

The bead slurry was transferred to a Bio-Rad Econoprep column and cleaved products were eluted by gravity flow. Beads were washed with 3 mL calmodulin binding buffer (10 mM Tris, pH 8; 1 mM MgOAc; 1 mM imidazole, 2 mM $CaCl_2$, 10% glycerol, 1X protease inhibitors (Sigma), and 0.5 mM DTT) and 3 µl $CaCl_2$ was added to the flow-through. A total of 500 µL calmodulin Sepharose resin was added to the flow-through and incubated at 4°C for 3 hours.

The flow-through was drained by gravity flow in a Bio-Rad Econoprep column, and the resin was washed with 10 mL calmodulin binding buffer for three times. TAP tagged proteins were eluted off calmodulin Sepharose with calmodulin elution buffer (10 mM Tris, pH 8; 0.3 M NaCl, 1 mM MgOAc, 1 mM imidazole, 2 mM EGTA, 10% glycerol, 1X protease inhibitors (Sigma), and 0.5 mM DTT.) Elutions were done by incubating resin in 1 mL calmodulin elution buffer for 5 minutes, then draining by gravity flow into microcentrifuge tubes. A total of 8 separate elutions were done.

A total of 20 µL aliquots from the above elutions were taken and mixed with 4X gel loading buffer. Samples were incubated at 100°C for 10 minutes and centrifuged down. The aliquots were loaded on a 15% precast Bio-Rad gel alongside a 1:10 diluted unstained marker and electrophoresed at 200 volts for approximately 45 minutes until the bromophenol blue dye front ran off the gel.

The gel was removed from the plates and incubated in 100 mL fixing solution (30% ethanol, 10% acetic acid, 60% MilliQ water) overnight at room temperature. Fixing solution was poured off and the gel was incubated sequentially with the following solutions: 100 mL ethanol wash (30% ethanol, 70% MilliQ water) for 10 minutes, 100 mL water, 100 mL sensitizer solution (0.02% sodium thiosulfate), 100 mL water, and 100 mL silver nitrate solution (0.1% silver nitrate, 0.02% formaldehyde, 99.9% water.) The gel was then quickly washed with 100 mL water then incubated with 100 ml developing solution (2.5% sodium carbonate, 0.05% formaldehyde, 0.005% sodium thiosulfate) until bands developed which took approximately 5 - 10 minutes. The

development was stopped by incubating the gel in 100 mL stop solution (0.5% glycine) for 10 minutes at room temperature.

One hundred microliters from elutions 1 and 2 from the TAP eluates were mixed with 200 µL cold 100 mM Tris, pH 8.5, and 100 µL trichloroacetic acid (TCA). The samples were incubated overnight at 4°C. The next day, the precipitate was centrifuged, and the pellets were washed with 500 µL cold acetone 3 times. The acetone was removed with a 1 mL pipette and any residual acetone was allowed to evaporate at room temperature prior to digestion.

The precipitated proteins were denatured in 8 M urea in 100 mM Tris, pH 8.5, and incubated in 0.1 M TCEP and 0.5 M CAM. Because histones do not contain cysteines, the use of TCEP and CAM was discontinued after sample 7 (see Table 4 for sample numbers). The denatured proteins in sample 1 (see Table 4) were digested with 0.06 µg endoproteinase LysC at 37°C overnight. The next day, the LysC digested samples were diluted to 2 M urea, then $CaCl_2$ was added to a final concentration of 2 mM. All other samples were not digested with LysC, but instead $CaCl_2$ was added after denaturing in urea. Next 0.5 µg trypsin was added and incubated at 37°C overnight. Digestion was quenched the next day with 7 µL formic acid.

In preparation for mass spectrometric analysis of the trypsin digested samples a Sutter P-2000 laser puller was used to pull 100 µm inner diameter x 365 µm outer diameter fused silica capillaries (FSC) (Florens and Washburn 2006). A pulled FSC (or column) is usually about 9 inches in length. The column was then packed using a pneumatic loading vessel as previously described

(Florens and Washburn 2006). The column was packed with 8 cm C18 (reverse phase) resin, followed by 2.5 cm strong cation exchange (SCX) resin, and finally 1.5-2 cm C18 resin (Figure 11A). The packed column was rinsed in Buffer A (5% HPLC grade acetonitrile, 0.1% HPLC grade formic acid) prior to loading the digested sample. Sample was loaded, washed in Buffer A for 5-10 minutes, then washed in Buffer B for 10 minutes for desalting.

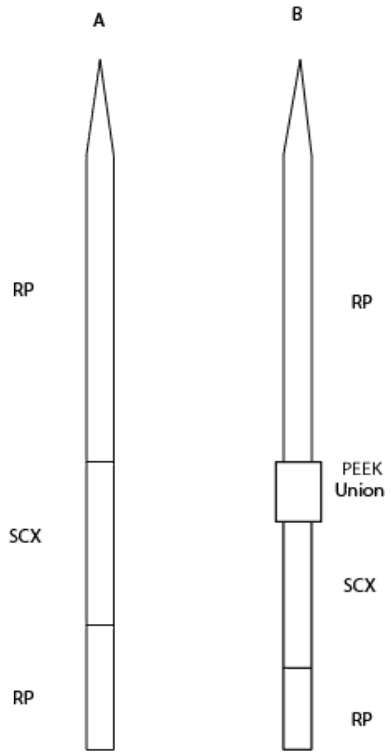

Figure 11. MudPIT columns. A) Three phase column (100 µm inner diameter) packed with RP, followed by SCX, then capped with RP. B) Three phase split coumn, which is a 100 µm column packed with RP, fitted into a PEEK union, and joined to a 250 µm column packed with SCX followed by RP.

The column was then placed in-line with a Proxeon nano-LC followed by an LTQ Velos linear ion trap mass spectrometer. The HPLC was set at a flow

rate of 500 nL/min, but the flow rate was decreased to 250-300 nL/min by time it reaches the tip of the column by using overflow tubing (Mosley, Florens et al. 2009). Xcalibur (Thermo) was used to control the gradient for the HPLC run as well as the mass spectrometric (MS) and tandem mass spectrometric (MS/MS) acquisition as previously described with a few modifications (Florens, Carozza et al. 2006). The first step was an 80 minute desalting step: a 70 minute gradient from 0% - 80% Buffer B (80% acetonitrile, 0.1% HPLC grade formic acid) followed by a 10 minute hold at 80% Buffer B. The next 8 steps were almost identical, only differing in the concentration of the salt, ammonium acetate. Each step was 110 minutes long: 5 minutes 100% Buffer A, 2 minutes with a set concentration of ammonium acetate from Buffer C (500 mM ammonium acetate, 5% acetonitrile, and 0.1% HPLC grade formic acid), 3 minutes 100% Buffer A, a 10 minute gradient from 0% - 10% Buffer B, and a 90 minute gradient from 10% - 45% Buffer B. Steps 2 - 9 increased in the ammonium acetate concentration as follows: 5, 10, 25, 50, 75, 100, 150, and 200 mM with 0.1% formic acid. The final two steps were identical: 5 minutes 100% Buffer A, 20 minutes 300 mM ammonium acetate from Buffer C, 5 minutes 100% Buffer A, a 10 minute gradient from 0% - 10% Buffer B, and a 90 minute gradient from 10% - 45% Buffer B (Florens, Carozza et al. 2006). Step 1 was eventually replaced with desalting on the pneumatic loading vessel in Buffer B for 5 minutes.

Xcalibur (Thermo) was set in data-dependent MS/MS acquisition. The dynamic exclusion time setting was initially at 50 seconds but was optimized to 90 seconds. Scans were initially done over the range of 400 to 1600 m/z, but the

range was lowered to 200 to specifically include smaller peptides from histone H3. As the sample eluted off the column, a charge of 1.5 kV was applied to ionize the sample. This charge was increased to 2.5 kV for samples 19 - 21 and 27 (see Table 4).

Data analysis was done using SEQUEST as a module of Proteome Discoverer 1.3 (PD1.3) (Thermo). A database consisting of 5815 proteins from *Saccharomyces cerevisiae* was downloaded from NCBI (10-27-09). A workflow analysis template was created in PD1.3 (Thermo) with the 10-27-09 database set as the default database to be searched against. The enzyme used for digestion (trypsin) was selected along with 2 - 5 missed cleavages. The default settings for minimum precursor mass (350 Da) and maximum precursor mass (5000 Da) were used until sample 12 when the minimum precursor mass was lowered to 200 Da. The minimum precursor mass was lowered because the m/z range was lowered in Xcalibur (Thermo) at this point to detect the smaller peptides in histone H3.

Static and dynamic modifications were also set at this point. Static modifications are the modifications that occur at every instance of the specified amino acid. Dynamic modifications may or may not be present on the amino acid. Samples 1 - 7 were searched with static modifications of carbamidomethylation from the chloroacetamide (+57 Da on cysteine) and methionine oxidation (+16 Da). Both of these static modifications were no longer used after sample 7, since the core histones do not contain cysteine and only contain 1 methionine in H2B. Dynamic modifications included propionylation of

lysine (+56 Da), carbamylation of lysine (+43 Da), acetylation of lysine (+42.0106 Da), or dimethylation of lysine (+28 Da).

PD1.3 (Thermo) also creates a database with reverse sequence peptides to help calculate the false discovery rate (FDR). The FDR is calculated by dividing the number of false positives identified by the sum of the false positives and real identifications. Our high confidence peptides have an FDR equal to or less than 2%, while our medium confidence peptides have an FDR equal to or less than 5%. SEQUEST® calculates a cross correlation score known as XCorr to identify the best peptide match to theoretical MS/MS fragment spectra. The XCorr is dependent on the length and charge of the peptide. Longer peptides will have higher XCorrs because they have more fragments that can be matched. As a default, SEQUEST® requires a peptide with a +1 charge to have an XCorr of at least 1.5, +2 peptides must be at least 2.5, and +3 peptides must be at least 3.5. However, we have found that PD1.3 incorrectly filters out many of the +1 charged spectra as a consequence of the false-discovery rate calculations (data not shown).

III. Propionylation

Propionylation of TCA precipitated samples was done as previously described with a few modifications (Garcia, Mollah et al. 2007). The propionylation reagent was at first mixed as 75 µL propionic anhydride and 25 µL methanol but updated to 25 µL propionic anhydride and 75 µL methanol (Plazas-Mayorca, Zee et al. 2009), and finally changed to 22.5 µL propionic anhydride

and 77.5 µL methanol. The propionylation reaction at first occurred at 51°C for 20 minutes but updated to 37°C for 15 minutes. Samples went through either a full four rounds of propionylation as previously described, or through only 1, 2, or 3 rounds of propionylation. Samples that went through the first round of propionylation are designated with an "A" in Tables 4, 5, 7, and 8. The samples that were propionylated twice before trypsin digestion are designated with a "B" in Tables 4, 5, 7, and 8. Samples that were propionylated twice before trypsin digestion and once after trypsin digestion are designated with "C" in Tables 4, 5, 7, and 8. Finally, the samples that were propionylated all four times are designated with "D" in Tables 4, 5, 7, and 8. Digested samples were quenched using 3 µL formic acid. Samples 2 and 3 were not denatured in urea, but all other samples were denatured in 8 M urea before propionylation and diluted to 2 M urea before trypsin digestion with 100 mM Tris, pH 8.5.

IV. Nuclei Prep and Acid Extraction

The nuclei prep was done as previously described with a few modifications (Plazas-Mayorca, Zee et al. 2009). Wild-type (BY4741) cells were grown in a 1 L culture, while *rtr1Δ* cells were grown in a 3 L culture. Forty milligrams of 100T zymolyase powder was used to digest the yeast cell wall yielding spheroplasts. The spheroplasts were washed twice in ice-cold YPD / 1 M sorbitol. A total of 40 mL Ficoll solution was initially used, but decreased to only 15 mL, while the subsequent 2.4 M sorbitol solution was decreased from 40

mL to 30 mL. βME was used in the Ficoll solution and 2.4 M sorbitol solution as a reducing agent instead of DTT.

After washing the nuclei, the histones were extracted using the histone extraction protocol from Abcam. Specifically, cells were resuspended in 20 mL TBS with 5 mM trichostatin A (a known histone deacetylase inhibitor) to maintain levels of histone acetylation (Yoshida, Kijima et al. 1990). Cells were pelleted by centrifugation at 4°C 4000 rpm for 5 minutes. Cells were again resuspended and centrifuged as above. The cells were then resuspended in 5 mL Triton Extraction Buffer (0.5% Triton X, 1X protease inhibitors (Sigma), 5 mM trichostatin A, and TBS). Cells were pelleted at 4°C 6500 rpm for 10 minutes. The cells were then washed with 2.5 mL Triton Extraction Buffer and centrifuged as above. The pellet was resuspended in 2 mL 0.2 N HCl and incubated at 4°C overnight with rotation to extract the basic proteins. The next day, samples were divided equally into 10 microcentrifuge tubes and dried down in a SpeedVac.

One sample was reconstituted in 30 µL 8 M urea and 100 mM Tris HCl pH 8.5. This sample was run on a gel and silver stained as already described. The histones were propionylated and digested with trypsin as described above. The columns used for MudPIT analysis of the acid extracted samples were initially the three phase columns, but changed to the three phase split columns after we experienced clogging of the three phase columns (Figure 11). The tips of a 100 µm inner diameter x 365 µm outer diameter were packed with 8 cm of C18 RP resin as already described. This RP tip was then attached to a filter union followed by a 250 µm inner diameter FSC. The 250 µm FSC was packed with 3

cm SCX resin followed by 1 - 1.5 cm C18 RP resin (Figure 11B). Columns were run and analyzed as already described above.

V. Carbamylation and Citraconylation

The nuclear proteins were purified using the nuclei prep followed by acid extraction as already described above. Acid extracted samples were resuspended in 8 M Urea and incubated at 60°C for 2 hours to carbamylate the lysines. Samples were digested and analysed by MudPIT as already described.

Samples for citraconylation were purified using a nuclei prep followed by acid extraction as described. Acid extracted samples were resuspended in 100 mM Tris-HCl pH 8.5 and urea was added to a final concentration of 2 M. To citraconylate the peptides 1 µL 98% citraconic anhydride was added to the resuspended sample. The sample was then incubated at room temperature for 30 minutes to citraconylate the lysines (Kadlik, Strohalm et al. 2003). $CaCl_2$ was added to a final concentration of 2 mM before trypsin digestion. Digestion and MudPIT analysis was done as already described.

VI. H3K36me3 Western Blot

Wild-type (BY4741) and mutant (*rtr1Δ*) cells were grown overnight at 30°C with shaking in 25 mL cultures. Cells were harvested the next day and washed with water. Cells were resuspended in 500 µL Nuclear Isolation Buffer (0.25 M sucrose, 14 mM NaCl, 60 mM KCl, 5 mM $MgCl_2$, and 0.8% Triton X.) Acid washed glass beads (5 mm diameter) were added to 100 µL volume. Lysis was

done at 4°C on a disruptor gene for 10 minutes. The supernatant was transferred to a new tube, while the beads were washed with 500 µL Nuclear Isolation Buffer. The wash was added to the supernatant in the new tubes. Cells were centrifigued and supernatant was pulled off. Cells were resuspended in water. This protocol was based on a personal communication from Kenneth Lee (Lee 2008).

Volumes of 0.25 µL, 2.5 µL, and 12.5 µL were mixed with 2X loading dye and boiled at 100°C for 10 minutes. Aliquots were run on a BioRad pre-cast 10-20% gradient gel at 200 volts for 30 minutes. The transfer was done at 100 volts for 1 hour. The membrane was blocked in 5% milk for 1 hour. The membrane was then cut above the 25 kD marker and separately incubated in 5 mL milk and 5 µL primary antibody. The membrane cut above the 25 kD band was incubated with anti-Pgk1 as a loading control, while the membrane containing the 25 kD band was incubated with anti-H3K36me3. Primary incubations were done for 1 hour. Membranes were washed with TBS three times for 10 minutes. Membranes were then incubated with 25 mL TBS and 5 µL secondary antibody for an hour and a half. The anti-Pgk1 membrane was incubated with anti-mouse, while the anti-H3K36me3 membrane was incubated with anti-rabbit. Membranes were washed again three times with TBS for 10 minutes. ECL Plus (GE Healthcare) was used to develop using the same procedure as already described above.

RESULTS

I. Histone H4-TAP Purification

A small scale lysis was done on TAP tagged histone H4 strains from the genes *HHF1* and *HHF2* to determine which gene had a higher level of protein expression. It was important to purify the histone H4 protein that had the most expression to maximize purification yield. Equal amounts of protein were loaded for gel separation based on the protein levels from the BCA assay. The Western blot in Figure 12 shows that histone H4 from *HHF2* is more highly expressed than histone H4 from *HHF1*. All further experiments using histone H4-TAP tagged cells used TAP tagged histone H4 from the *HHF2* gene product.

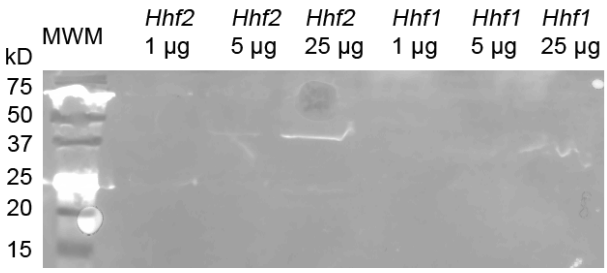

Figure 12. Western blot of TAP tagged histone H4 from *HHF2* and *HHF1*. Primary antibody used was anti-CBP. MWM is the molecular weight marker and kD stands for kiloDalton.

Tandem affinity purifications (TAP) utilize a tag on the protein of interest, in this case histone H4. This TAP tag allows for the protein to undergo two rounds of purification. The TAP tag used for these purifications consisted of a calmodulin binding sequence, a TEV protease cleavage sequence, and a protein A sequence. The protein A sequence binds to IgG beads for the first part of the purification. TEV protease cleaves and releases the bound protein from the IgG

resin, which is then bound to calmodulin beads in the presence of calcium. EGTA is then used to elute the bound protein from the calmodulin beads. The whole cell lysate was subjected to digestion with 100 units of DNase I to digest the DNA and release the bound histones (Mosley, Florens et al. 2009). The purified sample should contain the protein of interest (histone H4) along with the proteins bound to histone H4.

The first TAP purification done on histone H4-TAP tagged cells yielded the silver stain shown as Figure 13. A silver stained gel allows for the visualization of the proteins from the elutions off the calmodulin beads. The first elution contained the highest amount of protein. This silver stain shows proteins of varying size in the elutions, which should include histone H4-TAP and any proteins that were bound to histone H4 (see Table 1). Histone H4 is approximately 11.3 kD, and the TAP tag used is 20.7 kD. After cleavage with TEV protease, the TAP tag is approximately 3 kD, so the cleaved histone H4 should be approximately 14.3 kD.

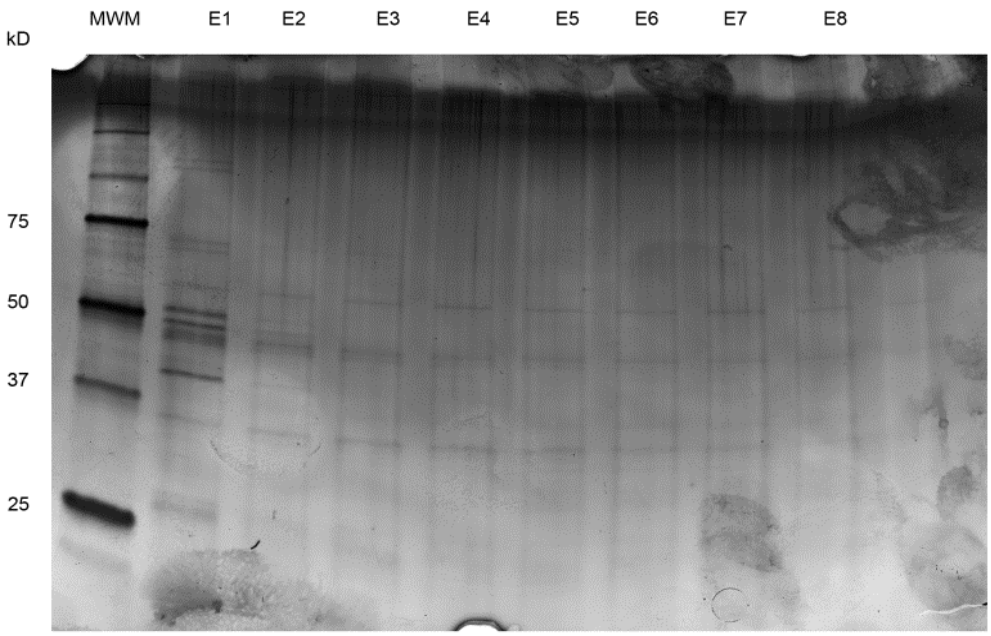

Figure 13. Silver stain of histone H4-TAP elutions from the calmodulin beads. MWM is the molecular weight marker and E1-E8 are the 8 elutions from the Calmodulin resin. The molecular weight of the MWM bands are indicated to the left of the figure in kiloDaltons (kD).

The proteins in the elutions were precipitated using trichloroacetic acid (TCA). The first two elutions were combined together to get a larger yield of protein for the TCA precipitation. The precipitated proteins were digested with both LysC and trypsin. LysC cleaves at the C-terminal end of lysines, while trypsin cleaves at the C-terminal end of both lysines and arginines. Histones are highly basic proteins full of lysines and arginines, so digestion with LysC/trypsin causes a problem for mass spec analysis. Trypsin digests the histones into small fragments that are highly charged which the linear ion trap cannot detect. After trypsin digestion, the sample was analyzed by MudPIT. MudPIT allows for

the digested sample to be separated by nanoscale liquid chromatography based on size and charge of the peptides before ionization and mass analysis.

Table 1 shows the proteins identified in this first histone H4-TAP purification. It lists the protein name, percent coverage of the sequence, and the number of spectral counts. Histones H2A, H2B, and H4 were detected in this first purification, while histone H3 was not detected likely due to overdigestion with trypsin. The two proteins identified with the most spectral counts were Hat1 and Hat2. These proteins are cytoplasmic histone acetyltransferases. Next highest are Ssa1, Ssa2, and Ssb2 which are ATPases that are involved in protein folding and transport of proteins. These 3 proteins have also been identified as common contaminants of TAP purifications (Gavin, Bosche et al. 2002; Krogan, Cagney et al. 2006). Hif1 forms a complex with Hat1 and Hat2 (Poveda, Pamblanco et al. 2004). All 4 of these chaperones have been shown to interact with Hat1 (Gong, Kakihara et al. 2009).

Eno2, Eno1, Pgk1, Fba1, and Tdh1 are all proteins involved in glycolysis and not known to interact with the histones. Eno2, Eno1, and Pgk1 have been shown to be common contaminants of TAP purifications in large-scale studies (Ng, Feng et al. 2002). Tef1 is a translation elongation factor, Act1 is actin, and Hsc82 is a chaperone, none of which have been shown to interact with the histones. Act1 has been shown to interact with Hif1, while the rest (Eno2, Eno2, Pgk2, Fba1, Tdh1, Tef1, and Hsc82) have been shown to interact with Hat1 (Krogan, Cagney et al. 2006). Set1 is a methyltransferase that is known to interact with histone H3 (Krogan, Cagney et al. 2006). Mam33 is a protein

involved in oxidative phosphorylation but has been shown to interact with histones H3 and H4 (Krogan, Cagney et al. 2006).

Table 1: Proteins Detected in First Histone H4-TAP Purification

Protein	PSM	Coverage (%)
Hat1	349	37.43
Hat2	279	22.44
Histone H4	225	33.33
Hif1	122	16.88
Ssa1	83	16.82
Eno2	82	23.8
Ssa2	78	16.9
Histone H2B	77	6.92
Eno1	66	14.65
Pgk1	61	20.43
Ssb2	50	17.62
Fba1	29	11.14
Histone H2A	27	6.87
Mam33	23	14.66
Tef1	20	2.4
Act1	20	6.93
Hsc82	18	7.66
Tdh1	10	15.36
Set1	10	1.11

Protein names are listed in the first column, PSM (peptide spectral matches) are in the second column, and sequence coverage (%) is in the final column. Histone H3 was tagged, so when it was purified all proteins associated with histone H4 should have also been purified. Proteins with PSMs of 10 or higher were included, while known contaminants were excluded. Contaminants were identified based on a TAP purification in a WT strain without a TAP tag (data not shown).

Table 2 shows the spectral counts for the majority of the purifications, including this first TAP purification. Histone H4 was the TAP tagged protein, so it

typically has the larger amount of spectral counts. Histone H3 is known to contain the lysine residues with modifications that correlate to transcription. Therefore, we want a large number of spectral counts for histone H3. This first prep yielded zero spectra for histone H3, so a second TAP purification was done for an untreated sample. Again, Table 2 shows that there were zero spectra for histone H3 in this second untreated TAP purification. Most likely histone H3 is being overdigested by trypsin in the untreated TAP purifications and therefore is not being detected by mass spectrometric analyses. Since histone H3 is the protein we are interested in, another approach is needed to be able to identify histone H3. Garcia et al. published a technique for blocking lysine residues before trypsin digestion, allowing for better coverage of all the histones, including histone H3 (Garcia, Mollah et al. 2007)

Table 2: Number of spectra detected for the histones from each preparation as indicated.

Sample	Histone	Untreated Spectral Counts	Propionylation Spectral Counts	Carbamylation Spectral Counts	Citraconylation Spectral Counts
Histone H4-TAP Purification #1	H2A	27	0	n/a	n/a
	H2B	77	0	n/a	n/a
	H3	0	0	n/a	n/a
	H4	225	0	n/a	n/a
Histone H4-TAP Purification #2	H2A	0	n/a	n/a	n/a
	H2B	214	n/a	n/a	n/a
	H3	0	n/a	n/a	n/a
	H4	66	n/a	n/a	n/a
Histone H4-TAP Acid Extraction #1	H2A	1088	31	n/a	n/a
	H2B	1085	30	n/a	n/a
	H3	1	32	n/a	n/a
	H4	5537	202	n/a	n/a
Histone H4-TAP Acid Extraction #2	H2A	0	166	158	50
	H2B	43	60	45	151
	H3	0	195	191	0
	H4	19	662	629	46
rtr1Δ Acid Extraction #1	H2A	99	n/a	8	n/a
	H2B	209	n/a	0	n/a
	H3	3	n/a	41	n/a
	H4	1150	n/a	864	n/a
rtr1Δ Acid Extraction #2	H2A	214	147	354	n/a
	H2B	162	407	629	n/a
	H3	0	180	352	n/a
	H4	1187	519	2509	n/a
BY4741 Acid Extraction #1	H2A	139	n/a	148	n/a
	H2B	207	n/a	252	n/a
	H3	62	n/a	503	n/a
	H4	318	n/a	1053	n/a

Sample name denotes the yeast strain genotype and the type of purification performed. The proteins listed are the 4 core histones. The spectral counts shown are how many times peptide spectral matches (PSMs) from that histone were detected by MudPIT analysis. The specific chemical treatments are listed at the top of the table.

II. Propionylation

Most likely histone H3 was overdigested by trypsin in sample 1, so we wanted to decrease the digestion of histone H3 to increase detection by MudPIT analysis. The technique from Garcia et al. blocked lysine residues with a propionyl group (+56 Da) followed by digestion with trypsin. This technique is more favorable than using ArgC to digest only at arginines, because trypsin is known to be more robust than ArgC. This would then increase the size of the digested peptides, along with decreasing the charge, and making them more ideal for mass spectrometric detection. In principle, this reaction requires propionic anhydride and methanol to be mixed to form the propionylation reagent (propionic acid). As propionic anhydride reacts with the methanol in the mixture, propionic acid is formed and the pH of the mixture decreases. Trypsin digestion requires the pH of the protein solution being digested to be at least at a pH of 8.0. This requires some pH adjustment with ammonium hydroxide to keep the solution at a desired pH following propionylation.

The original approach published by Garcia et al. used a total of 4 rounds of propionylation (Garcia, Mollah et al. 2007). The first round was performed to propionylate all unmodified lysine residues and the N-termini before digestion with trypsin. The second round of propionylation was perfromed to increase the efficiency of propionylation. The third round of propionylation was performed after trypsin digestion to propionylate the newly formed N-termini. The fourth round of propionylation was performed to again increase the propionylation efficiency. Subsequent studies have shown that using multiple rounds of

propionlyation decreases the ion abundance for detection by mass spectrometry (Drogaris, Wurtele et al. 2008). Based on these results, sample 2 (see Table 4) was propionylated for only the first 2 rounds, while sample 3 (see Table 4) went through the full 4 rounds of propionylation used by Garcia et al.

The two propionylated samples that underwent different rounds of propionylation were both from the first histone H4-TAP purification. Tables 2 and 3 show that after MudPIT analyses, no spectra were detected in either sample for any of the 4 histones. This was a significant decrease from the untreated samples, which detected a total of 329 spectra for 3 of the core histones in the first prep and 280 spectra for 2 of the core histones in the second prep.

Because no histones were detected in either of the propionylated samples, we decided to try to increase the amount of histones in the purified samples. This was initially attempted by changing the nuclease from DNase I to MNase or Benzonase. MNase is a nuclease that digests at the end of nucleosomes, while Benzonase is a non-specific nuclease that digests both DNA and RNA. The protocol from Garcia et al. did not call for denaturing the proteins in urea, which is commonly done before digesting with trypsin. We decided to include urea in the digestion to help increase digestion efficiency of the histones.

Table 3 shows the spectral counts for Benzonase and MNase treated samples 4 and 5 from the second histone H4-TAP purification. The MNase sample (5) shows 73 spectra for H2B, while the Benzonase sample (4) still does not show any spectra. We decided to use MNase as the nuclease for the rest of the experiment, since sample 5 resulted in detection of at least one histone.

Table 3: Number of spectra detected for the histones from each preparation as indicated. (miscellaneous treatments)

Sample	Histone	PRO (C,D)	Glycine PRO	0.5M PA	PRO (A) NDE	PRO (A) w/ Chy Rep 2	5x Trypsin PRO (B)	MNase PRO (B) Urea	Benz Urea PRO (B)	Utx Chy
Histone H4-TAP Purification #1	H2A	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	H2B	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	H3	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
	H4	0	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
Histone H4-TAP Purification #2	H2A	n/a	n/a	n/a	n/a	n/a	4	0	0	n/a
	H2B	n/a	n/a	n/a	n/a	n/a	50	73	0	n/a
	H3	n/a	n/a	n/a	n/a	n/a	16	0	0	n/a
	H4	n/a	n/a	n/a	n/a	n/a	65	0	0	n/a
Histone H4-TAP Acid Extraction #1	H2A	1	48	14	n/a	n/a	n/a	n/a	n/a	n/a
	H2B	0	1	138	n/a	n/a	n/a	n/a	n/a	n/a
	H3	489	4	0	n/a	n/a	n/a	n/a	n/a	n/a
	H4	248	390	345	n/a	n/a	n/a	n/a	n/a	n/a
BY4741 Acid Extraction #1	H2A	n/a	n/a	n/a	0	0	n/a	n/a	n/a	0
	H2B	n/a	n/a	n/a	118	0	n/a	n/a	n/a	0
	H3	n/a	n/a	n/a	0	0	n/a	n/a	n/a	5
	H4	n/a	n/a	n/a	2397	2	n/a	n/a	n/a	0

Sample name denotes the strain genotype and the type of purification performed. The proteins listed are the 4 core histones. The number of PSMs are shown in the table and indicate how many times peptides from that histone were detected by MudPIT analysis in each preparation. The treatments are listed at the top of the table. The letters following PRO indicate the rounds of propionylation done to the sample. PRO = propionylation, PA = propionic anhydride, NDE = no dynamic exclusion, Chy = chymotrypsin, Rep = replicate, Benz = Benzonase, Utx = untreated.

At this point, Benjamin Garcia suggested via a personal communication that there could be unreacted propionylation reagent in the samples, which was then propionylating trypsin and decreasing the digestion. So we decided to use five times the amount of trypsin usually used (25 µg rather than 5 µg) along with

the propionylation. Table 3 shows the result of increasing the amount of trypsin used. The 5x trypsin sample (6) was the first sample to detect all 4 histones, which confirmed Garcia's suggestion of unreacted propionylation reagent. The first peptides identified from histone H3 are listed in Table 4 along with the histone H3 peptides identified in all subsequent preparations. The histone H3 peptides identified in this sample contained some known acetylated lysines (K18, K23, and K56) along with K36, which is known to be methylated or acetylated.

Table 4: Histone H3 peptides identified from each preparation.				
Sample	Treatment (#)	Enzyme	Histone H3 Peptide Sequence	Highest XCorr
Histone H4-TAP TAP Purification #1	No treatment (1)	Trypsin	n/a	n/a
	Propionyl (2,B)	Trypsin	n/a	n/a
	Propionyl (3,D)	Trypsin	n/a	n/a
Histone H4-TAP TAP Purification #2	Benzonase/ Propionyl (4,B)	Trypsin	n/a	n/a
	MNase/ Propionyl (5,B)	Trypsin	n/a	n/a
	Soluble/5X Trypsin/ Propionyl (6,B)	Trypsin	VTIQkkDIkLAR	4.20
			kSAPSTGGVkkPHR	3.80
			RFQkSTELLIR	3.56
			kQLASkAAR	3.50
	No treatment (7)	Trypsin	n/a	n/a
Histone H4-TAP Acid Extraction #1	No treatment (8)	Trypsin	EIAQDFK	2.50
	Propionyl (9,C)	Trypsin	kQLASkAAR	3.74
			EIAQDFkTDLR	3.09
	Propionyl (10,B)	Trypsin	FQSSAIGALQESVEAYLVSLFE DTNLAAIHAKR	6.26
			KQLASKAAR	3.48
			FQKSTELLIR	2.99
			KSTGGKAPR	2.86
	Propionyl with Glycine (11,A)	Trypsin	kQLASkAAR	3.11
	0.5 M Propionic Acid (12,A)	Trypsin	n/a	n/a
Histone H4-TAP Acid Extraction #2	No treatment (13)	Trypsin	n/a	n/a
	2 Hr Carbamyl (14)	Trypsin	EIAQDFkTDLR	3.65
			FQkSTELLIR	3.23
			kQLASkAAR	3.08
			kSAPSTGGVkKPHR	3.05
			RFQkSTELLIR	3.91
	Citraconyl (15)	Trypsin	n/a	n/a
	Propionyl (16,B)	Trypsin	VTIQkkDIkLAR	4.45
			EIAQDFkTDLR	3.76
			kQLASkAAR	3.36
			kSAPSTGGVkkPHR	3.14
			kSTGGkAPR	3.08
			FQkSTELLIR	3.01

Table 4: Histone H3 peptides identified from each preparation. (CONT)				
Sample	Treatment (#)	Enzyme	Histone H3 Peptide Sequence	Highest XCorr
rtr1Δ Acid Extraction #1	No treatment (17)	Trypsin	FQSSAIGALQESVEAYLVSLFE DTNLAAIHAK	5.73
	2 Hr Carbamyl (18)	Trypsin	FQkSTELLIR	3.44
			kQLASkAAR	2.75
rtr1Δ Acid Extraction #2	No treatment (19)	Trypsin	n/a	n/a
	2 Hr Carbamyl (20)	Trypsin	EIAQDFkTDLR	3.83
			FQkSTELLIR	3.73
			RFQKSTELLIR	3.35
			kQLASkAAR	3.11
			kSTGGkAPR	3.00
			VTIQkkDIK	2.73
			EIAQDFK	2.41
	Propionyl (21,A)	Trypsin	EIAQDFK	2.55
			EIAQDFkTDLR	4.08
			FQkSTELLIR	3.13
			kDIkLAR	3.19
			kQLASkAAR	3.50
			kSAPSTGGVkkPHR	4.32
			kSTGGkAPR	3.18
			RFQkSTELLIR	3.30
			RVTIQkkDIK	3.08
			RVTIQKKDIkLAR	3.76
			SAPSTGGVkkPHR	3.35
			VTIQkkDIK	3.38
			VTIQkkDIkLAR	4.77
BY4741 Acid Extraction #1	No treatment (22)	Trypsin	EIAQDFKTDLR	3.31
			KSAPSTGGVK	2.72
			KSTGGKAPR	2.67
	2 Hr Carbamyl (23)	Trypsin	VTIQkkDIkLAR	4.55
			EIAQDFkTDLR	3.85
			kSTGGkAPR	3.68
			FQkSTELLIR	3.65
			kQLASkAAR	3.61

Table 4: Histone H3 peptides identified from each preparation. (CONT)				
Sample	Treatment (#)	Enzyme	Histone H3 Peptide Sequence	Highest Xcorr
BY4741 Acid Extraction #1 (cont)	No treatment (24)	Chymo	QSSAIGALQESVEAY	3.73
	Propionyl (25, A)	Chymo	n/a	n/a
	Propionyl NDE (26,A)	Trypsin	n/a	n/a
	Propionyl (27,A)	Chymo	n/a	n/a

Sample name denotes the strain genotype and the type of purification performed. Treatment means what type of modification was done to the purified proteins (untreated, propionyl, carbamyl, or citraconyl). The sample number is also listed in the treatment column followed by a letter designating the rounds of propionylation perfomed on the sample if applicable. "A" means the sample was propionylated once before digestion. "B" means the sample was propionylated twice before digestion. "C" means the sample was propionylated twice before digestion and once after digestion. "D" means the sample was propionylated twice before digestion and twice after digestion. Enzyme means what enzyme the proteins were digested with (Chymo = Chymotrypsin). The peptide sequences are the unique peptides detected from each preparation. The highest XCorr is listed, which is a calculation done by SEQUEST® to determine how well the spectra matches to a theoretical spectra for a given peptide. NDE = no dynamic exclusion.

Samples 1 - 5 and 7 were not able to detect any peptides from histone H3 after MudPIT analysis (Table 4). Overall, the TAP purifications were resulting in a low yield of the histones and high levels of histone associated proteins (Figure 13, Tables 1 - 3). Therefore, we sought to enrich the histones using a nuclei

prep followed by acid extraction as previously described (Garcia, Pesavento et al. 2007).

III. Nuclei Prep and Acid Extraction

Because histones are basic nuclear proteins, and because of the poor yield from the TAP purifications, we decided to use a nuclei prep followed by acid extraction to increase the yield of histones (Kizer, Xiao et al. 2006; Garcia, Pesavento et al. 2007). Figure 14 shows a silver stain of the acid extracted proteins. Comparing Figures 14 and 13 shows the difference in overall protein yield between the nuclei prep and the TAP purification. The nuclei prep shows more protein in the silver stain, so a nuclei prep was used from this point on to increase the histone abundance. The nuclei prep does not have as high a purity as the TAP purification, which is a drawback but should show enrichment for histone proteins due to their highly basic isoelectric point (pI). At this point, the increase in histone abundance was deemed to be more important to be able to detect more spectral counts for the histones.

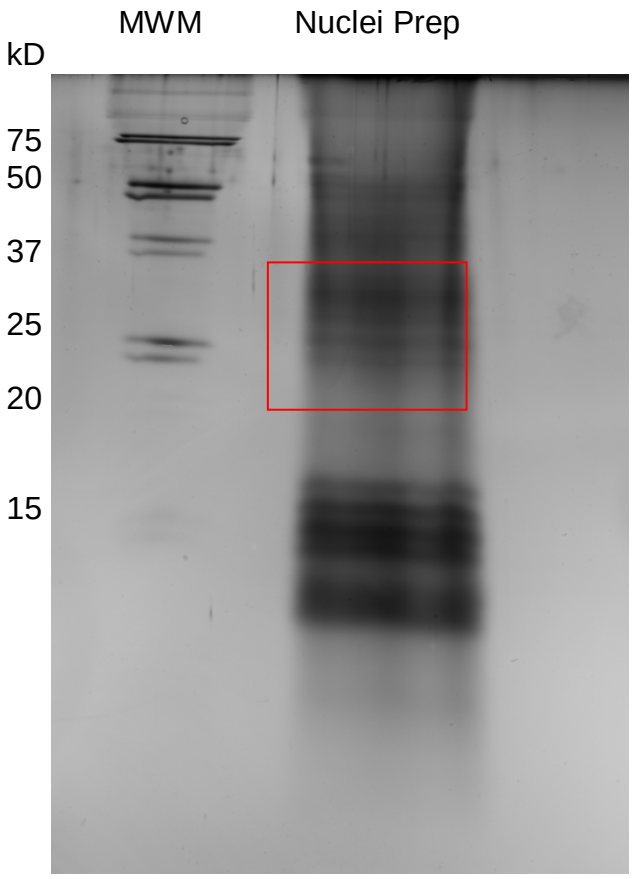

Figure 14. Silver stain of acid extracted proteins from a nuclei prep. Probable histones are boxed in red. The MWM marker is in the far left lane of the gel with the size of each band indicated in kD to the left of the figure.

Acid extracted samples were aliquoted equally into 10 microcentrifuge tubes and dried down in a vacuum centrifuge prior to individual chemical treatments. Table 2 shows the total number of spectra obtained for the core histones in the untreated sample from the first acid extraction of histone H4-TAP. A total of 7711 spectra for the core histones were observed for the first acid extraction, which is a dramatic increase in spectral counts compared to 329 spectra for the core histones in the first TAP purification. The next step was to try to increase the 1 spectrum for histone H3 in the acid extraction by

propionylating sample 10 following acid extraction. Table 2 shows the spectral counts for sample 10 for the core histones. There were 32 spectra for histone H3 detected by using 2 rounds of propionylation coupled with the acid extraction and a total of 295 spectra for all 4 core histones. The untreated acid extracted sample (10) had a total of 7711 spectra for the core histones, which decreased to 295 spectra for the core histones in the propionylated sample (10).

Another sample (9) from the acid extraction was propionylated, but this time it was propionylated for a total of 3 rounds. The spectral counts for sample 9 are shown in Table 3. There were a total of 738 spectra for all 4 core histones, but more important 489 of those spectra were for histone H3. This was the largest amount of spectra so far detected for histone H3 in our analyses. Through additional literature analysis it was determined that Garcia et al. had updated the 3:1 ratio of propionic anhydride to methanol to a ratio of 1:3 (Plazas-Mayorca, Zee et al. 2009). This could be the reason that we had unreacted propionylation reagent present in the sample. All propionylation reactions from the TAP purifications (samples 2 - 6) were done using the 3:1 ratio. Samples 9 and 10 from the first histone H4-TAP Acid Extraction also used the 3:1 ratio. Sample 11 in the first histone H4-TAP Acid Extraction and all subsequent propionylation reactions used the updated 1:3 ratio, with exception of the 0.5M propionic acid propionylation reaction (sample 12).

Tables 2 and 3 show the overall decrease in spectral counts for the core histones between untreated and propionylated samples. This is especially seen for histones H4, H2A, and H2B. MudPIT not only yields spectral counts for the

peptides in the sample but also ion intensities, along with other pieces of information regarding the peptide detection. Ion intensity is how abundant the ion is in a population of ions during a specific timepoint in the chromatography. Since there was a decrease in spectral counts for histones H4, H2A, and H2B in the propionylated samples, we also wanted to compare the ion intensities between the samples to see if the ions displayed a lower abundance. The best way to do this was to compare the intensity of a peptide that was highly sampled under both conditions, also known as a proteotypic peptide (Craig, Cortens et al. 2005). Table 5 shows the peptides from histone H4 that were identified from each preparation. The proteotypic peptide ISGLIYEEVR was chosen from histone H4, because it was present in both untreated and propionylated samples, plus it was responsible for almost a third of all of the spectral counts for H4 in the untreated samples. This particular peptide is present in the vast majority of the samples, whether treated or untreated.

Table 5: Histone H4 peptides identified from each preparation.				
Sample	Treatment (#)	Enzyme	Histone H4 Peptide Sequence	Highest XCorr
Histone H4-TAP TAP Purification #1	No treatment (1)	Trypsin	TVTSLDVVYALK	4.11
			DNIQGITKPAIR	3.87
			ISGLIYEEVR	3.82
	Propionyl (2,B)	Trypsin	n/a	n/a
	Propionyl (3,D)	Trypsin	n/a	n/a
Histone H4-TAP TAP Purification #2	Benzonase/ Propionyl (4,B)	Trypsin	n/a	n/a
	MNase/ Propionyl (5,B)	Trypsin	n/a	n/a
	Soluble/5X Trypsin/ Propionyl (6,B)	Trypsin	kTVTSLDVVYALkR	5.09
			AVLkSFLESVIR	4.02
			ISGLIYEEVR	3.81
			GkGGkGLGkGGAkR	3.76
	No treatment (7)	Trypsin	KTVTSLDVVYALK	4.17
			ISGLIYEEVR	4.09
			TVTSLDVVYALK	3.76
			DNIQGITKPAIR	3.61
Histone H4-TAP Acid Extraction #1	No treatment (8)	Trypsin	SFLESVIRDSVTYTEHAK	5.62
			KTVTSLDVVYALKR	5.29
			ISGLIYEEVR	4.41
			RKTVTSLDVVYALK	4.31
			TVTSLDVVYALK	4.29
			TVTSLDVVYALKR	4.10
			RISGLIYEEVR	4.06
			ILRDNIQGITKPAIR	3.66
			DSVTYTEHAK	3.40
			AVLKSFLESVIR	3.37
			DNIQGITKPAIR	2.88
			SFLESVIR	2.77
	Propionyl (9,C)	Trypsin	ISGLIYEEVR	4.14
			DNIQGITkPAIR	3.17
			AVLKSFLESVIR	4.35
			ISGLIYEEVR	4.01
			AVLKSFLESVIRDSVTYTEHAKR	3.72

Table 5: Histone H4 peptides identified from each preparation. (CONT)				
Sample	Treatment (#)	Enzyme	Histone H4 Peptide Sequence	Highest XCorr
Histone H4-TAP Acid Extraction #1 (CONT)	Propionyl (10,B)	Trypsin	KTVTSLDVVYALKR	5.46
			DNIQGITKPAIR	2.99
			TLYGFGG	2.16
	Propionyl/ Glycine (11,A)	Trypsin	kTVTSLDVVYALkR	4.98
			GkGGkGLGkGGAkR	4.60
			AVLkSFLESVIR	4.41
			ISGLIYEEVR	4.07
			DNIQGITKPAIR	3.27
			TLYGFGG	1.77
	0.5 M Propionic Acid (12,A)	Trypsin	kTVTSLDVVYALkR	4.49
			TVTSLDVVYALkR	4.45
			AVLkSFLESVIR	4.12
			ISGLIYEEVR	4.05
			TVTSLDVVYALK	3.68
			SFLESVIR	2.19
			TLYGFGG	2.19
Histone H4-TAP Acid Extraction #2	No treatment (13)	Trypsin	DSVTYTEHAK	2.97
	2 Hr Carbamyl (14)	Trypsin	AVLkSFLESVIR	4.15
			DNIQGITkPAIR	3.84
			DNIQGITKPAIR	3.44
			DSVTYTEHAkR	3.23
			GkGGkGLGkGGAkR	3.35
			ISGLIYEEVR	4.19
			kTVTSLDVVYALkR	5.10
	Citraconyl (15)	Trypsin	TLYGFGG	2.11
	Propionyl (16,B)	Trypsin	kTVTSLDVVYALkR	4.74
			AVLkSFLESVIR	4.28
			ISGLIYEEVR	4.19
			DNIQGITkPAIR	3.98
			GkGGkGLGkGGAkR	3.00
			DSVTYTEHAkR	2.69

Table 5: Histone H4 peptides identified from each preparation. (CONT)				
Sample	Treatment	Enzyme	Histone H4 Peptide Sequence	Highest XCorr
rtr1Δ Acid Extraction #1	No treatment (17)	Trypsin	SFLESVIRDSVTYTEHAK	5.20
			KTVTSLDVVYALK	5.16
			TVTSLDVVYALK	4.32
			KTVTSLDVVYALKR	4.29
			ISGLIYEEVR	4.17
			TVTSLDVVYALKR	3.83
			RISGLIYEEVR	3.70
			DNIQGITKPAIR	3.55
			ILRDNIQGITKPAIR	3.47
			DSVTYTEHAK	3.01
	2 Hr Carbamyl (18)	Trypsin	ISGLIYEEVR	4.17
			AVLkSFLESVIR	3.85
			DNIQGITkPAIR	3.07
			GkGGkGLGkGGAkR	2.76
rtr1Δ Acid Extraction #2	No treatment (19)	Trypsin	AVLKSFLESVIR	3.30
			DNIQGITKPAIR	3.76
			ILRDNIQGITKPAIR	3.80
			ISGLIYEEVR	4.14
			ISGLIYEEVRAVLK	3.60
			KTVTSLDVVYALK	5.17
			KTVTSLDVVYALKR	5.51
			RISGLIYEEVR	3.81
			SFLESVIRDSVTYTEHAK	5.78
			SFLESVIRDSVTYTEHAK R	5.22
			TVTSLDVVYALK	4.60
			TVTSLDVVYALKR	3.94
	2 Hr Carbamyl (20)	Trypsin	AVLkSFLESVIR	4.24
			DNIQGITkPAIR	3.98
			DSVTYTEHAK	2.88
			DSVTYTEHAkR	3.38
			DSVTYTEHAKRk	3.33
			GLGkGGAkR	3.39
			ILRDNIQGITkPAIR	4.75
			ISGLIYEEVR	4.27
			kILRDNIQGITkPAIR	3.59
			KTVTSLDVVYALK	4.70
			kTVTSLDVVYALkR	4.92

Table 5: Histone H4 peptides identified from each preparation. (CONT)				
Sample	Treatment	Enzyme	Histone H4 Peptide Sequence	Highest XCorr
rtr1Δ Acid Extraction #2 (CONT)	2 Hr Carbamyl (20) (CONT)	Trypsin (CONT)	RISGLIYEEVR	3.25
			SFLESVIR	2.89
			SFLESVIRDSVTYTEHAK	4.97
			TVTSLDVVYALK	4.28
			TVTSLDVVYALKR	3.97
	Propionyl (21,A)	Trypsin	AVLkSFLESVIR	4.40
			DNIQGITkPAIR	3.87
			GGKGLGkGGAkR	3.78
			GkGGkGLGK	2.81
			GkGGkGLGkGGAK	3.97
			GkGGkGLGkGGAkR	5.28
			ILRDNIQGITkPAIR	5.00
			ISGLIYEEVR	4.27
			kILRDNIQGITkPAIR	3.16
			kTVTSLDVVYALK	4.35
			kTVTSLDVVYALkR	5.41
			RISGLIYEEVR	3.18
			SFLESVIRDSVTYTEHAK	4.29
			SGRGKGGKGLGKGGAkR	3.11
			TVTSLDVVYALK	4.23
			TVTSLDVVYALkR	4.95
BY4741 Acid Extraction #1	No treatment (22)	Trypsin	ISGLIYEEVR	4.08
			TVTSLDVVYALK	3.70
			DNIQGITKPAIR	3.38
			DSVTYTEHAK	2.97
			DSVTYTEHAKR	2.96
			SFLESVIR	2.91
			TVTSLDVVYALKR	2.86
	2 Hr Carbamyl (23)	Trypsin	kTVTSLDVVYALkR	5.25
			AVLkSFLESVIR	4.46
			ISGLIYEEVR	4.37
			GkGGkGLGkGGAkR	4.02
			DNIQGITkPAIR	3.79
			DSVTYTEHAkR	3.67
			TLYGFGG	2.30

Table 5: Histone H4 peptides identified from each preparation. (CONT)				
Sample	Treatment	Enzyme	Histone H4 Peptide Sequence	Highest XCorr
BY4741 Acid Extraction #1 (CONT)	No treatment (24)	Chymo	n/a	n/a
	Propionyl (25,A)	Chymo	n/a	n/a
	Propionyl NDE (26,A)	Trypsin	ISGLIYEEVR	4.13
			TLYGFGG	2.48
	Propionyl (27,A)	Chymo	TLYGFGG	1.95

Sample name denotes the strain genotype and the type of purification performed. Treatment indicates the type of modification performed for the purified proteins (untreated, propionyl, carbamyl, or citraconyl). The sample number is also listed in the treatment column followed by a letter designating the rounds of propionylation perfomed on the sample if applicable. "A" means the sample was propionylated once before digestion. "B" means the sample was propionylated twice before digestion. "C" means the sample was propionylated twice before digestion and once after digestion. "D" means the sample was propionylated twice before digestion and twice after digestion. Enzyme means what enzyme the proteins were digested with. The peptide sequences are the unique peptides detected from each preparation. The highest XCorr is listed, which is a calculation done by SEQUEST® to determine how well the spectra matches to a theoretical spectra for the peptide. NDE = no dynamic exclusion.

Figure 15 shows an extracted ion chromatogram that compares the ion intensity obtained for the proteotypic peptide ISGLIYEEVR of histone H4 from a propionylated sample and an untreated sample from the same purification. This peptide was chosen because it was one of the few peptides present in both samples. The extracted ion chromatogram allows a comparison of ion intensity for this peptide in both samples across the MudPIT steps. Figure 15 shows there

is a significant decrease in ion intensity for this proteotypic peptide from the untreated sample to the propionylated sample. The untreated sample shows a peak in ion intensity at 16 million counts, while the peak for the propionylated sample cannot be visualized properly at this scale.

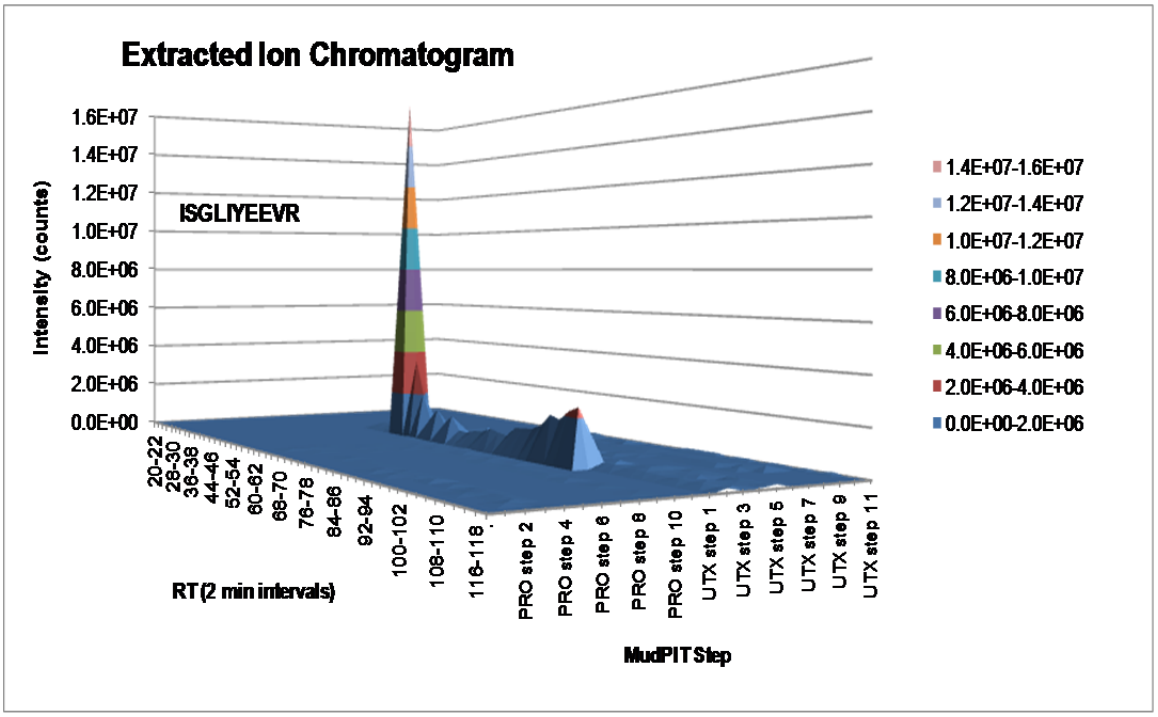

Figure 15. Extracted ion chromatogram of a proteotypic peptide from histone H4. Comparison of ion intensity between a propionylated (PRO) sample and an untreated (UTX) sample by MudPIT step (z-axis). Retention time (RT) is plotted as the x-axis in 2 minute intervals starting at 20 minutes, before the organic gradient starts. The lowest ion intensity (y-axis) mark is at 2 million counts and goes up to 16 million counts.

The lowest mark on the intensity axis is at 2 million intensity counts, so Figure 16 allows for visualization of the ion intensity peak for the propionylated sample which is around 100,000 intensity counts. This is a clear example of the significant (160-fold) decrease in the ion intensity caused by the propionylation

reaction. It is also important to note that this propionylated sample was one of the original samples propionylated with the 3:1 ratio for the propionylation reagent. Figure 15 has a large peak towards the beginning of the organic gradient where the majority of this peptide eluted in the untreated sample. The peptide continued to elute through the rest of this step at a lower level. Figure 16 shows jagged peaks for the elution of this peptide in the propionylated sample. The intensity counts for the peptide in the propionylated sample are near where the background or "noise" is typically shown resulting in a jagged extracted ion chromatogram. The peptide also peaks closer to the end of the MudPIT run rather than at the beginning like the untreated sample shows.

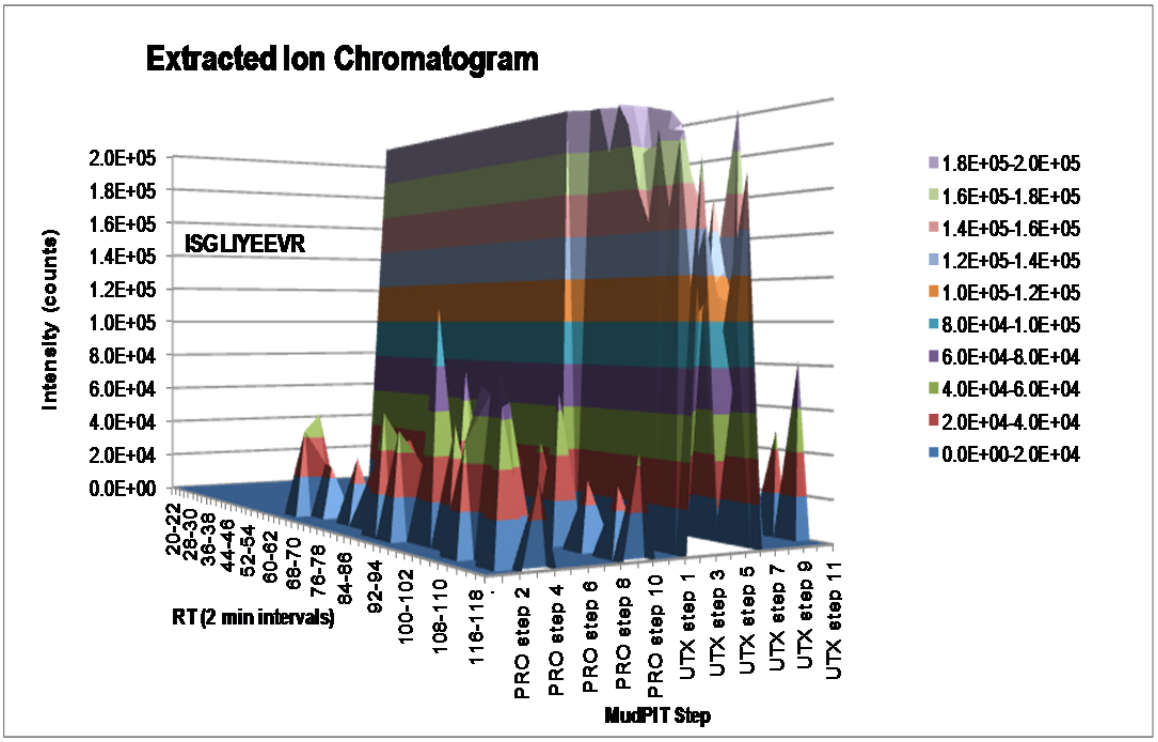

Figure 16. Extracted ion chromatogram of same histone H4 peptide as Figure 15. The only difference is the intensity scale (y-axis). The lowest mark on the intensity scale is 50,000 counts and goes up to 200,000 counts allowing for the propionyl intensity to be visualized.

IV. Carbamylation and Citraconylation

Because of the low spectral counts (Table 2 column 4 and Table 3 columns 3 and 4) and ion intensities (Figures 15 and 16) using the propionylation method, we were eager to find other similar methods that we could test. After additional literature analysis and a suggestion by a peer, citraconylation and carbamylation were chosen. Carbamylation was the most straightforward approach, since it involved just heating the sample in urea for 2 hours to carbamylate the lysine residues. The citraconylation reaction was very similar to the propionylation reaction, although there was an additional perk for using the citraconylation. This perk was the fact that the citraconyl group could be removed by decreasing the pH by addition of acid, which was already done to quench the trypsin digestion.

A comparison was done for the second histone H4-TAP acid extraction to see how these two new methods compared to the propionylation reaction. Table 2 shows the spectral counts for all 3 treatments (columns 4-6) and the untreated sample (column 3). The untreated sample yielded poor results, only identifying spectral counts from 2 histones (43 for histone H2B and 19 for histone H4). This was most likely due to a poorly packed column or a bad LC/MS run, which we had insufficient material to repeat. The column could have gotten clogged halting the separation and ionization of the peptides from the sample. Also, unfortunately, the citraconylation reaction did not help to detect any histone H3 spectra. Since the blocking reactions were supposed to increase the detection for histone H3, this technique was not used again. Both the propionlyation (with

the 1:3 propionylation reagent ratio) and carbamylation reactions were able to yield almost 200 spectra for histone H3, along with similar spectral counts for the other 3 histones.

We used SEQUEST® through Proteome Discoverer to search for lysine acetylation and dimethylation in the untreated, carbamylated, and propionylated samples from the second histone H4-TAP acid extraction. Table 6 shows the results of this search. We did not search for lysine trimethylation, because we used a linear ion trap mass spectrometer, which is not a high mass accuracy instrument. A linear ion trap has a mass resolution of 0.1 - 1 Dalton, which means that it cannot differentiate between a difference of less than 1 Dalton (Mann and Kelleher 2008). Acetylation is the addition of 42.0105 Daltons, and trimethylation is the addition of 42.0469 Daltons. Because of the mass resolution of this instrument, the residues in Table 6 that are listed as acetylated could actually be acetylated or trimethylated.

Table 6: Histone H3 peptides with modifications from histone H4TAP acid extraction #2				
Treatment (#)	Peptide Sequence	Modifications	Spectra	XCorr
None (13)	kQLASkAAR	K1(Acetyl); K6(Acetyl)	14	3.50
	KQLASkAAR	K6(Acetyl)	7	3.18
2 Hr Carbamyl (14)	TKQTARKSTGGKAPR	K2(Carbamyl); R6(Dimethyl)	1	2.93
	KQLASKAAR	K1(Carbamyl); K6(Carbamyl)	4	3.08
	KQLASKAAR	K1(Carbamyl); K6(Acetyl)	3	2.97
	KQLASKAAR	K1(Acetyl); K6(Acetyl)	2	3.12
	KSAPSTGGVKKPHR	K1(Acetyl); K10(Carbamyl)	7	3.10
	KSAPSTGGVKKPHR	K1(Carbamyl); K10(Carbamyl)	5	3.05
	RFQKSTELLIR	K4(Carbamyl)	152	3.92
	RFQKSTELLIR	K4(Acetyl)	62	3.74
	FQKSTELLIR	K3(Carbamyl)	60	3.11
	FQKSTELLIR	K3(Acetyl)	17	2.99
	EIAQDFKTDLR	K7(Carbamyl)	13	3.39
	EIAQDFKTDLR	K7(Dimethyl)	5	3.08
Propionyl (16,B)	ARTKQTARkSTGGKAPR	K9(Acetyl)	1	2.81
	kSTGGkAPR	K1(Propionyl); K6(Propionyl)	37	3.08
	kSTGGkAPR	K1(Acetyl); K6(Propionyl)	8	3.36
	kSTGGkAPR	K1(Acetyl); K6(Acetyl)	4	3.08
	kQLASkAAR	K1(Propionyl); K6(Propionyl)	13	3.36
	kQLASkAAR	K1(Acetyl); K6(Acetyl)	4	3.36
	kSAPSTGGVkkPHR	K1(Propionyl); K10(Propionyl); K11(Propionyl)	2	3.14
	FQkSTELLIR	K3(Acetyl)	9	3.10
	FQkSTELLIR	K3(Propionyl)	9	3.01
	EIAQDFkTDLR	K7(Propionyl)	26	3.76
	EIAQDFkTDLR	K7(Dimethyl)	2	3.06
	VTIQkkDIkLAR	K5(Propionyl); K6(Propionyl); K9(Propionyl)	11	4.45

Modifications listed are either from treatments (propionyl and carbamyl) or PTM searches (dimethyl and acetyl). A linear ion trap cannot tell the difference between aceyl and trimethyl, so the residues identified with acetylation could actually be trimethylation. All samples were digested with trypsin. The numbers in the parentheses in the treatment column designate what number sample. The “B” in the treatment column for the propionylated sample indicates that this sample was propionylated twice before trypsin digestion.

The carbamylation reaction results in the addition of 43 Daltons to lysine residues. The difference between carbamylation and acetylation/trimethylation is also less than 1 Dalton. This means that the linear ion trap may not be able to differentiate between these three modifications in the carbamylated samples. This can be illustrated by looking at the carbamylated peptides in Table 6 column 3. The majority of the peptides for the carbamylated sample are shown as either carbamylated or acetylated. These peptides have similar spectral counts and XCorrs, showing that there is most likely no distinction between carbamylation and acetylation/trimethylation in these samples. A higher mass accuracy instrument would need to be used to identify which residues were actually carbamylated, acetylated, or trimethylated. Propionylation does not have this same problem, because the propionyl group is +56 Daltons, which a linear ion trap can distinguish from acetylation/trimethylation.

V. BY4741 and *rtr1Δ* Acid Extractions

Because the TAP tag was no longer being utilized in the nuclei preps, we proceeded to perform a nuclei prep followed by an acid extraction for wild-type (BY4741) yeast cells. The initial untreated sample (22) for the BY4741 acid extraction yielded a total of 726 spectra for all 4 histones and 62 spectra for histone H3. This was promising, because this was the largest amount of spectra for histone H3 in any untreated sample. The carbamylated sample yielded 1956 total spectra for all histones and 503 for histone H3. This was the first time that a

treated sample was able to increase detection of histone H4, which had always been higher in the untreated samples.

Four other treatments (24 - 27) were done to the aliquots from the BY4741 acid extraction. The spectral counts from 3 of these samples are shown in Table 3. Sample 26 was propionylated for 1 round with 1M propionylation reagent with no dynamic exclusion on the mass spectrometer, sample 24 was untreated and digested with chymotrypsin, and samples 25 and 27 were propionylated for 1 round with 1M propionylation reagent and digested with chymotrypsin. The propionylated sample that was digested with chymotrypsin yielded zero spectral counts for any of the histones, and has thus been left out of Table 3.

Dynamic exclusion limits the amount of times that a peptide is selected by the mass spectrometer for fragmentation. Without any dynamic exclusion time setting, the mass spectrometer will only select the most abundant ions for fragmentation. Using a dynamic exclusion time setting allows for further sampling of less abundant ions in the sample. Since the histones should be some of the most abundant proteins in the acid extraction, we decided to turn off the dynamic exclusion to see if this would increase the spectral counts for the histones. Table 3 (column 6, sample 26) shows that the spectral counts for histone H4 increased from the untreated sample in Table 2 (column 3, sample 22) with the normal 90 second dynamic exclusion setting.

Chymotrypsin differs from trypsin in that it digests at the C-terminal end of tryptophan, tyrosine, leucine, and phenylalanine. Histone H3 only contains 7 chymotrypsin cleavage sites, so the peptide fragments would be larger than the

trypsin digested fragments. Also, the propionylation would still block the charge on the lysine residues. The goal was that the chymotrypsin digested sample would be able to help increase the coverage of histone H3. Table 3 columns 7 and 11 show that the chymotrypsin digested samples yielded very low spectral counts.

A nuclei prep followed by acid extraction and carbamylation reaction was performed on an *rtr1Δ* sample to determine the differences between post-translational modifications (PTMs) between wild-type and a *RTR1* deletion mutant strain. Unfortunately, the first *rtr1Δ* acid extracted sample that was carbamylated yielded poor results (i.e. only 41 spectra for histone H3.) A second acid extraction was done for *rtr1Δ* to increase the spectral counts in the carbamylated sample and also to have a biological replicate. Table 2 includes the spectral counts for the untreated (sample 19, column 3), propionylated (sample 21, column 4), and carbamylated (sample 20, column 5) samples. A propionylation reaction was also performed to show the difference between the two modifications and compare the ion intensities with the correct 1:3 ratio for the propionylation reagent. The untreated sample did not yield any spectra for histone H3, but did have a total of 1563 spectra for all 4 histones. The propionylated sample was able to increase detection of histone H3 to 180 spectra, but decreased the overall spectra to 1253. Finally, the carbamylated sample increased the detection of histone H3 to 352 spectra and a total of 3844 spectra for all 4 histones.

Figure 17 shows an extracted ion chromatogram for the second *rtr1Δ* acid extraction and the untreated, propionylated, and carbamylated samples. This figure uses the same proteotypic peptide from histone H4 (ISGLIYEEVR) shown in Figures 15 and 16. The untreated sample shows a maximum ion intensity of over 700 million counts, the peak for the propionylated sample is at around 100 milllion, and the carbamylated sample is at 400 million counts as shown in Figure 17. It is important to note that the first ion extracted chromatagram used a propionylated sample that used the original 3:1 propionylation reagent, while this figure uses the updated 1:3 ratio. This corrected ratio helped to increase the maximum intensity of detected peptides, but it is still considerably lower than the untreated sample. Even the carbamylated sample is almost half the maximum intensity of the untreated sample, but the overall ion intensity is still more intense than the ion intensity for the propionylated sample.

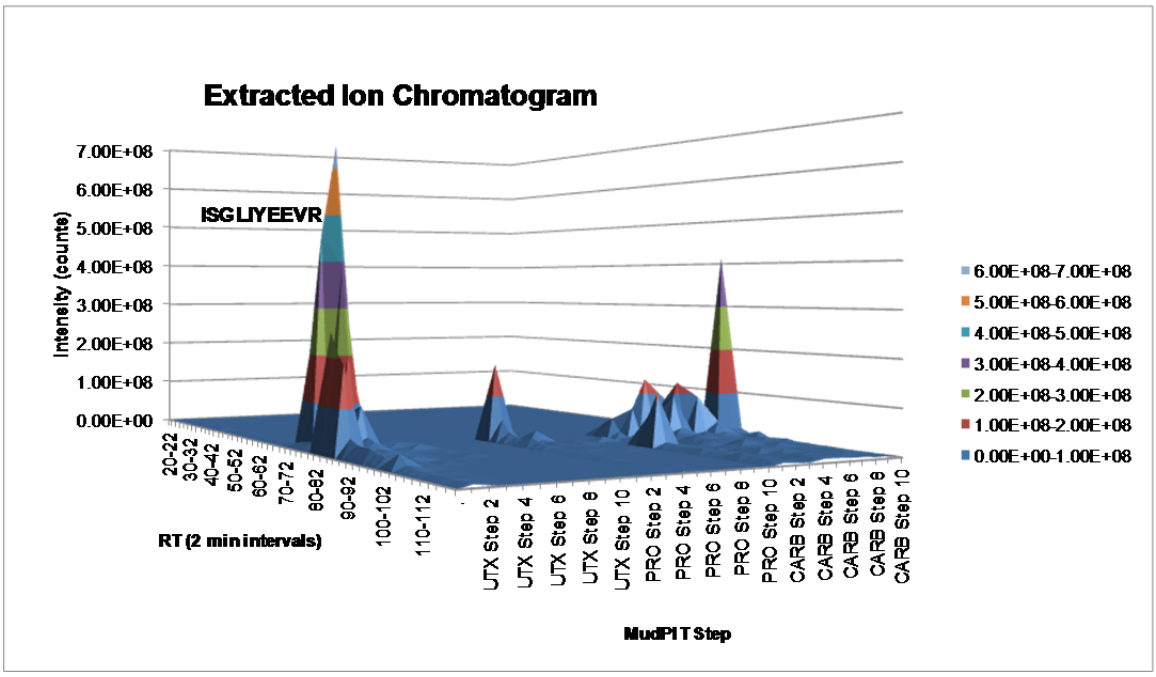

Figure 17. Extracted ion chromatogram for histone H4 proteotypic peptide from *rtr1Δ* nuclei prep #2. The lowest intensity mark shown is at 100 million counts going up to 700 million counts (y-axis). RT in 2 minute intervals starts at 20 minutes, around when the organic gradient begins (x-axis). The MudPIT steps for the untreated sample are shown first, followed by the MudPIT steps for the propionylated sample, and lastly the carbamylated sample (z-axis). UTX = untreated, PRO = propionylated, and CARB = carbamylated.

VI. Post Translational Modifications

Figure 4 shows the most well characterized yeast histone modifications for histone H2A. The N-terminus of histone H2A contains three acetylated lysines (K4, K7, and K21), while the C-terminus of histone H2A contains two phosphorylated serines (S121 and 128) and a sumoylated lysine (K126). All six of these histone H2A residues have only been detected while using the propionylation method in different samples (6, 9, 10, 11, 12, 16, and 21) as shown in Table 7. H2AK21ac is detected in at least one sample from untreated,

carbamylated, propionylated, and citraconylated. H2AK4ac and K7ac are only detected in the propionylated and carbamylated samples. The propionylation and citraconylation methods both help to detect the C-terminus of histone H2A.

Table 7: Histone H2A peptides identified from each preparation.

Sample	Treatment (#)	Enzyme	Histone H2A Peptide Sequence	Highest XCorr
Histone H4-TAP TAP Purification #1	No treatment (1)	Trypsin	AGLTFPVGR	3.07
	Propionyl (2, B)	Trypsin	n/a	n/a
	Propionyl (3,D)	Trypsin	n/a	n/a
Histone H4-TAP TAP Purification #2	Benzonase / Propionyl (4,B)	Trypsin	n/a	n/a
	MNase / Propionyl (5,B)	Trypsin	n/a	n/a
	Soluble/5X Trypsin / Propionyl (6,B)	Trypsin	sAKAGLTFPVGR	3.78
	No treatment (7)	Trypsin	n/a	n/a
Histone H4-TAP Acid Extraction #1	No treatment (8)	Trypsin	NDDELNKLLGNVTIAQGGVLPNIHQNLLPK	6.76
			LLGNVTIAQGGVLPNIHQNLLPK	6.05
			LLGNVTIAQGGVLPNIHQNLLPKK	4.21
			HLQLAIRNDDELNK	3.12
			AGLTFPVGR	3.06
	Propionyl (9,C)	Trypsin	sGGkGGkAGSAAkASQSR	2.92
	Propionyl (10,B)	Trypsin	IGSGAPVYLTAVLEYLAAEILELAGNAAR	6.44
			SAKAGLTFPVGR	4.04
	Propionyl with Glycine (11,A)	Trypsin	IGSGAPVYLTAVLEYLAAEILELAGNAAR	5.76
			sAKAGLTFPVGR	3.29
	0.5 M Propionic Acid (12,A)	Trypsin	LLGNVTIAQGGVLPNIHQNLLPK	4.47
			sAKAGLTFPVGR	3.45
Histone H4-TAP Acid Extraction #2	No treatment (13)	Trypsin	n/a	n/a
	2 Hr Carbamyl (14)	Trypsin	IGSGAPVYLTAVLEYLAAEILELAGNAAR	6.41
			SAkAGLTFPVGR	4.05
			HLQLAIR	2.61
	Citraconyl (15)	Trypsin	SAKATKASQEL	3.47
			AGSAAKASQSR	3.16
			AGSAAKASQSRSAK	3.13
			ATKASQEL	2.81
			AGLTFPVGR	2.73
	Propionyl (16,B)	Trypsin	SGGkGGkAGSAAkASQSR	5.22
			SAkAGLTFPVGR	3.90

Table 7: Histone H2A peptides identified from each preparation. (CONT)				
Sample	Treatment	Enzyme	Histone H2A Peptide Sequence	Highest XCorr
rtr1Δ Acid Extraction #1	No treatment (17)	Trypsin	NDDELNKLLGNVTIAQGGVLPNIHQNLLPK	6.61
			LLGNVTIAQGGVLPNIHQNLLPK	5.07
			AGLTFPVGR	2.91
			HLQLAIRNDDELNK	2.84
			HLQLAIR	2.77
	2 Hr Carbamyl (18)	Trypsin	SAkAGLTFPVGR	3.18
			HLQLAIR	2.80
rtr1Δ Acid Extraction #2	No treatment (19)	Trypsin	AGLTFPVGR	3.23
			HLQLAIRNDDELNK	4.92
			LLGNVTIAQGGVLPNIHQNLLPK	5.72
			NDDELNKLLGNVTIAQGGVLPNIHQNLLPK	7.41
			SAKAGLTFPVGR	3.82
	2 Hr Carbamyl (20)	Trypsin	NDDELNKLLGNVTIAQGGVLPNIHQNLLPK	6.27
			NDDELNkLLGNVTIAQGGVLPNIHQNLLPkK	4.84
			GGkAGSAAkASQSR	4.56
			SAkAGLTFPVGR	4.21
			HLQLAIRNDDELNK	3.50
			AGLTFPVGR	3.18
			AGSAAkASQSR	3.09
			LLGNVTIAQGGVLPNIHQNLLPK	3.04
			HLQLAIR	2.91
	Propionyl (21,A)	Trypsin	AGLTFPVGR	3.29
			GGkAGSAAkASQSR	4.49
			HLQLAIRNDDELNK	3.76
			KSAkTAkASQEL	3.65
			LLGNVTIAQGGVLPNIHQNLLPK	5.17
			NDDELNKLLGNVTIAQGGVLPNIHQNLLPK	7.63
			NDDELNKLLGNVTIAQGGVLPNIHQNLLPKk	5.26
			NDDELNkLLGNVTIAQGGVLPNIHQNLLPkkSAK	4.83
			SAkAGLTFPVGR	4.29
			SAkTAkASQEL	3.08
			SGGkGGkAGSAAkASQSR	5.02

Table 7: Histone H2A peptides identified from each preparation. (CONT)				
Sample	Treatment	Enzyme	Histone H2A Peptide Sequence	Highest XCorr
BY4741 Acid Extraction #1	No treatment (22)	Trypsin	LLGNVTIAQGGVLPNIHQNLLPK	3.31
			AGLTFPVGR	4.15
	2 Hr Carbamyl (23)	Trypsin	SGGkGGkAGSAAkASQSR	4.01
			SAkAGLTFPVGR	n/a
	No treatment (24)	Chymo	n/a	n/a
	Propionyl (25,A)	Chymo	n/a	n/a
	Propionyl NDE (26,A)	Trypsin	n/a	n/a
	Propionyl (27,A)	Chymo	n/a	5.34

Sample name denotes the strain genotype and the type of purification performed. Treatment refers to the type of chemical modification performed for the purified proteins (untreated, propionyl, carbamyl, or citraconyl). The sample number is also listed in the treatment column followed by a letter designating the rounds of propionylation perfomed on the sample if applicable. "A" means the sample was propionylated once before digestion. "B" means the sample was propionylated twice before digestion. "C" means the sample was propionylated twice before digestion and once after digestion. "D" means the sample was propionylated twice before digestion and twice after digestion. Enzyme means what enzyme the proteins were digested with. The peptide sequences are the unique peptides detected from each preparation. The highest XCorr is listed, which is a calculation done by SEQUEST® to determine how well the spectra matches to a theoretical spectra for the peptide. NDE = no dynamic exclusion.

Figure 4 also shows the most well characterized yeast histone modifications for H2B. The N-terminus of histone H2B is highly acetylated (K6, K11, K16, K17, K21, and K22) and is phosphorylated at S10. There is one known site of methylation on histone H2B at K37. Finally, there is a site of

ubiquitination at K123 on histone H2B. Table 8 lists all of the histone H2B peptides identified from each preparation. It is important to notice that all of these histone H2B modifications were detected in the propionylated and carbamylated *rtr1Δ* acid extraction #2 samples only. This is consistent with Table 2 showing that the highest amount of histone H2B spectra were for these two samples (407 and 629). Also, Table 8 shows that all of the lysines were not propionylated or carbamylated, probably due to the large yield of histone H2B from this purification and/or incomplete lysine modification.

Table 8: Histone H2B peptides identified from each preparation.

Sample	Treatment	Enzyme	Histone H2B Peptide Sequence	Highest XCorr
Histone H4-TAP TAP Purification #1	No treatment (1)	Trypsin	SmSILNSFVNDIFER	4.94
			KETYSSYIYK	2.95
			LILPGELAK	2.65
			VLKQTHPDTGISQK	2.63
	Propionyl (2,B)	Trypsin	n/a	n/a
	Propionyl (3,D)	Trypsin	n/a	n/a
Histone H4-TAP TAP Purification #2	Benzonase / Propionyl (4,B)	Trypsin	n/a	n/a
	MNase / Propionyl (5,B)	Trypsin	LILPGELAkHAVSEGTR	4.49
	Soluble/5X Trypsin / Propionyl (6,B)	Trypsin	IATEASkLAAYNkkSTISAR	5.63
			LILPGELAkHAVSEGTR	4.62
	No treatment (7)	Trypsin	SmSILNSFVNDIFER	4.94
Histone H4-TAP Acid Extraction #1	No treatment (8)	Trypsin	SMSILNSFVNDIFER	5.11
			VLKQTHPDTGISQK	4.01
			KETYSSYIYK	3.64
			QTHPDTGISQKSMSILNSFVNDIFER	3.32
			LILPGELAK	2.85
	Propionyl (9,C)	Trypsin	n/a	n/a
	Propionyl (10,B)	Trypsin	IATEASKLAAYNKKSTISAR	5.98
			AVTKYSSSTQA	3.25
	Propionyl with Glycine (11,A)	Trypsin	IATEASkLAAYNkkSTISAR	5.14

Table 8: Histone H2B peptides identified from each preparation. (CONT)				
Sample	Treatment	Enzyme	Histone H2B Peptide Sequence	Highest XCorr
Histone H4-TAP Acid Extraction #1 (CONT)	0.5 M Propionic Acid (12,A)	Trypsin	QTHPDTGISQkSMSILNSFVNDIFER	5.58
			IATEASkLAAYNkkSTISAR	5.25
			kETYSSYIYkVLK	4.75
			SMSILNSFVNDIFER	4.22
			ETYSSYIYkVLK	3.93
			kETYSSYIYK	3.09
Histone H4-TAP Acid Extraction #2	No treatment (13)	Trypsin	SmSILNSFVNDIFER	3.53
			KETYSSYIYK	3.46
			QTHPDTGISQK	2.91
	2 Hr Carbamyl (14)	Trypsin	LILPGELAkHAVSEGTR	3.50
	Citraconyl (15)	Trypsin	AVTKYSSSTQA	3.65
			HAVSEGTRAVTK	3.36
			EIQTAVR	2.01
	Propionyl (16,B)	Trypsin	IATEASkLAAYNkkSTISAR	5.07
			AVTkYSSSTQA	2.63
rtr1Δ Acid Extraction #1	No treatment (17)	Trypsin	SmSILNSFVNDIFER	4.96
			IATEASKLAAYNKK	4.06
			KETYSSYIYK	3.56
	2 Hr Carbamyl (18)	Trypsin	n/a	n/a
rtr1Δ Acid Extraction #2	No treatment (19)	Trypsin	EIQTAVRLILPGELAK	3.60
			ETYSSYIYKVLKQTHPDTGISQK	3.95
			KETYSSYIYK	3.61
			KETYSSYIYKVLK	3.99
			SMSILNSFVNDIFER	5.07
			VLKQTHPDTGISQK	4.52
	2 Hr Carbamyl (20)	Trypsin	AEkkPASkAPAEK	2.97
			APAEkKPAAK	3.51
			APAEkKPAAkK	2.97
			AVTkYSSSTQA	3.13
			ETYSSYIYK	2.76
			ETYSSYIYkVLK	4.05
			ETYSSYIYkVLkQTHPDTGISQK	3.76
			IATEASkLAAYNK	3.94
			IATEASKLAAYNKK	4.34
			KETYSSYIYK	3.76
			KETYSSYIYkVLK	4.73
			KPAAkkTSTSTDGK	4.24
			kPASKAPAEkkPAAK	4.09
			LAAYNkkSTISAR	4.38
			LILPGELAk	2.88
			LILPGELAKHAVSEGTR	3.89
			QTHPDTGISQkSMSILNSFVNDIFER	4.29

Table 8: Histone H2B peptides identified from each preparation. (CONT)				
Sample	Treatment	Enzyme	Histone H2B Peptide Sequence	Highest XCorr
rtr1Δ Acid Extraction #2 (CONT)	2 Hr Carbamyl (20) (CONT)	Trypsin	SAKAEKkPASK	2.75
			SMSILNSFVNDIFER	4.95
			VLkQTHPDTGISQk	3.80
	Propionyl (21,A)	Trypsin	AEkKPASkAPAEK	3.59
			AEkkPASKAPAEkkPAAK	5.09
			APAEkkPAAkK	3.82
			APAEkKPAAkkTSTSTDGK	4.16
			APAEkKPAAkkTSTSTDGkK	4.63
			APAEkKPAAkkTSTSTDGkKR	6.45
			AVTkYSSSTQA	3.45
			ETYSSYIYkVLK	4.27
			ETYSSYIYkVLkQTHPDTGISQK	3.90
			IATEASkLAAYNK	4.22
			IATEASkLAAYNkk	4.77
			IATEASkLAAYNkkSTISAR	6.08
			kETYSSYIYkVLK	4.82
			KETYSSYIYkVLkQTHPDTGISQK	5.67
			KPAAkkTSTSTDGK	4.34
			kPAAkkTSTSTDGkK	5.00
			KPAAkkTSTSTDGkkR	5.48
			kPASkAPAEK	3.36
			kPASkAPAEkkPAAK	5.12
			kTSTSTDGkkR	3.46
			LAAYNkkSTISAR	4.78
			LILPGELAkHAVSEGTR	4.75
			SAkAEkkPASK	3.13
			SAKAEkKPASkAPAEK	3.44
			SMSILNSFVNDIFER	4.95
			VLKQTHPDTGISQK	4.37

Table 8: Histone H2B peptides identified from each preparation. (CONT)				
Sample	Treatment	Enzyme	Histone H2B Peptide Sequence	Highest XCorr
BY4741 Acid Extraction #1	No treatment (22)	Trypsin	SmSILNSFVNDIFER	4.64
			KETYSSYIYK	3.80
			APAEKKPAAK	3.34
			LILPGELAK	2.77
			ETYSSYIYK	2.75
			LAAYNKK	2.60
	2 Hr Carbamyl (23)	Trypsin	IATEASkLAAYNkkSTISAR	6.09
			SSAAEkkPASkAPAEkkPAAkkTSTSVDGkkR	4.63
			AVTkYSSSTQA	4.02
			LILPGELAkHAVSEGTR	3.78
	No treatment (24)	Chymo	n/a	n/a
	Propionyl (25,A)	Chymo	n/a	n/a
	Propionyl NDE (26,A)	Trypsin	EIQTAVR	2.26
	Propionyl (27,A)	Chymo	n/a	n/a

Sample name denotes the strain genotype and the type of purification performed. Treatment means what type of modification was done to the purified proteins (untreated, propionyl, carbamyl, or citraconyl). The sample number is also listed in the treatment column followed by a letter designating the rounds of propionylation perfomed on the sample if applicable. “A” means the sample was propionylated once before digestion. “B” means the sample was propionylated twice before digestion. “C” means the sample was propionylated twice before digestion and once after digestion. “D” means the sample was propionylated twice before digestion and twice after digestion. Enzyme refers to the proteolytic enzyme the proteins were digested with. The peptide sequences are the unique peptides detected from each preparation. The highest XCorr is listed, which is a calculation done by SEQUEST® to determine how well the spectra matches to a theoretical spectra for the peptide. NDE = no dynamic exclusion.

As already mentioned, methylation at H3K4 and H3K36 have been shown to correlate with transcription. Table 6 shows histone H3 peptides identified in the histone H4-TAP acid extraction #2 with modifications (propionyl, carbamyl, acetyl, or dimethyl). The untreated sample does not result in identification either of these lysine residues. Both the carbamyl sample and the propionyl sample identify unmodified peptides with K36. The propionyl sample also shows one spectra for a peptide containing K4 with a low XCorr of 2.81.

K36 was not identified in any of the untreated samples (Table 4). Both the propionylation and carbamylation treatments help to identify K36. The propionyl sample only identifies the K36 containing peptide (kSAPSTGGVkkPHR) where all three lysine residues are propionylated. This means that the identified peptide was unmethylated at K36 when propionylated. It is difficult to determine if this is the case for the carbamyl sample, since the linear ion trap cannot distinguish between acetyl, trimethyl, and carbamyl. This means that the identified acetylated K36 could actually be just a carbamyl. This does not help with the identification of the methylation state of K36 in wildtype versus *rtr1Δ* cells.

VII. Peptides Specific to Treatments

Table 2 shows how low the spectral counts were for histone H3 for all of the untreated samples. Histone H3 had zero spectra for the majority of the untreated samples and always had the least amount of spectra compared to the other histones. The purpose of the propionylation, carbamylation, and citraconylation treatments, plus all of the derivations of these treatments was to

increase the spectral counts specifically for histone H3. Tables 2 and 3 show the results of these treatments on the spectral counts. Both propionylation and carbamylation increased the spectral counts for histone H3. Table 9 shows a comparison of peptides that were only detected in the untreated, propionylated, carbamylated, and citraconylated samples. It is important to know if the treatments help to detect specific peptides that other treatments are not able to detect.

Table 9: Unmodified Histone Peptides Specific to Treatments

Histones	Untreated	Propionylated	Carbamylated	Citraconylated
H2A	LLGNVTIAQGGVLPNIHQNLLPKK	NDDELNKLLGNVTIAQGGVLPNIHQNLLPKKSAK	n/a	AGSAAKASQSRSAK
		KSAKTAKASQEL		ATKASQEL
H2B	QTHPDTGISQK	SAKAEKKPASKAPAEK	SSAAEKKPASKAPAEKKPAAKKTSTSVDGKKR	HAVSEGTRAVTK
	LAAYNKK	AEKKPASKAPAEKKPAAK		
	EIQTAVRLILPEGELAK	KPASKAPAEK		
		APAEKKPAAKKTSTSTDGK		
		APAEKKPAAKKTSTSTDGKK		
		APAEKKPAAKKTSTSTDGKKR		
		KPAAKKTSTSTDGKK		
		KPAAKKTSTSTDGKKR		
		KTSTSTDGKKR		
		KETYSSYIYKVLKQTHPDTGISQK		
H3	KSAPSTGGVK	SAPSTGGVKKPHR	n/a	n/a
	FQSSAIGALQESVEAYLVSLFEDTNLAAIHAK	FQSSAIGALQESVEAYLVSLFEDTNLAAIHAKR		
		RVTIQKKDIK		
		RVTIQKKDIKLAR		
		KDIKLAR		
H4	ISGLIYEEVRAVLK	SGRGKGGKGLGKGGAKR	GLGKGGAKR	n/a
	SFLESVIRDSVTYTEHAKR	GKGGKGLGK	DSVTYTEHAKRK	
	RKTVTSLDVVYALK	GKGGKGLGKGGAK		
		AVLKSFLESVIRDSVTYTEHAKR		

The four core histones are listed on the left of the table, while the treatments are listed at the top. Representative propionylated samples were performed with the corrected 1:3 propionylation reagent ratio.

As shown in Table 9, the propionylated samples have the most unique peptides specific to a chemical treatment. Specifically, the propionylated sample is able to help detect sequences that are rich in lysine residues. This is because the propionyl group blocks the lysine residues and prevents cleavage by trypsin. The untreated samples have unique peptides that are lost when the sample undergoes chemical treatments. After identification of the unmodified peptides specific to each chemical treatment, the modified peptides would also be helpful to compare the usefulness of each treatment.

Table 10 lists the modified peptides specific to each treatment. The citraconylated sample did not have any unique modified peptides, so it was left out of the table. The untreated, propionylated, and carbamylated samples are the only ones listed in Table 10. Modifications are listed by the mass change of the modification after the modified residue (dimethyl +28 Da, acetyl/trimethyl +42 Da, carbamyl +43 Da, and propionyl +56 Da). For instance, K(+28) means that particular lysine residue was dimethylated.

Table 10: Modified Histone Peptides Specific to Treatments

Histones	Untreated	Propionylated	Carbamylated
H2A	MSGGK(+42)GGK(+28)AGSAAK	SGGK(+42)GGK(+56)AGSAAKASQSR	MSGGKGGKAGSAAK(+42)ASQSR
		SGGK(+42)GGK(+56)AGSAAK(+56)ASQSR	SGGK(+42)GGK(+43)AGSAAK(+43)
		SGGK(+56)GGK(+56)AGSAAK(+28)ASQSR(+28)	SGGK(+42)GGK(+43)AGSAAK(+43)ASQSR
		GGK(+42)AGSAAK(+56)ASQSR	GGK(+28)AGSAAK(+43)ASQSR
		GGK(+56)AGSAAK(+42)ASQSR	GGK(+42)AGSAAK(+43)ASQSR
		NDDELNK(+28)LLGNVTIAQGGVLPNIHQNLLPK	K(+28)SAK(+43)ATK(+43)ASQEL
		NDDELNKLLGNVTIAQGGVLPNIHQNLLPK(+42)K	
		NDDELNK(+56)LLGNVTIAQGGVLPNIHQNLLPK(+28)K(+28)	
H2B	KPASK(+42)APAEK	APAEK(+56)KPAAK(+56)K(+42)STSVDGK(+56)K	KETYSSYIYKVLK(+42)
	LILPGELAK(+42)HAVSEGTR	K(+42)ETYSSYIYK(+56)VLK	QTHPDTGISQK(+28)SMSILNSFVNDIFER
		K(+42)ETYSSYIYK(+56)VLK(+56)QTHPDTGISQK	LAAYNK(+42)K(+28)STISAR
		QTHPDTGISQK(+42)SMSILNSFVNDIFER	
		IATEASK(+56)LAAYNK(+28)K(+28)	
		IATEASK(+56)LAAYNK(+42)K(+56)STISAR	
		IATEASK(+56)LAAYNK(+56)K(+28)STISAR(+28)	
H3	RFQK(+42)STELLIR	ARTKQTARK(+42)STGGKAPR	TK(+43)QTAR(+28)KSTGGKAPR
H4	AVLK(+42)SFLESVIR	SGRGKGGKGLGKGGAK(+42)R	SGRGKGGKGLGK(+28)GGAKR
	K(+28)TVTSLDVVYALK	GGKGLGKGGAK(+28)	SGRGK(+28)GGK(+42)GLGKGGAKR
		GGK(+56)GLGK(+42)GGAK(+42)R	GKGGKGLGK(+28)GGAK(+28)R
		ISGLIYEEVR(+28)	
		K(+56)TVTSLDVVYALK(+28)R(+28)	

The core histones are listed on the left of the table, while treatments are listed at the top. The propionylated samples were done using the corrected 1:3 propionylation reagent ratio. Modifications are listed by their mass change after the residue. Dimethyl (+28 Da), acetyl/trimethyl (+42 Da), carbamyl (+43 Da), and propionyl (+56 Da).

Table 10 shows that the propionylation treatment helps to detect the most unique modified peptides. The carbamylated treatment also detects unique modified peptides. Both the carbamylated and propionylated treatments were able to detect a H3K4 containing peptide, though neither shows H3K4 as mono-, di-, or trimethylated. Tables 9 and 10 together show that the best way to increase the overall coverage of the core histones is to use both the propionylation and carbamylation treatments along with no treatment at all.

Figure 18 shows the sequence coverage for the core histones for all untreated samples (1, 7-8, 13, 17, 19, and 22), propionylated samples (2-6, 9-12, 16, 21, and 26), and carbamylated samples (14, 18, 20, and 23). The untreated samples show the lowest amount of overall coverage for the core histones. The propionylated sample shows the most coverage for the core histones which is consistent with the fact that the propionylation resulted in the highest number of unique peptides identified. Both the propionylated and carbamylated samples almost reach 100% coverage of histone H2B.

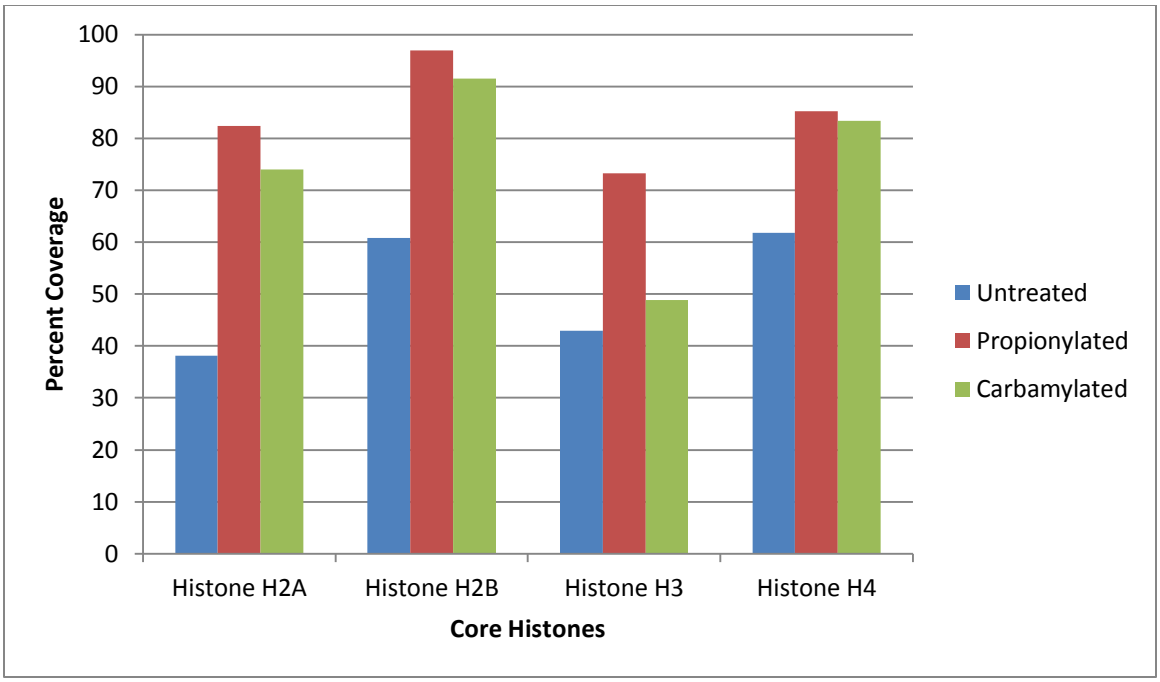

Figure 18. Sequence coverage for core histones. Core histones are indicated at the bottom of the graph. Percent coverage is indicated to the left of the graph.

VIII. H3K36me3 Western Blot

Figure 10 shows some example ChIP-chip data obtained for BY4741 and *rtr1Δ* cells using an antibody against H3K36me3. This figure shows that there is a 3' shift of H3K36me3 past the 3' end of multiple genes in *rtr1Δ.* Specifically, the H3K36me3 mark shifts past the transcription termination site. This then led us to question whether or not there was a change in the overall abundance of H3K36me3 in *rtr1Δ.* The Western blot in Figure 19 was performed to answer this question.

A small scale nuclei prep was done to enrich for nuclear proteins present in the sample, thus enriching the histone proteins. Because the protein was resuspended in SDS the exact amount of protein was not determined, but

instead increasing volumes of each sample were loaded. Pgk1 was used as a loading control, because this protein is known to remain constant in the wildtype compared to the mutant (data not shown). Based on the levels of Pgk1 present in both samples, the *rtr1Δ* sample has slightly more protein present. Taking this into account, it seems that there is not a significant change in the total levels of H3K36me3 between BY4741 and *rtr1Δ.* This suggests that there is a change in the pattern of H3K36me3 in *rtr1Δ* yet no change in overall H3K36me3 levels.

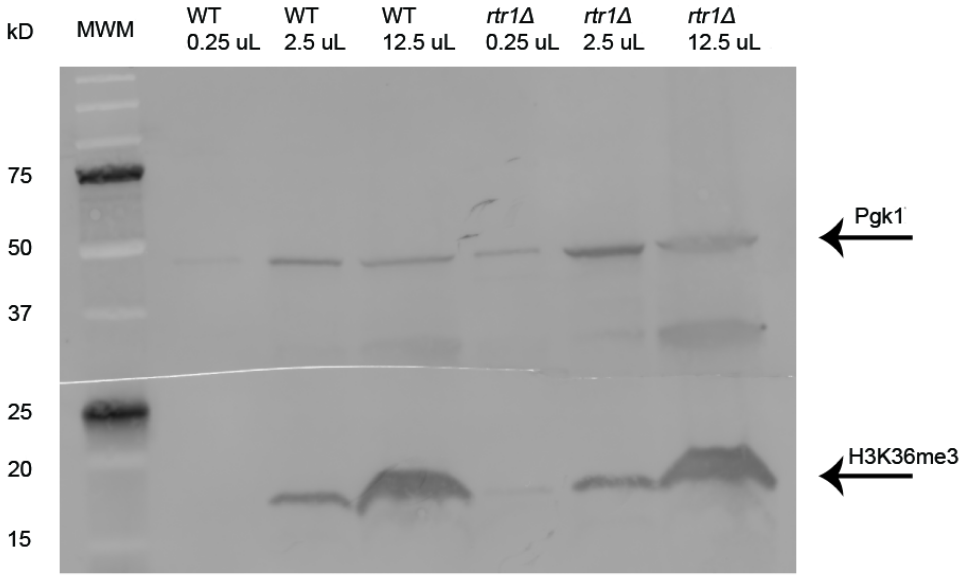

Figure 19. Histone H3K36me3 Western blot. The level of H3K36me3 was examined between BY4741 and *rtr1Δ* by loading increasing volumes of a small scale nuclei preparation for each sample. Pgk1 was used as a loading control. The molecular weight marker (MWM) is loaded in the first lane with labels to the left of the figure to indicate the protein size in kiloDalton (kD). The total volume of lysate loaded in each lane is given at the top of the figure.

DISCUSSION

It has long been known that histones serve to compact DNA into nucleosomes thereby reducing the size of the genetic material, but studies in recent years have shown that histones also play a major role in the regulation of transcription (Strahl, Ohba et al. 1999; Litt, Simpson et al. 2001; Noma, Allis et al. 2001). Histones are modified post-translationally with a variety of modifications as shown in Figures 4 and 5 (Murray 1964; Gershey, Vidali et al. 1968; Nathan, Ingvarsdottir et al. 2006; Nelson, Santos-Rosa et al. 2006). Acetylation of histone lysine residues has been well characterized, but the specific role of lysine methylation in transcriptional regulation has not been fully elucidated (Gershey, Vidali et al. 1968; Takahashi, McCaffery et al. 2006; Li, Carey et al. 2007). To understand how lysine methylation helps to regulate transcription, better techniques need to be developed to identify these modifications and changes in modification abundance in different cellular conditions.

MudPIT allows for separation and analysis of complex peptide samples and has been used to help identify previously unknown protein PTMs (Florens and Washburn 2006; Guillemette, Drogaris et al. 2011). Histones present a problem for the standard MudPIT approach, because of the abundance of lysine and arginine residues. Before MudPIT analysis, proteins are typically digested with trypsin, which cleaves C-terminally to arginine and lysine residues, resulting in small, highly charged histone fragments. These small, highly charge fragments are not ideal for mass spectrometry analysis. Blocking lysine residues

with chemical modifications can be used to hinder trypsin digestion at these residues (Butler, Harris et al. 1969; Peters, Kubicek et al. 2003). This then allows for trypsin to digest only at arginine residues, increasing the size of the histone fragments and neutralizing the charge at lysines. Propionylation of lysine residues has recently been used to help identify histone modifications (Peters, Kubicek et al. 2003; Garcia, Mollah et al. 2007). Coupling lysine propionylation and MudPIT analysis has the potential to increase the identification of histone modifications and help to determine the role of these modifications in different cellular conditions.

In order to develop our novel approach to identify the histone modifications, we first attempted to obtain a highly purified histone sample. Tandem affinity purifications use a TAP tag to doubly purify the protein of interest and any associated proteins (Rigaut, Shevchenko et al. 1999). One of the core histone proteins (histone H4) was TAP tagged and purified. Table 1 shows that in sample 1 there were some cytoplasmic histone associated proteins also purified (Hat1, Hat2, Hif1) (Krogan, Cagney et al. 2006; Gong, Kakihara et al. 2009). Because we were interested in studying posttranslational modifications on the core histones that are associated with transcription (a nuclear process) we did not want to include cytoplasmic modifications and proteins.

Since histones are basic nuclear proteins, we proposed that a nuclei prep followed by acid extraction would enrich the histones allowing for more spectra to be detected. Table 2 shows the increase in spectra for histones H2A, H2B, and H4 in an untreated acid extraction for histone H4-TAP. However, we also were

aware that this would result in decreased purity since acid extraction will enrich for all basic proteins in our isolated nuclei (Kizer, Xiao et al. 2006). The TAP purification for sample 1 yielded 19 proteins with at least 10 spectral counts detected by MudPIT analysis (Table 1). Sample 8 was the first untreated acid extraction which yielded about 160 proteins with at least 10 spectral counts detected by MudPIT analysis. At this point we decided that increasing the abundance of the histones was more important than analysing a more purified sample since it would facilitate our efforts to optimize the chemical modification procedure for histone detection using MudPIT.

Adding the propionylation reaction to the acid extraction allowed for detection of histone H3, but also led to decreases in the spectral counts for the rest of the core histones; a phenomena that has also been reported by other groups (Drogaris, Wurtele et al. 2008). Figures 15 and 16 also illustrate another problem we discovered that was a result of the propionylation reaction. An extracted ion chromatogram allows for the visualization of the ion intensities for a particular ion across all of the MudPIT steps (Wong, Sullivan et al. 2008). Figures 15 and 16 clearly show that there was a 160-fold decrease in the maximum ion intensity for the proteotypic peptide ISGLIYEEVR from histone H4 (Craig, Cortens et al. 2005). The elution pattern also differs between the untreated and propionylated samples. The untreated sample shows a clear peak towards the beginning of the organic gradient (Figure 15), while the propionylated sample's main peak is towards the end of the organic gradient (Figure 16). The propionylation reaction changes how the peptide elutes off the chromatography,

which is something that could be further investigated to determine exactly how and why this changes considering that the peptide we used for this analysis ISGLIYEEVR does not contain a lysine. Because of the problems with the propionylation reaction lowering the overall spectral counts and ion intensities of the histone peptides other chemical blocking methods were also researched.

Carbamylation and citraconylation have also been used to chemically block lysine residues (Kadlik, Strohalm et al. 2003; Piscopo, De Petrocellis et al. 2006). Both techniques were used to compare to the propionylation reaction. Table 2 shows the comparison of the spectral counts for all three treatments and no treatment in the second histone H4-TAP acid extraction. This was also the first time for the corrected propionylation reagent ratio (1:3) (Plazas-Mayorca, Zee et al. 2009). Both propionylation and carbamylation increased the spectral counts for histone H3, while citraconylation did not help with detection of histone H3. Our studies show that carbamylation was an additional option to increase detection of histone H3 though chemical modification whereas citraconylation is not.

The goal of increasing the spectral counts of histone H3 was to better identify K4 and K36 methylation, since they have been shown to correlate with transcription (Yuan, Liu et al. 2005). Table 6 shows the histone H3 peptides with modifications from the second histone H4-TAP acid extraction. K4 and K36 were identified in both treatments in this prep, but they were blocked by the chemical modifications. These results indicate that neither of these residues were identified as methylated in these samples. These data indicate that the addition

of chemical modifications was not sufficient to identify methylation of K4 or K36 using MudPIT.

H3K4 still causes problems for detection by mass spectrometry even with the chemical blocking of lysine residues. Figures 3 and 5 show the location of H3K4 in the amino acid sequence for histone H3. The first 8 amino acids of H3 are “ARTKQTAR.” It is important to notice that when cleaved with trypsin, the fragments would be “AR”, “TK”, and “QTAR.” Blocking the lysine residue and cleaving with trypsin would yield “AR” and “TKQTAR.” This yields a larger peptide fragment containing K4, which would hopefully increase detection. The mass of this unmodified fragment would be approximately 685 Daltons. Unblocked, the fragment would be charged on the N-terminus, C-terminus, and lysine side chain yielding a +3 overall charge. Blocking the lysine residue reduces the charge to +2 and the m/z ratio to 342.5 Daltons. The original m/z range that was being searched would have ignored this peptide, which is why the m/z range was lowered to 200 Daltons.

Even with lowering the m/z range and blocking the lysine residues, H3K4 was not confidently detected as methylated. SEQUEST® assigns XCorr values based on how well the theoretical and experimental spectra match (Eng, McCormack et al. 1994). Methylated H3K4 was never assigned an XCorr of above 2.1, which means that the experimental spectra matched the theoretical spectra but not extremely well. More peptide-spectrum match confidence would be needed to say that any of these spectra actually represented methylated H3K4 peptides.

Table 6 also illustrates a problem created by the carbamylation reaction. Lysine residues that were identified as acetylated were also identified as carbamylated. Acetylation is an addition of approximately 42 Daltons, while carbamylation is an addition of 43 Daltons. The linear ion trap mass spectrometer used for these experiments has a mass resolution of approximately plus or minus 1 Dalton (Mann and Kelleher 2008). This means that carbamylation, acetylation, and even trimethylation cannot be differentiated using our mass spectrometer which lacks a high mass accuracy analyzer such as an Orbitrap or an FT.

The carbamylated and propionylated peptides in Table 6 show the detection of an H3K36 containing peptide. This peptide is "KSAPSTGGVKKPHR." Just like K4, K36 was only detected as blocked by either the carbamylation or propionylation. As just mentioned above, the linear ion trap mass spectrometer is not a high mass accuracy instrument, so the assignment of K36 being carbamylated could actually be acetylation or trimethylation. This would need to be further investigated using a high mass accuracy instrument that could confidently assign the correct modification (Zhang, Yau et al. 2004).

Tables 8 and 9 show the unique unmodified and modified peptides detected using no treatment, propionylation, and carbamylation. Reviewing these two tables, it is apparent that each treatment helps to detect different peptides. We see high levels of histone protein sequence coverage when combining the data from all of the propionylated samples, which is not increased

when adding in the data from the untreated and carbamylated samples (Figure 18). Though combining all of the propionylated samples together yields the highest histone protein sequence coverage, the propionylated samples do not consistently help to detect all of the amino acid residues that were detected in at least one of the propionylated samples. We recommend combining all three approaches with the use of a high mass accuracy mass spectrometer to increase the detection of the most histone peptides with and without modifications. This approach would give confidence to the assignment of H3K4 as methylated and determine if H3K36 was carbamylated, trimethylated, or acetylated because of the higher resolving power of a high mass accuracy mass spectrometer (Mann and Kelleher 2008).

The importance of H3K36me3 is emphasized by the ChIP-chip data shown in Figure 10. The methyltransferase responsible for H3K36 methylation is Set2, which is thought to bind to the serine 2 and serine 5 phosphorylated CTD of RNAPII (Kizer, Phatnani et al. 2005; Vojnic, Simon et al. 2006). Rtr1 is a serine 5 phosphatase that is thought to remove the serine 5 phosphorylation from the RNAPII CTD, which would in turn displace Set2 from the CTD (Mosley, Pattenden et al. 2009). Figure 10 shows that when *RTR1* is deleted, H3K36me3 shifts past the TTS, which indicates that Set2 is associated with RNAPII past its normal termination site when Rtr1 is not present. It is also important to determine if the levels of H3K36me3 are overall changed when *RTR1* is deleted. Figure 18 shows that the overall levels of H3K36me3 were not changed when *RTR1* was deleted. This suggests that Set2 could be mislocalized in the absence of Rtr1

resulting in higher levels of H3K36me3 past the normal TTS. This hypothesis will be tested by our laboratory in the future.

CONCLUSION

Histones are highly basic proteins which when digested by trypsin are hard to analyze using mass spectrometry. Because histones are basic nuclear proteins, a nuclei prep followed by acid extraction is the best purification strategy to increase overall abundance of purified histones. Blocking the lysine residues and cleaving with trypsin is a useful technique to increase detection of histone peptides using MudPIT. In particular, carbamylation and propionylation are the best two methods to block lysine residues. Using both propionylation and carbamylation along with no treatment has been shown to increase the identification of unmodified and modified histone peptides when coupled with MudPIT analysis.

Both carbamylation and propionylation help to identify key lysine residues of H3, while both still cannot help to confidently identify K4. More work would need to be done with these techniques to identify K4 and increase the spectral counts for modified K36.

Our laboratory has identified a 3' shift of H3K36me3 localization at multiple RNAPII target genes in *rtr1Δ*. It has now been shown that there is no change in the overall cellular abundance of H3K36me3 in *rtr1Δ.* More work will be performed to determine if the 3' shift of H3K36me3 in *rtr1Δ* is because of a change in the recruitment pattern of Set2 across RNAPII target genes in yeast.

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