“OPEN” CHROMATIN:

HISTONE ACETYLATION, LINKER HISTONES

& HISTONE VARIANTS

by

Jennifer Kristel Choi

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Abstract

DNA in the eukaryotic cell is packaged into a structure called chromatin. Chromatin is a dynamic structure that regulates access to DNA in response to environmental stimuli. Two widely conserved mechanisms that influence chromatin structure are the addition of post-translational modifications (PTMs) to histones and other chromatin-associated proteins, and the replacement of canonical histones with histone variants.

Histone acetylation is catalyzed by histone acetyltransferases (HATs). HATs are comprised of a catalytic subunit, and associated proteins. Genetic analysis of the yeast HATs has shown that the combined deletion of the two HAT genes, *GCN5* and *SAS3*, results in an inviable strain of yeast. In this thesis, I show that the inviability of the *gcn5Δsas3Δ* mutant is due to a combined failure to acetylate both histone H3 and the chromatin-remodeler protein Rsc4. Further, I show that acetylation of Rsc4 is catalyzed by Gcn5 in a HAT complex-independent manner.

The linker histone, H1, is associated with higher-order chromatin structure; it has been shown that removal of H1 is required to allow access to DNA. In this work, I show that deletion of the linker histone rescues the growth of a conditional *gcn5Δ sas3Δ* mutant expressing a temperature-sensitive version of Sas3. Further, I present the incorporation of the histone variant Htz1 as an additional mechanism for mobilizing the linker histone away from the +1 nucleosome. I, also, provide data that corroborates evidence suggesting that the yeast linker histone binds a single nucleosome.

Another histone variant found in many eukaryotes is histone H3.3, which is primarily incorporated into transcriptionally active regions in chromatin. In this dissertation, we created a

series of human-yeast histone hybrids and tested their ability to rescue yeast lacking both endogenous copies of histone H3. Our data shows that the two human histone H3 variants, H3.1 and H3.3, are functionally interchangeable for growth in most nutrient conditions, confirming that the four amino acids that are different between H3.1 and H3.3 are not necessary to create transcriptionally permissive chromatin. Finally, we present evidence that three yeast H3 C-terminal domain amino acids play an important role in regulating the interactions of yeast H3.

Preface

Chapter 3: "Acetylation of Rsc4 by Gcn5 is essential in the absence of histone H3 acetylation" is based on a first author paper published in the journal Molecular and Cellular Biology in 2008. "Acetylation of Rsc4p by Gcn5p is essential in the absence of histone H3 acetylation." Choi JK, Grimes DE, Rowe KM, Howe LJ. Mol Cell Biol. 2008 Dec;28(23):6967-72. doi: 10.1128/MCB.00570-08. All of the experiments were designed by myself and Dr. LeAnn Howe. I conducted all of the experiments. Keegan Rowe, Daniel Grimes, and Dr. LeAnn Howe helped to make strains and plasmids. I helped to edit the manuscript, which was written by Dr. LeAnn Howe.

Chapter 4: "The loss of the histone Htz1 leads to the increased binding of Hho1to nucleosomes" is based on a first author manuscript currently in preparation. All experiments were designed by myself and Dr. LeAnn Howe. I conducted all of the experiments with the help of the following individuals: Dr. Barry Young helped with the SDL screen and analysis using "Balony;" Dr. Julie Brind'Amour helped with the library preparation for the ChIP-Seq experiment; and Benjamin Martin and Nicolas Coutin helped with the read mapping and data analysis of the ChIP-Seq data.

Chapter 5: "Human-yeast hybrid H3 histones rescue the loss of endogenous yeast H3" is based on ongoing research. All of the experiments were designed by myself and Dr. LeAnn Howe. I conducted all of the experiments. The collaborative work with Dr. Chris Nelson's lab is ongoing and I did all of the experiments and created all of the strains shown in this thesis.

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List of Abbreviations

Δ	Delta- signifies deletion
°C	Degree Celsius
5-FOA	5-Fluoroorotic Acid
µg	Microgram
µM	Micromolar
A	Alanine
Ac	Acetylation
Ada2	Transcriptional Adaptor
Asf	Anti-Silencing Factor
bp	Base pair
C	Cysteine
CENP-A	Centromere Protein A
ChIP	Chromatin immunoprecipitation
ChIP-Seq	ChIP followed by high throughput sequencing
co-IP	Co-immunoprecipitation
Cse4	Chromosome Segregation
DNA	Deoxyribonucleic acid
DNaseI	Deoxyribonucleic acid nuclease 1
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EtBr	Ethidium Bromide
FACT	FAcilitates Chromatin Transactions
g	Gram
GAL	Galactose
Gcn5	General Control Nonderepressible
GST	Glutathione S-Transferase
h	Hour
H2A.Z	Histone Two A Z1
H3K9ac	Acetylation of Lysine 9 on Histone H3
H3K14ac	Acetylation of Lysine 14 on Histone H3
H3K18ac	Acetylation of Lysine 18 on Histone H3
H3K23ac	Acetylation of Lysine 23 on Histone H3
H3K4me3	Trimethylated Lysine 4 on Histone H3
H3K4me3	Trimethylation of Lysine 4 on Histone H3
H3K56ac	Acetylation of Lysine 56 on Histone H3
H3K79me3	Trimethylation of Lysine 79 on Histone H3
HA	HemAgglutinin
HAT	Histone Acetyltransferase
HDA	Histone DeAcetylase Complex
Hho1	Histone H One

HDAC	Histone deacetylase complex
HFD	Histone fold domain
HMG	High-Mobility Group
Htz1	Yeast Histone Two A Z1
HU	hydroxyurea
ING	Inhibitor of Growth
IP	Immunoprecipitation
kb	Kilo base pair
kg	Kilogram
L	Litre
L	Leucine
LTR	Long Terminal Repeat
m	Metre
M	Molarity
Me	Methylation
mg	Milligram
min	Minute
mL	Millilitre
mM	Millimolar
MMS	Methyl methanesulfonate
MNase	Micrococcal nuclease
MS	Mass spectrometry
MYST	MOZ, YBF2/SAS3, SAS2, Tip60
ncRNA	Non-coding RNA
NFR	Nucleosome Free Region
ng	Nanogram
nM	Nanomolar
NP	Nucleoplasmin
nt	Nucleotide
NTD	N-Terminal Domain
NuA3	Nucleosome Acetyltransferase 3
O/N	Overnight
OD	Optical Density
ORF	Open reading frame
ORF	Open Reading Frame
PCR	Polymerase chain reaction
pH	Potential of Hydrogen
PH	Pleckstrin Homology
PHD	Plant HomeoDomain
PIC	Pre-Initiation Complex
Pob3	Polymerase 1 Binding

PTM	Post-Translational Modification
PWWP	Proline-Tryptophane-Tryptophane-Proline
Q	Glutamine
QPCR	Quantitative PCR
R	Arginine
RNA	Ribonucleic acid
RNAPII	RNA Polymerase II
rpm	Revolutions per minute
rRNA	Ribosomal RNA
RSC	Remodel the Structure of Chromatin
Rsc2	Remodel the Structure of Chromatin subunit 2
Rsc4	Remodel the Structure of Chromatin subunit 4
Rtt	Regulator of Ty1 Transposition
*rtt109*Δ	Rtt109 gene deletion
s	Second
S	Serine
SAGA	Spt-Ada-Gcn5 Acetyltransferase
Sas3	Something About Silencing
SDL	Synthetic Dosage Lethality
SDR	Synthetic Dosage Rescue
SDS	sodium dodecylsulfate
SET	Su (var) Enhancer of Zeste and Trithorax
Set1/2	SET domain-containing
Spt16	SuPpressor of Ty
SSRP	Single Structure Recognition Protein
STDEV	Standard deviation
T	Threonine
TA	Transcription Activator
TAP	Tandem Affinity Purification
TBP	Tata binding protein
TE	Buffer consisting of Tris base, EDTA, and water
TEV	Tobacco Etch Virus Protease
TF	Transcription Factor
tRNA	Transfer RNA
TSS	Transcription Start Site
Ub	Ubiquitination
UTR	UnTranslated Region
w/o	Without
WCE	Whole Cell Extract
WT	Wild Type
yFACT	Yeast FACT

Yng1	Yeast homolog of mammalian Ing1
α	Alpha-signifies anti

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Dedication

I would like to dedicate this thesis to my parents Gorlieo and Jean and brother Michael whose unending love, support, and encouragement have enabled me to pursue my goals.

Chapter 1. Introduction

The fundamental element of DNA organization, in the eukaryotic cell, is the nucleosome: a DNA and protein ensemble comprised of 2 copies of 4 histone proteins that form an octamer, and 147 bp of DNA (Finch et al., 1977). Histones and other associated proteins enable the cell to compact DNA over 10,000-fold. This compaction, however, comes at the cost of accessibility. Thus, the eukaryotic cell has evolved to have a host of mechanisms to coordinate the unfolding and refolding of this structure, called chromatin, in response to environmental and cellular stimuli to allow for transcription. The processes that modulate access to DNA include DNA methylation, covalent modification of histones, incorporation of histone variants, nucleosome remodeling, nuclear positioning, and the interaction of regulatory proteins and RNAs. These processes create the chromatin landscape of the cell, which contributes to the control of genes that determine cell fate.

One of the most remarkable aspects of the development of multicellular organisms is the capacity to generate a range of different cell types in a highly regulated and reproducible manner from a single progenitor cell. These differentiated cells all have the same genotype as the progenitor cell, yet they have can have very different proteomes, transcriptomes, and epigenetic traits, which are due to their different chromatin landscapes. The mechanisms that control the chromatin landscape of the cell are generally conserved among eukaryotes, and the study of chromatin in model organisms including *Saccharomyces cerevisiae* and *Drosophila melanogaster* has been, and remains, key to growing our understanding of DNA organization.

More recently, the Encyclopedia of DNA Elements (ENCODE) project embarked upon the ambitious goal of creating a "user manual" for the human genome using and developing

cutting edge technologies that would elucidate chromatin patterns across large regions of DNA (The ENCODE Project Consortium, 2004). The current findings of the Constortium, however, only start to catalogue the many features of human chromatin in a limited number of cell types, and its members have been criticized for their choice of analytical techniques and for having fallen for the genetic equivalent of apophenia (i.e. creating meaningful patterns from random data) (Brugger, 2001; Fyfe et al., 2008; Niu and Jang, 2013; Eddy, 2012; Graur et al., 2013). Thus, we have only started to scratch the surface of epigenetic regulation and to unravel how chromatin functions.

1.1 The Nucleosome

The nucleosome is the fundamental repeating unit of chromatin. It is composed of a histone octamer, containing two copies each of the four core histones—H2A, H2B, H3, and H4—which is wrapped by 147 bp of sharply bent DNA (Luger et al., 1997; Richmond and Davey, 2003). Histone octamers are organized on DNA by proteins called histone chaperones (Burgess and Zhang, 2012). Neighbouring nucleosomes are separated by linker DNA, with the length of the linker varying between organisms and cell types (McGhee and Felsenfeld, 1980). The fifth histone protein, the linker histone, binds to the nucleosome at the linker DNA, creating a structure known as a chromatosome (Allan et al., 1980). Due to the vitally important nature of chromatin, the histone proteins and their modifying complexes are some of the most conserved across all eukaryotes.

Nucleosomes enwrap 75-90% of the DNA in a nucleus, and are found at favoured positions in the genome. High-resolution techniques, that have allowed characterization of

genome wide patterns of nucleosome occupancy, reveal that nucleosomes are generally depleted at promoters and terminators, which are called the 5' and 3' nucleosome free regions (NFRs), and occupy preferred positions within genes (Yuan et al., 2005; Lee et al., 2007; Mavrich et al., 2008; Schones et al., 2008). *In vitro* studies have shown that nucleosome occupancy can be greatly influenced by the octamer's affinity for the underlying DNA sequence (Thastrom et al., 1999). Using purified yeast genomic DNA and salt dialysis, scientists were able to reconsititute chromatin that had highly similar nucleosome positioning as *in vivo* nucleosome maps, suggesting that nucleosome positioning was somewhat influenced by the underlying DNA sequence (Liu et al., 2006; Kaplan, et al., 2009). However, there are many aspects of *in vivo* positioning that cannot be replicated in reconstitution assays using purified DNA and histones, such as the strong positioning of the +1 nucleosome, which is the first nucleosome after the NFR at the start of a gene (Kaplan et al., 2009; Zhang et al., 2009).

Access to DNA wrapped in a nucleosome is occluded for proteins that are involved in transcription, DNA replication, or DNA repair (Kornberg and Lorch, 1995); thus cells have devised ways of recruiting chromatin remodelers and histone modifying complexes to specific nucleosomes in order to effect modifications to the nucleosome that enable access to the underlying sequence. This remodeling of nucleosome positioning in transcribed chromatin is achieved by the actions of ATP-dependant protein complexes, known as chromatin remodelers, and by the interaction of the general transcription machinery (Weiner et al., 2010; Zhang et al., 2011; Hughes et al., 2012).

Nucleosomes can either be well positioned, or "fuzzily" positioned depending on the factors that contribute to their positioning (Mavrich et al., 2008; Barski et al., 2007; Lee et al., 2007; Jiang and Pugh, 2009). These positions are influenced by the underlying DNA sequence,

chromatin remodelers, and barriers that prevent nucleosome occupancy at a particular loci (Jiang and Pugh, 2009; Gaffney et al., 2012). In yeast, the +1 nucleosome, which is the first nucleosome on the gene coding region after the 5' NFR, displays the tightest positioning (Mavrich et al., 2008). All of the subsequent, downstream nucleosomes are then positioned in arrays relative to the +1 nucleosome through packing principles, whereby nucleosomes further downstream display statistical positional decay (Mavrich et al., 2008; Barski et al., 2007). Data from studies looking at the human genome, has shown that infrequently expressed genes have more nucleosome position "fuzziness" at the 5' NFR and downstream than highly expressed genes, and have correlated well positioned nucleosomes to increased RNA Pol II occupancy (Schones et al., 2008; Andersson et al., 2009).

In addition to ATP-dependent chromatin remodeling of nucleosome position, there are two additional ways that a cell modifies a nucleosome: histone post-translational modification (PTM), and histone variant incorporation (Talbert and Henikoff, 2010; Hargreaves and Crabtree, 2011; Zentner and Henikoff; 2013). Unlike chromatin remodeling, which repositions nucleosomes on DNA, histone modification and histone variant incorporation change the chemical structure, or composition, of a nucleosome. These changes regulate the interactions of nucleosomes with additional protein complexes to facilitate transcription, repair, or further condensation of chromatin into higher order chromatin structures, and can change the stability of the nucleosome structure (Allfrey and Mirsky, 1964; Jenuwein and Allis, 2001; Altaf et al., 2008; Bonish and Hake, 2012; Jin and Felsenfeld, 2007).

1.2 Chromatin Structure

In order to balance compaction with regulated access for transcription, chromatin is folded into a series of increasingly compact structures. Traditionally, chromatin has been separated into two distinct structures: the transcriptionally active euchromatin, and the transcriptionally silent heterochromatin (Grewal and Jia, 2007; Huisinga et al., 2006). These distinctions were originally based upon differential chromosome staining (Heitz, 1928). Darkly stained chromatin was dense and labeled heterochromatic, and the less stained chromatin was more open and labeled euchromatic. Additionally, studies done on lampbrush chromosomes suggested that for transcription to occur this condensed chromatin, the heterochromatin, had to first be unfolded back into euchromatin (Callan, 1981).

For decades, chromatin has been thought of as a hierarchically folded structure in which the 10 nm chromatin fiber, comprised of tandem arrays of nucleosomes, was folded into the 30 nm fiber, and then further condensed through the actions of other chromatin proteins. The 30 nm fiber is proposed to be the first level of folding beyond the nucleosome. Its formation requires the binding of the linker histone, H1, which is conserved in most eukaryotes (Yan et al., 1994). The 30 nm fiber has been proposed to be either of a zig-zag or solenoid structure (Robinson and Rhodes, 2006; Dorigo et al., 2004); however, whether the 30 nm fiber is a bona fide structure *in vivo* is a subject of debate (Tremethick, 2007; Fussner et al., 2011). A transition from the 30 nm fiber to the 10 nm fiber has been assumed to be a factor in the ability of transcription factors to access DNA. This was proposed due to experiments where repressed genes were less susceptible than active genes to nuclease digestion (Levy and Noll, 1981). However, recent studies have shown that transcription occurs, *in vivo,* in a chromatin state that is up to ~25-50 times more compact than the 30 nm fiber (Hu et al., 2009). Additionally, by electron microscopy, the

chromatin in the nucleus appears to be predominantly made up of 10 nm sized fibers (Eltsov et al., 2008; Fussner et al., 2011). This has led to the idea that chromatin compaction is the result of the interdigitation of many nucleosomal arrays (Eltsov et al., 2008; Luger et al., 2012). All of these data suggest that the traditional idea of heterochromatin and euchromatin may not represent the nature of transcribed and repressed chromatin, and that heterochromatin or euchromatin structure is defined by regional factors that enable inter-convertible states rather than compaction.

Euchromatin is the transcriptionally active form of chromatin, and, in both *S. cerevisiae* and other eukaryotes, is generally enriched in nucleosomes that are hyperacetylated, and tri-methylated at H3K4 and H3K36 (Bannister et al., 2005; Barski et al., 2007; Roth et al., 2001; Kouzarides, 2007). It is depleted for such modifications, or marks, associated with heterochromatin, such as tri-methylation at H3K9, H3K27, and H4K20 (Kouzarides, 2007; Schneider and Grosshedl, 2007; Yang et al., 2009; Barski et al., 2007). In yeast, the euchromatin makes up the majority of the genome, and heterochromatin, or silent chromatin, is restricted to the telomeres, regions of the ribosomal-RNA encoding DNA, and the mating-type loci, *HMR***a** and *HMLα* (Rusche et al., 2003). In mammals, euchromatin is enriched for genes and depleted in repetitive sequences such as transposons, and satellite repeat elements (Richards and Elgin, 2002). Due to the mechanisms that position nucleosomes around and over gene regions, the nucleosomes in euchromatin tend to be arranged in irregular arrays, with wider spaces at the NFRs and closer internucleosome spacing over coding regions (Weiner et al, 2010; Radman-Livaja and Rando, 2010). This irregular spacing allows increased access to the DNA by DNA-binding proteins like transcription factors, and by nucleases such as DNase1 (Richards and Elgin, 2002; Rando and Winston, 2012). Additionally, in mammals, origins of replication that are

located in euchromatin often fire earlier in S-phase than origins in heterochromatin (Hatton et al., 1998; Raghuraman et al., 2001).

The traditional view of heterchromatic portions of the genome was that heterochromatin was made up of large regions of mainly repetitive DNA that remained transcriptionally silent throughout the life of the cell (Brown, 1966; Grewal and Moazed, 2003). However, research over the past two decades has revealed that some regions of heterochromatin contain genes that are important for cell viability and fertility, and that it is important for chromosome organization and inheritance (Elgin, 1996; Karpen et al., 1996; Dimitri and Junakovic, 1999; Henikoff et al., 2001; Coulthard et al., 2003; Dimitri et al., 2005). Heterochromatin is functionally categorized into two different groups: constitutive heterochromatin, which silences repetitive elements, and facultative heterochromatin, which is responsible for silencing genes during development (Brown, 1966; Plath et al., 2002; Trojer and Reinberg, 2007; Margueron et al., 2005; Beck et al., 2010).

Like transcriptionally active chromatin, heterochromatin is associated with certain chromatin modifications. It needs to be noted that heterochromatin in the yeast *S. cerevisiae* has a distinct molecular composition, compared to the heterochromatin of other eukaryotes, including the yeast *Schizosaccharomyces pombe* (Hickman et al., 2011; Rusche et al., 2003). Analysis of *S. cerevisiae* and other yeasts of the *Saccharomycotina* sub-phylum show that they have lost proteins that are important for heterochromatin formation in other eukaryotes, and have evolved other proteins to silence chromatin (Hickman et al., 2011; Nakayashiki, 2005; Brachmann et al., 1995; Bell et al., 1995; Zill et al., 2010). Budding yeast heterochromatin is formed by the binding of the Sir proteins to nucleosomes, of which only the deacetylase Sir2 has homologues in other species (Rusche et al., 2003; Hickman et al., 2011; Greiss and Gartner,

2009; Suave et al., 2006). Another interesting feature of budding yeast heterochromatin, is that it represents very small regions of the genome, which are bound by barrier-elements such as Htz1-containing nucleosomes (Rusche et al., 2003; Meneghini et al., 2003). However, it does share some heterochromatic characteristics with other eukaryotes, for example hypoacetylated nucleosomes (Braunstein et al., 1993; Rusche et al., 2002). Due to these differences, for the remainder of this thesis, I will use the term heterochromatin to refer to heterochromatin in eukaryotes other than budding yeast, unless otherwise indicated.

Heterochromatin is hypoacetylated, and can be enriched in DNA methylation, H3K9me3, H3K27me3 and members of the heterochromatin protein 1 (HP1) family (Richards and Elgin, 2002; Trojer and Reinber, 2007; Almouzini and Probst, 2011, Rando and Winston, 2012). Heterochromatic regions are also characterized by having stretches of highly ordered nucleosomes, which are thought to help fold the arrays into higher order structures (Sun et al., 2001; Grewal and Moazed, 2003). These ordered arrays are thought to be the result of chromatin remodeling and are particularly prevalent around regions that are important for chromosome stability, such as the centromere and telomeres, which are generally highly repetitive and transcriptionally silent (Neves-Costa et al., 2009; Mueller and Bryk, 2007; Yu et al., 2011).

A noteworthy example of heterochromatin formation is the silencing of the second copy of the X-chromosome in female mammalian somatic cells, which is necessary for balancing X-linked gene dosage between males and females. X-chromosome silencing, called X-chromosome inactivation (XCI), was first observed by cytological staining in cat neurons due to its completely heterochromatic character (Barr and Bertram, 1949; Lyon, 1961). XCI has been called the perfect epigenetic event, since once an X-chromosome is inactivated after the start of differentiation it almost always remains completely silenced in subsequent cell divisions (Grant

et al., 1988; Heard et al., 1997; Basu and Zhang, 2011). A critical feature of XCI is the coating of the silent X-chromosome with the non-coding RNA (ncRNA) *Xist,* which has been shown to be important for creating, but not maintaining, the silent state (Brown et al., 1991; Maharens et al., 1997; Penny et al., 1996; Costanzi et al., 2000; Csankovski et al., 2001). Further, the inactive X shares all of the marks associated with autosomal heterochromat in, such as DNA methylation and enrichment of the histone PTMs H3K27me3 and H3K9me2 (Boggs et al., 2002; Heard et al., 2001; Bartlett et al., 1991; Lucchesi et al., 2005).

1.3 Histones and Histone Variants

Histones are small highly basic proteins that contain three distinct structural domains: the central "histone fold" domain, histone fold extensions, and long unstructured N-termainal tails (Luger et al., 1997; Arents and Moudrianakis, 1995). The "histone fold" domain (HDF) is conserved across all eukaryotes, and is also found in all the major branches of archaea (Sandman and Reeve, 2006). The assembly of histones into the octamer is thought to begin with the heterodimerization of histones H3 and H4, which then form a $(H3\text{-}H4)_2$ tetramer held together by interactions between the two H3 molecules (Luger et al., 1997). The octamer is completed with the addition of two H2A-H2B dimers.

The majority of nucleosomes in the cell have octamers that are composed of the canonical core histones, which is the classification of the histones that make up the majority of octamers in chromatin. The genes encoding the canonical histones are transcribed in a cell cycle-dependent manner and deposited by DNA replication-dependent pathways, during S-phase (Osley, 1991; Stein et al., 2006; Marzluff et al., 2002; Marzluff et al., 2008). In humans, the

canonical histones are H3.1/H3.2, H4, H2A, and H2B. In yeast, the canonical nucleosomes are H3, H4, H2A, and H2B.

Outside of DNA replication, specific nucleosomes are constructed with variant histones, which can result in a change in the stability of nucleosomes or the recruitment of additional chromatin modifying complexes (Figure #I1.2-1) (Bonish and Hake, 2012). Mammals appear to have the greatest number of histone variants. To date, in human cells, six non-allelic histone variants have been found for histone H3 (Ederveen et al., 2011), four for histone H2A (Millar, 2013), and two testis specific variants of H2B (Gonzales-Romero et al., 2010). Histone H4 is unique in having no known variants. Many of the human histone variants may not exist or are yet to be discovered in other species. However, there are four variants that occur across multiple species, which can be considered universal variants: the centromeric H3 variant (CENP-A in humans, and Cse4 in *S. cerevisiae*), H3.3, H2A.Z, and H2A.X. In yeast, the only histone variants are H2A.Z, called Htz1, and Cse4, while the roles of the variants H2A.X and H3.3 are considered to be played by the canonical histones.

The centromeric variant, CENP-A/Cse4, is essential for proper chromosome segregation and the creation of the unique chromatin structure around the centromere that allows for the formation of the kinetochore (Greaves et al., 2007, Meluh et al., 1998). In human cells, CENP-A and H2A.Z form distinct 3D domains, over a large region of centromeric chromatin, which interact with the many inner and outer kinetochore proteins (Greaves et al., 2007). Yeast Cse4, in contrast, forms a point centromere comprised of a single nucleosome-like structure (Avamudham et al., 2013).

H2A.Z is the other universal histone variant, in most eukaryotes. Vertebrates have two copies of this variant (H2A.Z1 and H2A.Z2) that differ by 3 residues and are non-redundant,

although H2A.Z2 has not been shown to have a function as of yet (Eirin-Lopez et al., 2009). H2A.Z is required for viability in most organisms, and in mice and *Drosophila* creation of a homozygous null for the gene that encodes H2A.Z results in developmental lethality (van Daal and Elgin, 1992; Clarkson et al., 1999; Faast et al., 2001). Interestingly, in yeast, Htz1 is non-essential and although loss of Htz1 results in chromosome segregation defects, cells that are null for *HTZ1*behave very similarly to wild type (WT) (Krogan et al., 2004).

H2A.Z has been shown to be specifically deposited at the 5' ends of some genes, where it has seemingly contradictory functions in transcription (Zlatanova and Thakar, 2008). In yeast, Htz1 is generally incorporated into the two nucleosomes flanking the 5' NFR of a gene, and it has been estimated that ~75% of all Htz1-loci are at annotated genes (Raisner et al., 2005; Guillemette et al., 2005; Watanabe et al., 2013). Although there is no correlation between transcription rate and the amount of Htz1 at a promoter (Raisner et al., 2005), studies of Htz1's role in chromatin has revealed that it both positively and negatively regulates transcription (Meneghini et al., 2003; Santisteban et al., 2000, Adam et al., 2001; Zlatanova and Thakar, 2008). Genome-wide studies of Htz1 and its modified form, Htz1 acetylated at lysine 14, show that the unmodified histone variant is present predominantly at inactive genes, while acetylated Htz1 is found at transcriptionally active genes (Millar et al., 2006). This spatial distinction between modified and unmodified Htz1 points to a functional distinction between the two forms of yeast H2A.Z, which is supported by a study looking at yeast growth in galactose-containing media, showing that an unacetylatable-mutant of Htz1 has a defect in the induction of the gene *GAL1* compared to the wild type histone variant (Halley et al., 2010). The purpose of Htz1 at the promoters of inactive genes is not clear, however, it has been proposed that the incorporation of Htz1 could mark these nucleosomes for eventual eviction and replacement (Zhang et al., 2005).

Alternately, the prevalence of Htz1-nucleosomes at inactive genes could be due to the lack of H3K56 acetylation at those promoters (Watanabe et al., 2013). H3K56ac is a mark that is associated with newly assembled nucleosomes which are deposited in a replication independent manner (Rufiange et al., 2007). A recent study showed that nucleosomes that had an H3K56Q mutant, which mimics acetylated H3K56, triggered the removal of Htz1 by SWR1-C; whereas nucleosomes that had H3K56R, which mimics an unacetylable lysine 56, or unacetylated H3K56 did not (Watanabe et al., 2013). Interestingly, in humans H2A.Z enrichment at promoters is correlated with the transcription rate, and nucleosomes that contain both the histone variants H2A.Z and H3.3 have been shown to be less stable than nucleosomes with either one or neither histone variant (Jin et al., 2009; Jin and Felsenfeld, 2007; Barski et al., 2007).

Htz1 is also found at boundary regions, such as at the boarders of telomeres, where Htz1-nucleosomes occupy a larger region than two nucleosomes and prevent heterochromatin spreading (Meneghini et al., 2003; Guillemette et al., 2005; Zlatanova and Thakar, 2008). In plants, H2A.Z has been shown to play a part in regulation of DNA methylation, although the exact mechanism of this cross-talk was not discovered (Zilberman et al., 2008; Kobor and Lorincz, 2009). H2A.Z has, further, been shown to be important for the suppression of antisense RNAs, and DNA repair (Zofall et al., 2009; Attikum et al., 2007). In human cells, H2A.Z has been shown to accumulate at seemingly non-targeted sites within the genome, and may have a function in creating facultative heterochromatin (Hardy and Robert, 2010; Hardy et al., 2009). A role for H2A.Z in the creation of heterochromatin in mammals, rather than the prevention of its spread, is supported by data showing that H2A.Z knock-down does not change the profile of the heterochromatin mark H3K9me2 and that monoubiquitylated H2A.Z is enriched on the inactive X-chromosome (Sarcinella et al., 2007; Hardy et al., 2009).

Finally, in mammals, H2A.Z is enriched at and plays an important role in the organization of the centromeres (Greaves et al., 2007). In *S. pombe,* experiments using ChIP-chip show that H2A.Z is not associated with any centromeric DNA; however, the same study showed that H2A.Z is important for expression of the protein CENP-C which is a centromere protein required for maintaining centromere silencing (Hou et al., 2010). Finally, in *S. cerevisiae,* Htz1 is not found at the centromeric nucleosome, although its regulation around the centromere has been shown to be important for centromere function (Durand-Dubief et al., 2012).

The other histone variants, H3.3 and H2A.X, are not required for cell viability and are necessary only for specific functions during meiosis or development (Arkady et al., 2002; Hodl and Basler, 2009). H2A.X's major role is during DNA repair, where its phosphorylated form is localized to DNA double stranded breaks (DSBs) (Rogakou et al., 1998). It has also been shown to be important for proper condensation and pairing of the X and Y chromosomes during male meiosis in mice (Fernandez-Capatillo et al., 2003). The variant H3.3 has also been shown to be important during meiosis (Hodl and Basler, 2009). H3.3 differs from the canonical histone by only four or five amino acids (H3.2 or H3.1, respectively) yet its localization is highly correlated with transcription and post-translational modifications that are associated with transcription, compared to either H3.1 or H3.2 (Loyola and Almouzni, 2007). Interestingly, correlation to transcription, where it has been the most studied, may be a result of expression timing rather than any particular characteristic of H3.3 that enables transcription, since it can be replaced by the canonical H3.2 in flies for everything but gamete production (Hodl and Basler, 2009).

Generally, variant histones are expressed through-out the cell cycle and these proteins are deposited outside of replication. Histone variant deposition is controlled by specific chromatin remodelers and chaperones. H3.3 is deposited by the chaperones HIRA and DAXX/ATRX, and

it is enriched at locations of active transcription (Tagami et al., 2004; Goldberg et al., 2010). H2A.Z is incorporated into the nucleosome by the chromatin remodeling complex SWR1-C and is targeted by histone H3 and H4 N-terminal tail acetylation, but antagonized by H3K56 acetylation (Krogan et al., 2003; Kobor et al., 2004; Mizuguchi et al., 2003; Wanatabe et al., 2013). These variant chaperones and chromatin remodelers deposit the histones to specific regions in the genome: H3.3 to actively transcribed genes at their 5' region, and H2A.Z to various regions, including around the NFR and at chromatin boundary regions (Zlatanova and Thakar, 2006; Ahmad and Henikoff, 2002).

Table 1.1 Human subtypes and their characteristics

H3 Subtype	
H3.1	Major H3 subtype, S-phase expression, replication-dependent incorporation
H3.2	Major H3 subtype, S-phase expression, replication-dependent incorporation
H3.3	H3 variant, cell cycle independent expression, replication-independent incorportation
CENA-P	Centromeric H3 variant
H3.1t/H3.4	Testis specific H3 variant
H3.5	Testis specific H3 variant, similar to H3.3
H3.Y	mRNA detected, cell line dependent expression
H3.X	mRNA detected, expression unknown

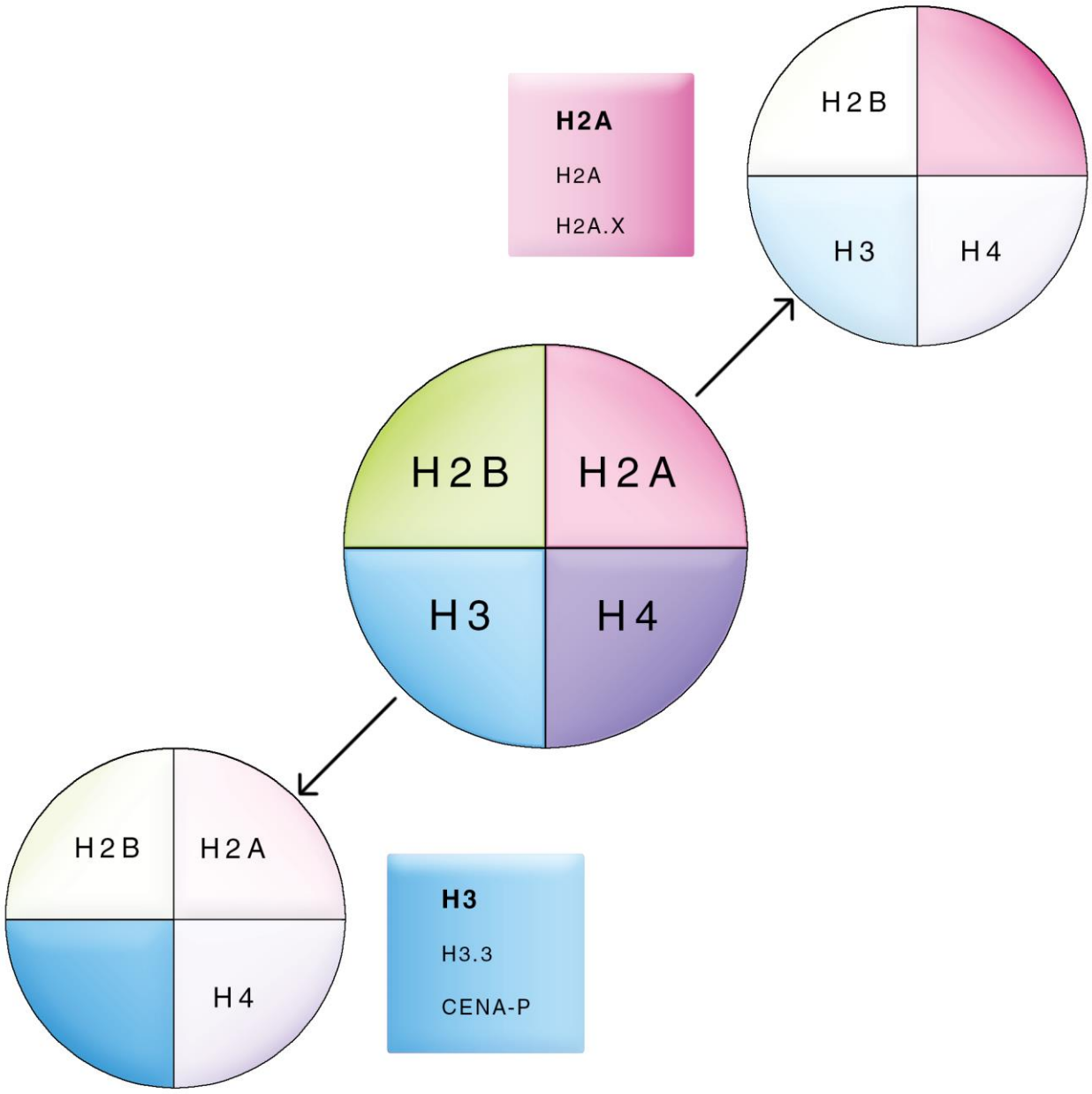


Figure 1.1. Schematic of histone variant incorporation into a nucleosome for the four universal variants.

1.4 The Linker Histone

The linker histone, unlike the core histones, binds to the linker DNA outside of a nucleosome to form the chromatosome (Hayes et al., 1994; Woojin et al., 1998). In metazoan somatic cells, all linker histones belong to the histone H1 family; however, there is another class of linker histone, H5, found in the nucleated erythrocytes of avian and amphibian organisms (Koutzamani et al., 2002; Thomas et al., 1992; Ramakrishnan et al., 1993; Duggan and Thomas, 2000). For simplicity, while acknowledging that this other class of linker histones exists, this thesis will use the term linker histone to refer only to members of the histone H1 family of proteins.

In general, with *S. cerevisiae* being a notable exception, histone H1 is present in near stoichiometric amounts to nucleosomes, such that each nucleosome has one associated linker histone (Bates and Thomas, 1981, Woodcock et al., 2006). Interestingly, the increased presence of linker histone in a cell is correlated with a larger nucleosome repeat length (Woodcock et al., 2006). *S. cerevisiae* which has a low H1 to nucleosome ratio, which has been variously reported to be 1:37 or 1:4, has a nucleosome repeat of 165 bp (Freidkin and Katcoff, 2001; Downs et al., 2003). By comparison, mouse thymus cells have been shown to have an H1 to nucleosome ratio of 1:1.15 and a nucleosome repeat length of 196 bp (Fan et al., 2003); and *S. pombe*, which has no identified linker histone, and a shorter nucleosome repeat of 156 bp (Godde and Widom, 1992).

Similar to the core histones, H1 is a positively charged protein that has a tripartite structure comprised of a long C-terminal tail, a globular domain, and a shorter N-terminal tail region. Its globular and C-terminal domains have been shown to bind to DNA, protecting an additional 20 bp of DNA beyond the nucleosome (Allan et al., 1996; Lu and Hansen, 2004; Goytisolo et al.,

1996). The C-terminal domain of H1 is an unstructured domain that is rich in lysine, serine, and proline, and has been characterized as being "dynamically fuzzy," meaning that it exists in an ensemble of different conformations (McBryant and Hansen, 2012). It has been shown to be necessary for binding of the linker histone and its sequence is a major determinant of linker histone residency time at a nucleosome (Hendzel et al., 2004; Th'ng et al., 2005).

The *S. cerevisiae* linker histone, which is called Hho1, is a non-canonical linker histone. Unlike the linker histones of multicellular eukaryotes, Hho1 has a second globular domain in place of the C-terminal tail domain (Kasinsky et al., 2001, Ushinski et al., 1997; Landsman, 1996). *In vitro*, both of globular domains of Hho1 fold into similar secondary and tertiary structures, albeit with different stabilities, leading to the suggestion that the yeast linker histone binds to two nucleosomes simultaneously (Ono et al., 2003; Ali et al., 2004; Ali and Thomas, 2004; Schafer et al., 2005). Binding experiments done using electrophoretic mobility shift assays, however, showed that the yeast linker histone bound to di-nucleosome arrays in a 1:1 ratio like canonical histone H1 (Patterton et al., 1998). Further, experiments studying the effects of Hho1 on transcriptional silencing showed that a mutant Hho1 protein, which lacked the second globular domain, was capable of recapitulating the phenotype of full-length Hho1 (Yu et al., 2009). Therefore, although it is non-canonical in structure and is present in a lower H1:nucleosome ratio, the yeast linker histone is thought to interact with nucleosomes similarly to the linker histones in other eukaryotes (Woodcock et al., 2006; Yu et al., 2009).

The number of linker histones across species is highly irregular. In mammals, there are 11 variants of the linker histone: four germline specific and seven somatic (Happel and Doenecke, 2009). Budding yeast and fruit flies have a single linker histone, *Arabadopsis thaliana* has three, *Xenopus laevis* has five, and *Caenorhabditis elegans* has eight (Ushinsky et

al., 1997; Nagel and Grossbach, 2000; Ascenzi and Gantt, 1999; Risley and Eckhart, 1981; Jedrusik and Schultz, 2001). Deletion of individual somatic subtypes in conjunction with deletion of H1.0 in mice has shown that the different linker histone variants are functionally interchangeable (Fan et al., 2001). However, at the chromatin level, the different variants have been shown to be mobilized from nucleosomes after different lengths of time, called the residency time (Th'ng et al., 2005; Conn et al., 2008). Further, studies have shown that the different linker histone sub-types are expressed at different times during the cell cycle, and that depletion of individual variants leads to unique changes in gene expression (Happel et al., 2009; Sancho et al., 2008). Thus, like the core histone variants, the linker histone variants appear to regulate specific chromatin events.

Linker histones are important factors that mediate chromatin structure. Early studies showed that loss of the linker histone changed the morphology of chromatin fibers under different salt conditions (Thoma et al., 1979). Since then, the linker histone has been shown to play a key role in the formation of the 30 nm fiber and higher order chromatin structure, *in vitro* (Hansen, 2002; Robinson and Rhodes, 2006). However, due to the debate around the existence of the 30 nm fiber *in vivo,* exactly how linker histones mediate higher order chromatin remains to be determined. The depletion of the embryonic linker histone in *Xenopus laevis* egg extracts, which can go through chromosome replication *in vitro,* showed that chromosomes lacking H1 are dramatically lengthened, and have morphological defects that prevent their proper alignment (Maresca et al., 2005). Additionally, mouse ES cells lacking three H1 genes, and having only 50% of the normal H1 content, have a shorter nucleosome repeat length and other structural changes (Fan et al., 2005). Further, mouse embryos that are missing the same three linker histones die during mid-gestation (Fan et al., 2003). Thus, while linker histones are clearly

important for the formation of higher order chromatin structure, the mechanisms by which they regulate this structure remain to be explored.

The various linker histones play specific functions in chromatin. Deletion of the non-essential gene encoding the yeast linker histone—which is considered to be non-canonical in structure because it has a second globular domain instead of a long unstructured C-terminal tail—changes the expression of a small subset of genes, showing that it is not a general repressor of transcription (Hellauer et al., 2001). This is supported by the fact that mouse ES cells missing half their H1 content go through the cell cycle normally with the expression of only a few genes altered (Fan et al., 2005). Also, work looking at the interaction between mouse linker histones and gene silencing through DNA methylation and H3 methylation has shown that the different linker variants do not have the same ability to silence two loci in mouse ES cells (Yang et al., 2012). Genome-wide studies to look at the patterns of linker histone binding in the cell, in addition to the ones that have already been performed, will help to elucidate the difference in function between the linker variants (Li et al., 2012; Cao et al., 2013).

One aspect of linker histones that is not well understood is how the linker variants are regulated with regards to their positioning in the genome. Studies assaying linker histone binding to different nucleosomal structures have shown that linker histones preferentially bind certain nucleosomes. Linker histones are known to reside for less time at nucleosomes that are highly acetylated (Misteli et al., 2000; Raghuram et al., 2010). *In vitro* studies assaying linker histone binding to the nucleosomes containing either the histone variant H3.3 or H2A.Z show that linker histones do not bind well to H2A.Z nucleosomes, but bind normally to H3.3 nucleosomes (Thakar et al., 2009). Conversely, *in vivo* studies in *Drosophila* using DamID to map linker histone binding show an anti-corelation between the DamID generated profile and a map of H3.3

(Braunschweig et al., 2009). However, how linker histone binding to these different nucleosomes is affected by their structure is yet to be determined.

1.5 Histone Post-translational Modification

Over a half century ago, it was discovered that highly transcribed genes are associated with histones that were highly methylated and acetylated (Allfrey et al., 1964). Since then dozens of histone PTMs have been identified. Phosphorylation, acetylation, methylation, and ubiquitination are all modifications that have been widely studied on all four core histones, and as mass-spectroscopy techniques have become more refined scientists have been able to identify many new sites for known modifications, as well as, new types of modification, like lysine crotonylation (Tan et al., 2011). Many histone PTMs are correlated with defined chromatin states, such as euchromatin or heterochromatin, and it has long been known that within those regions histone PTMs are non-randomly distributed (Rando and Winston, 2012; Zentner and Henikoff, 2013). Despite this, the "how" and "why" of many modifications at specific loci remain unknown; however, the development of genome-wide chromatin immunoprecipitation techniques (e.g. ChIP-chip and ChIP-Seq) has led to the generation of a plethora of PTM maps that highlight patterns of modification across the genome (Liu et al., 2005; Wang et al., 2008; Kharchenko et al., 2011).

Histone modifications occur on all parts of the histone proteins, but the sites of modification are the densest on the histone N-terminal tail (Figure I1.3-1). The flexible N-terminal tails of the histones protrude beyond the wrapped DNA of the nucleosome, and are accessible to protein complexes after incorporation into chromatin (Luger et al., 1997). Histones,

however, do not need to be part of a nucleosome to become modified, and many histones are modified before deposition. For example, in yeast, acetylation of newly synthesized H3 is crucial for proper S-phase nucleosome assembly (Li et al., 2008); in humans, H3.1 is sometimes mono-methylated at H3K9, prior to deposition, which leads to further methylation in chromatin (Loyola et al., 2006); and H4 is di-acetylated during its chaperone mediated transport into the nucleus (Varreault et al, 1997; Campos et al., 2010).

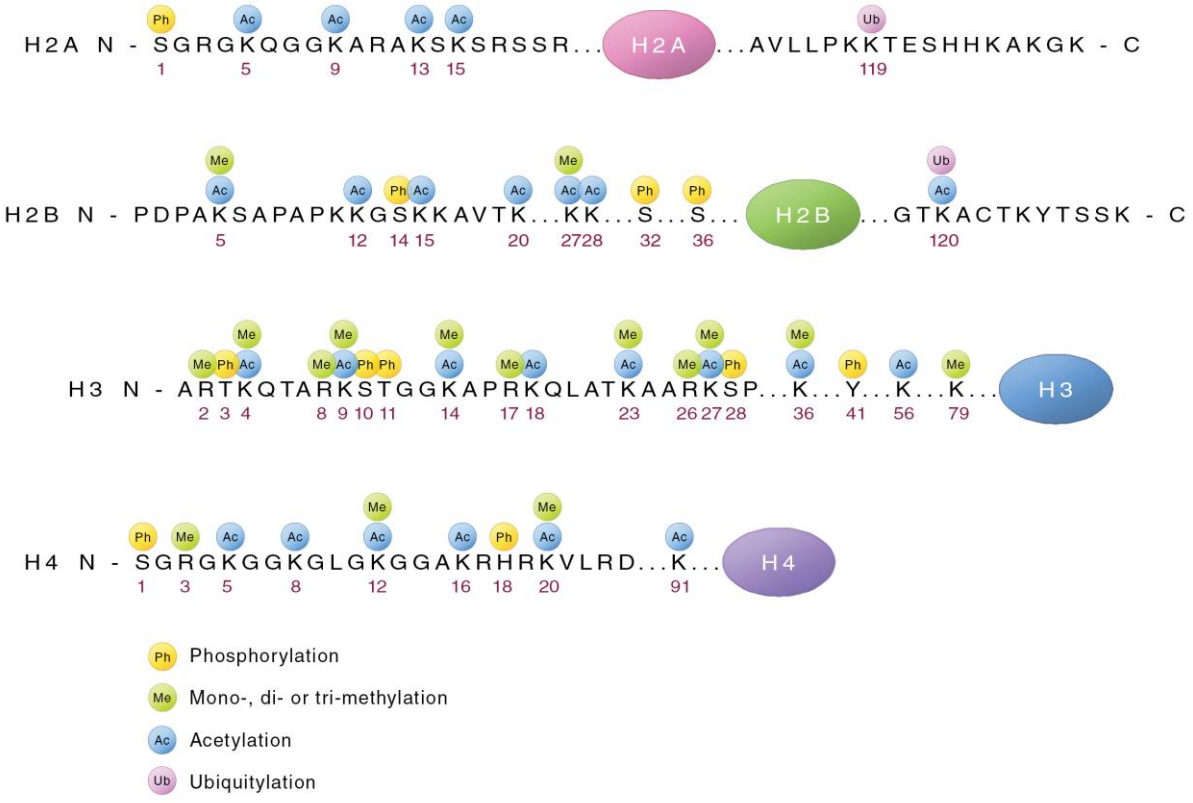


Figure 1.2. Schematic of a selection of post-translational modification on the core histones. If two possible modifications can occur on the same residue, the different modifications are depicted stacked on top of each other.

Post-translational modifications on histones have at least three major functions. First, acetylation and phosphorylation can alter the electrostatic interactions between histones and histones, or histones and DNA, creating nucleosomes that are more permissible to transcription (Choi and Howe, 2009; Banerjee and Chakravartee, 2011). Second, histone modifications can affect the modification status of nearby histones, a phenomenon called histone cross-talk, by promoting or blocking the further catalysis of PTMs (Maltby et al., 2012; Lee et al., 2010). Third, histone modifications can act as a molecular beacon, whereby they create binding motifis for chromatin-associated proteins with domains called 'readers'. 'Reader' domains are highly specific, and they are proposed to be a major mechanism for targeting chromatin-modifying complexes to certain areas of the genome (Musselman et al., 2012).

One of the best studied processes, with regards to histone PTMs, is transcription. Acetylation of lysines on the H3 and H4 N-terminal tails is highly correlated with transcriptional activity, however, whether the different acetylations are causal or consequential has yet to be determined (Alfrey et al., 1964). The best characterized 'reader' motif for acetylated lysines is the bromodomain, which forms a highly conserved four helix domain (Dalluin et al., 1999; Filippakoppolous et al., 2012). Bromodomains are found in many different proteins; the human genome encodes 46 bromodomain-containing proteins, including histone acetyltransferases and chromatin remodelers (Filippokopolous and Knapp, 2012). However, only a few histone acetylations have been definitively associated with a specific bromodomain.

In addition to acting as a binding motif, acetylation neutralizes the positive charge of the lysine residue it modifies. This causes a change in the electrostatic interaction between histone and DNA in a nucleosome, which increases the access of transcriptional machinery (Hong et al., 1993). Interestingly, the various sites of acetylation appear to function redundantly on either H3

and H4 during normal transcriptional processes (Choi et al., 2008; Dion et al., 2005; Martin et al., 2004). Therefore, either acetylation is more important for charge neutralization than as a binding motif, or bromodomains recognize a variety of different acetylations on the histone tails *in vivo*, rendering each acetylation interchangeable with its neighbor in regards to binding motif identity.

Acetylation of histone lysines is catalyzed by a class of proteins known as histone acetyltransferases (HATs). The first HAT identified was Gcn5, which was subsequently shown to be the catalytic subunit in two large protein complexes: SAGA, and SLIK/SALSA (Grant el al., 1997; Pray-Grant et al., 2002, Sterner et al., 2002). SAGA is the largest and best characterized of the Gcn5 containing HAT-complexes, it has been shown to have a role in transcription initiation and elongation, and is conserved in eukaryotes (Koutelou et al., 2010; Brownell et al., 1996; Nagy and Tora., 2007; Kou et al., 1996; Rodriguez-Navarro, 2009). In addition to Gcn5, other HATs have been identified in yeast including the MYST-family HATs Sas2, Sas3, and Esa1, which are part of the HAT-complexes SAS, NuA3, and NuA4, respectively (Grant et al., 1997; Reifsnyder et al., 1996; Smith et al., 1998; Clarke et al., 1999; Osada et al., 2001). In yeast, Esa1 is the only essential HAT; however, yeast lacking both Sas3 and Gcn5 are inviable (Smith et al., 1998; Clarke et al., 1999; Howe et al., 2001).

In addition to histone substrates, HATs also modify non-histone proteins; for example Gcn5 acetylates subunits of the chromatin remodeling complexes RSC and SWI/SNF (Choi et al., 2008; VanDemark et al., 2007; Kim et al., 2010), and NuA4 has been shown to have many non-histone targets, including Pck1 (Lin et al., 2009). In order to determine substrate specificity, HAT-complexes contain a number of associated proteins which direct the HAT-complexes to the appropriate genomic locus or target protein (Balasubramanian et al., 2002; Candau et al., 1997;

Grant et al., 1997; Allard et al., 1999; Boudreault et al., 2003). These subunits are sometimes shared with other chromatin modifying complexes, which is another mechanism through which cells regulate complex targeting (Kimura et al., 2005; Lin et al., 2008). NuA4, which acetylates histones H4, H2A, and H2A.Z, shares the subunit Tra1 with SAGA/SLIK; Arp4 with RSC, SWI/SNF, and SWR-1C; and Yaf9, Swc4, and Act1 with SWR1-C (Lu et al., 2009; Auger et al., 2008).

The tri-methylation of H3K4 (H3K4me3) is also a PTM associated with transcription and is found predominantly at the 5' ends of genes (Maltby et al., 2012); however, deletion of the gene encoding Set1, the sole H3K4 methyltransferase in yeast, does not greatly impair transcription (Miller et al., 2001). One of the 'reader'-domains that recognizes H3K4me3 is the PHD (plant homeodomain) finger domain of the Yng1 protein, which is a yeast homolog of the ING-family of proteins, and a subunit of the histone acetyltransferase NuA3 (Martin et al., 2006; Taverna et al., 2006). Persistence of H3K4me3 at the 5' end of a gene is an example of cross-talk between histone PTMs, where the removal of H3K4me3 by the demethylase Jhd2 is regulated by the presence of an acetylation at H3K14 (Maltby et al., 2012).

Unlike acetylation, lysine methylation does not change the charge of histone proteins. Therefore, its function appears to be only that of a binding motif in chromatin (Zentner and Henikoff, 2013). Methylation can occur on a protein in three different forms (mono-, di-, and tri-methylation) each which can have a different regulatory effect in chromatin. For example, in mammalian cells, mono- and di-methylated H3K9 is enriched in euchromatin, whereas tri-methylated H3K9 is enriched in pericentric heterochromatin (Rice et al., 2003). Unfortunately, due to the structural similarities between the different methylation states, generating quality

antibodies has consistently been a challenge, and much about the each methylation state remains to be discovered.

Histone PTMs are found in all chromatin locations, and have been characterized in relation to many DNA functions including transcription, DNA repair, silencing and heterochromatin formation, and replication. The large number of modifications and target sites suggests a high degree of combinatorial complexity, which led to the idea that there was a "histone code" made up of patterns of PTMs (Strahl and Allis, 2000). A pervasive problem with the "histone code"-hypothesis is one of semantics, since over the past decade the term "histone code" has been used in different contexts by different investigators. One of the most widely accepted interpretations of the "histone code" refers to the concept that a given combination of modification would be "read" by a protein complex and thus lead to a downstream event specific to that combination, and distinct from the effect of either modification alone (Rando, 2012). However, investigations into some of these combinations, through the systemic mutation of modified amino acids on the N-terminal tails of histone H3 and H4, have shown that most of the lysine residues do not each have a unique effect on gene expression (Martin et al., 2004; Dion et al., 2005). A particularly puzzling histone PTM is H3K4me3, which is found at the 5' end of nearly all transcribed genes, yet deletion of its methyltransferase only affects the regulation of 50-100 genes (Lenstra et al., 2011; Liu et al., 2005; Barski et al., 2007; Guenther et al., 2007; Ng et al., 2003; Pokholok et al., 2005; Rando et al., 2012). Additionally, many of the effector proteins have a much less specific genome-wide binding pattern than their perturbation signatures suggest that they should (Venters et al., 2011; Lenstra et al., 2011). Data from genome-wide studies have shown that relatively few PTM combinations actually occur *in vivo*, and this simplicity begets the question of whether combinatorial binding by chromatin-regulating

complexes leads to discrimination between loci or is required for allosteric regulation (Rando, 2012; Watanabe et al., 2013; Li et al., 2007; Dourin et al., 2010). In many eukaryotes, a further complexity is that genes are regulated 3-dimensionally by long-distance interactions with enhancers and insulators, meaning that the PTMs at a gene are not the only ones that could affect transcription (Marsman and Horsfield, 2012; Lomvardas et al., 2006; Sanyal et al., 2012; Lieberman-Aiden et al., 2009). Due to these complexities, it has been suggested that the "histone code" is closer to a "chromatin language" (Rando, 2012; Berger, 2007) which will require much more research to understand.

1.6 Chromatin Remodeling Complexes

Histone-modifying complexes and chromatin remodeling complexes are the two categories of enzyme-containing protein machinery that are involved in regulating access to nucleosomal DNA. Histone-modifying complexes catalyze the addition of post-translational modifications which, as discussed above, alter the chemical structure of a histone protein. Chromatin remodelers use the power of ATP to move nucleosomes along DNA, and are large multi-subunit complexes that have been categorized into families by their ATPase subunits. The first chromatin remodeler to be identified was yeast SWI/SNF, which was found to be an activator of transcription, and is the best characterized of the SWI/SNF family of remodelers (Peterson and Herskovwitz, 1992, Laurent et al., 1991). Yeast SWI/SNF mobilizes nucleosomes by displacing them to allow transcription to occur (Biggar and Crabtree, 1999). Its sibling complex RSC, whose catalytic subunit Sth1shares homology with SWI/SNF's Swi2, is another remodeler that regulates gene expression (Cairns et al., 1996). SWI/SNF and RSC have two

proteins in common, Arp7 and Arp9, and have a number of homologous subunits. Despite this, the two complexes are not functionally redundant—unlike SWI/SNF, subunits of RSC are essential for cell viability (Du et al., 1998; Cao et al., 1997). RSC occurs in two distinct isoforms, RSC1 and the more abundant RSC2, which have been shown to share some functional overlap (Cairns et al., 1999). The combined deletion of both *RSC1* and *RSC2* is lethal in yeast, suggesting that RSC is involved in transcription at essential genes through the complex's actions remodeling nucleosomes to maintain the NFR at promoters (Cairns et al., 1999; Hartley and Madahani, 2009; Badis et al., 2008). Further, RSC has been shown to be required for sister chromosome cohesion and kinetochore function (Baetz et al., 2003; Hsu et al., 2003). In mammals, SWI/SNF and RSC functions are incorporated into the polymorphic BAF complex. BAF, which has a large capacity for subunit substitutions, has only started to be characterized, and the context and consequences of its different forms is still largely unknown (Euskirchen et al., 2012; Hargreaves and Crabtree, 2011).

A sub-family of the SWI/SNF chromatin remodelers is the INO80 family which, in yeast, includes INO80 and SWR1-C. Like the other chromatin remodelers, INO80 is able to slide nucleosomes on DNA and is important for spacing (Shen et al., 2003; Udugama et al., 2011). INO80 has been shown to be important for the regulation of stress response genes (Klopf et a., 2009). It is also recruited to DNA double strand breaks, stalled replication forks, and telomeres (van Attikum et al., 2004; Papamichos-Chronakis and Peterson, 2008; Yu et al., 2007). Interestingly, INO80 has recently been shown to be involved in the regulation and localization of the histone variant Htz1 through replacement of Htz1:H2B dimers with H2A:H2B dimers (Papamichos-Chronakis et al., 2011). SWR1-C also has the ability to replace histone dimers: its main function is to replace H2A:H2B dimers with Htz1:H2B dimers (Kobor et al., 2004; Krogan

et al., 2003; Mizuguchi et al., 2004). Unlike all of the other chromatin remodelers, SWR1-C does not have nucleosome sliding activity (Mizuguchi et al., 2004; Wanatabe and Peterson, 2013). In humans, there are two complexes that incorporate H2A.Z into a nucleosome that are analogous with yeast SWR1-C. The first is Tip60, which is a combination of SWR1-C and the histone acetyltransferase NuA4 (Doyon et al., 2004; Lu et al., 2009), and the second is SCRAP (Cai et al., 2005; Ruhl et al., 2006).

Despite understanding the subunit composition of chromatin remodelers and their patterns of recruitment in the genome, how remodelers are recruited and the mechanism by which they mobilize histones are not completely clear. For example, it has been shown that SWR1-C does not deposit Htz1 effectively in strains where the HATs NuA4 and SAGA/NuA3 have been compromised, indicating that histone acetylation is important for SWR1-C function (Raisner et al., 2005; Zhang et al., 2005). SWR1-C, like all chromatin remodelers, has subunits containing "reader" motifs, and in this case the double bromodomain of Bdf1 is able to recognize and bind to the acetylated, target nucleosomes (Raisner et al., 2005). However, how the double bromodomain acts in recruitment, complex regulation, or both is still uncertain (Wanatabe and Peterson, 2013). Similarly, how all of the bromodomains of the RSC complex, which contains 5 of the 14 bromodomains in yeast, help the complex recognize its targets and regulate its function remains to be discovered (Zhang et al., 2010; Chambers and Downs, 2012).

1.7 Chapter Summaries

1.7.1 The Acetylation of Rsc4K25 by Gcn5 is Essential in the Absence of Histone H3 Acetylation

Genetic analysis of the yeast HATs has shown that the combined deletion of two non-essential HAT genes *GCN5* and *SAS3* results in an inviable strain of yeast (Howe et al., 2001). In Chapter 2 of this dissertation, I present data that demonstrates that the inviability of the *gcn5Δsas3Δ* double mutant strain is due to a combined failure to acetylate both histone H3 and Rsc4. These results provide insight into the function of histone H3 acetylation, and demonstrated that a failure to neutralize the charge on H3 in combination with impaired RSC function is lethal. Additionally, I provide evidence that acetylation of Rsc4 is catalyzed by Gcn5 in a HAT complex-independent manner, which is the first indication that Gcn5 functions independently of its HAT complex-associated proteins *in vivo.*

1.7.2 The Interaction of the Yeast Linker Histone, Hho1, with Nucleosomes is Regulated by the Presence of the Histone Variant Htz1.

The linker histone, H1, is associated with higher order chromatin structure. Among the questions still to be answered regarding the yeast linker histone, is how Hho1 binding is regulated in the cell. In Chapter 3 of this dissertation, I show that yeast strains that over-produce Hho1 have a growth defect that is exacerbated by the loss of Htz1. Further, I present data that Htz1 both directly interferes with Hho1 binding to the +1 nucleosome, and indirectly interferes with linker histone binding to downstream nucleosomes. Finally, I provide data that corroborates

the current evidence that the yeast linker histone binds to a single nucleosome, and only protects one nucleosomal repeat length of DNA.

1.7.3 The Human-yeast Histone H3 Hybrids can Rescue the Loss of Endogenous Yeast H3.

In human cells, most of the DNA is associated with nucleosomes containing two copies of either H3.1, H3.2, or H3.3. The variant H3.3, is constitutively expressed throughout the cell cycle and is primarily incorporated into transcriptionally active regions in chromatin, leading to the suggestion that H3.3 is important for creating open nucleosome structures that contribute to the transcriptionally permissive chromatin structure (Tagami et al., 2004; Goldberg et al., 2010; Jin and Felsenfeld, 2007). In Chapter 4 of this dissertation, I show that yeast strains in which the sole copy of H3 is a hybrid human-yeast protein, are viable. Additionally, I show that the two human histone H3 variants, H3.1 and H3.3, are functionally interchangeable during transcription and replication in a number of different nutrient conditions, but not in galactose containing media. Finally, I present evidence that the yeast H3 C-terminal domain amino acids H3M120, H3K121, and H3K125 play an important role in histone H3 function, possibly by regulating the interaction of yeast H3 with the other octamer proteins.

Chapter 2. Materials and Methods

2.1 Yeast Strains and Plasmids

All strains used in this study are isogenic to S288C and are listed in Table 2.1-1. Yeast culture and genetic manipulations were performed using standard protocols (Ausubel et al. 1987). Genomic deletions were verified by PCR analysis and whole-cell extracts were generated as previously described (Ausubel et al. 1987, Kushnirov 2000).

The strains carrying the histone H3 mutations were derived from FY2162, which has deletions of the *HHT1-HHF1* and *HHT2-HHF2* genes, and carries *HHT2-HHF2* on a *URA3* plasmid (Duina and Winston, 2004). A complete list of yeast strains can be found in Table 2.1-1.

Table 2.1 Yeast strains used in this thesis

Name	Mating Type	Genotype	Plasmid
YLH101	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63*	
YLH115	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 gcn5::HIS3 sas3::HISMX6*	*pLP1364*
YLH119	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 gcn5::HIS3 nto1::HISMX6*	
YLH146	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ada2::HIS3 sas3::HISMX6*	
YLH224	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912d35::HIS4*	*pHHT2-HHF2.416*
YLH315	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912d35::HIS4 sas3::KANMX6*	
YLH316	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 gcn5::KAN sas3::KAN*	*pGCN5.HHF2.HHT2*
YLH320	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912d35::his4 gcn5::KAN*	

Name	Mating Type	Genotype	Plasmid
YLH410	Mat a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RSC2.Tap::HIS*	
YLH414	Mat a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RSC2.Tap::HIS gcn5::KAN*	
YLH415	Mat a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RSC2.Tap::HIS ada2::KAN*	
YLH417	Mat a	*his3Δ1 leu2Δ0 met15Δ0? lys2Δ0? ura3Δ0 rsc4::KAN*	*pRSC4.URA3*
YLH426	Mat α	*his3Δ1 leu2Δ0 met15Δ0? lys2Δ0? ura3Δ0 rsc4::KAN ada2::HISMX6*	
YLH434	Mat a	*his3Δ1 leu2Δ0 met15Δ0? lys2Δ0? ura3Δ0 rsc4::KAN Rsc2TAP::HIS*	
YLH443	Mat a	*his3Δ1/his3Δ200? leu2Δ0/leu2Δ1? met15Δ? lys2Δ0/lys2-128Δ? ura3-52 trp1D63 ura3Δ0 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912d35::HIS4 rsc4::KAN*	*pRSC4.HHT2.HHF2.416*
YLH443	Mat a	*his3Δ1/his3Δ200? leu2Δ0/leu2Δ1? met15Δ? lys2Δ0/lys2-128Δ? ura3-52 trp1D63 ura3Δ0 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912d35::HIS4 rsc4::KAN*	*pRSC4.HHT2.HHF2.416*
YLH446	Mat α	*can1Δ::STE2pr-Sp_his5 lypΔ1; his3D1; leu2D0; met15dD; ura3D0; LYS2+; S288c*	
YLH517	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 (hht1-hhf1)::LEU2 (hht2-hhf2)::KANMX6*	*pHHT2-HHF2.416*
YJC004	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 gcn5::HIS3 sas3::HISMX6 hho1::TRP1*	
YJC058	Mat a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 swr1::KANMX4*	
YJC062	Mat a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 htz1::KANMX4*	
YJC065	Mat a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 chz1::KANMX4*	

The *TRP1* plasmid expressing wild-type *HHT2* and*HHF2* (pLH305) was constructed by ligation of the SpeI restricted fragment from pDM18 (Duina and Winston, 2004) into the SpeI site of pRS414. Plasmids expressing lysine-to-arginine (K14R [pLH307], K9,14,18,23R [pLH311], K9,14,18,23,27R [pLH353], K9,14,18,23,27,36R [pLH354]), and lysine to glutamine (K9,14,18,23Q [pLH434]) mutant versions of histone H3 were described previously (Martin et al., 2006) or prepared for the present study by ligating annealed oligonucleotides into the BamHI and AgeI sites of pLH305.

Plasmids expressing the yeast-codon optimized histones were constructed for the present study by ligation of codon optimized DNA fragments produced by either GenScript of Integrated DNA Technologies into the BamHI and XhoI sites of pLH305. The gene encoding *RSC4*, including 122 bp of upstream and 250 bp of downstream sequences, was cloned into the SalI and BamHI sites of both pRS416 (pLH372) and pRS415 (pLH373). Mutation of Rsc4p lysine 25 to alanine was done by megaprimer-based mutagenesis to generate pLH374. For simultaneous expression of *RSC4*, *HHT2*, and *HHF2* from the same plasmid, the SpeI restricted fragments from pLH305, pLH307, pLH311, and pLH434 were ligated into the SpeI sites of pLH373 and pLH374. Plasmids expressing wild-type Gcn5p (pLH185), $Gcn5p_{1-261}$ (pLH385), wild-type Sas3p (pLH141), and temperature-sensitive Sas3C357Y/P375A (pLH157) were described previously (Candau et al., 1997; Howe et al., 2001). The *HHO1* ORF, with no upstream or downstream sequences, was cloned into the BamHI and XhoI sites of pLH249 plasmid, which is a pRS416 plasmid with the *GAL1* promoter and *CYC1* terminator (Mumberg et al., 1994).

Table 2.2 Plasmids used in this thesis.

Name	**Vector**
pLH121	*pRS414 (Trp) (Vector control)*
pLH141	*pSAS3FLG.415*
pLH137	*psas3.ts.415*
pLH143	*pSas3FLG.416*
pLH185	*pGcn5HA.414*
pLH188	*pgcn5KQL.414*
pLH249	*pGAL.416*
pLH282	*pHHF2.hht2Δ3-29.414*
pLH285	*pGCN5.HHF2.HHT2.414*
pLH286	*pGCN5.HHF2.hht2Δ3-29.414*

Name	Vector
pLH305	*pHHT2.HHF2.414 (pH3.A)*
pLH307	*pHHF2.hht2K14R.414*
pLH311	*pHHF2.hht2K9,14,18,23R.414*
pLH320	*pHHT2.HHF2.414(NheI/AgeI)*
pLH345	*pHHO1.415*
pLH353	*pHHF2.hht2K9,14,18,23,27R.414*
pLH354	*pHHF2.hht2K9,14,18,23,27,36R.414*
pLH372	*pRSC4.416*
pLH373	*pRSC4.415*
pLH374	*prscK25A.415*
pLH378	*pRSC4.HHT2.HHF2.416*
pLH382	*pRSC4.314*
pLH383	*prsc4K25A.314*
pLH384	*pHHF2.hht2K9,18,23,27R*
pLH387	*pRSC4.HHT2.HHF2.314*
pLH388	*prsc4K25A.HHT2.HHF2.314*
pLH389	*pRSC4.hht2K14R.HHF2.314*
pLH390	*prsc4K25A.hht2K14R.HHF2.314*
pLH391	*pRSC4.hht2Δ3-39.HHF2.314*
pLH392	*prsc4K25A.hht2Δ3-39.HHF2.314*
pLH393	*pRSC4.hht2K9,14,18,23,27,36R.HHF2.314*
pLH394	*prsc4K25A.hht2K9,14,18,23,27,36R.HHF2.314*
pLH499	*phht2K9,14,18,23Q.HHF2*
pJC027	*prsc4K25A.hht2K9,14,18,23Q.314*
pJC090	*pHHF2.hht2Δ.hH3.1.414 (pH3.B)*
pJC092	*pHHF2.hht2Δ.hH3.3.414 (pH3.C)*
pJC093	*pHHF2.hht2(61-134).hH3.1(1-60).414 (pH3.D)*
pJC094	*pHHF2.hht2(61-134).hH3.3(1-60).414 (pH3.E)*
pJC095	*pHHF2.hht2(61-119).hH3.1(1-60, 120-134).414 (pH3.F)*
pJC096	*pHHF2.hht2(61-119).hH3.3(1-60, 120-134).414 (pH3.G)*
pJC097	*pHHF2.hht2(120-134).hH3.1(1-119).414 (pH3.H)*
pJC098	*pHHF2.hht2(120-134).hH3.3(1-119).414 (pH3.I)*
pJC099	*pGAL.HHO1.416*
pJC103	*pHHF2.hht2Q120M.414*
pJC104	*pHHF2.hht2K121P.414*
pJC105	*pHHF2.hht2K125Q.414*
pJC106	*pHHF2.hht2L130I.414*

Name	Vector
pJC107	*pHHF2.hht2S134A.414*
pJC117	*pHHF2.hht2Q120MK121P.414*
pJC118	*pHHF2.hht2Q120MK125Q.414*
pJC119	*pHHF2.hht2K121PK125Q.414*
pJC120	*pHHF2.hht2(120-134).hH3.1(1-119).414 (pH3.J)*
pJC121	*pHHF2.hht2(120-134).hH3.3(1-119).414 (pH3.K)*

2.2 Calmodulin Affinity Purification and Western Blot Analysis

Strains expressing Rsc2 with a tandem affinity purification (TAP) tag (Puig et al., 2001) were cultured in yeast extract, peptone, and dextrose (YPD) media to mid-log phase. Lysates from cells were prepared in extraction buffer (50 mM HEPES [pH 7.5], 350 mM NaCl, 2 mM $CaCl_2$, 0.1% Tween 20, 10% glycerol, protease inhibitor cocktail [P8215; Sigma-Aldrich Co.]) by bead beating. Approximately 75 mg of extract was incubated with 10 µl of calmodulin affinity resin (Stratagene) for 2 h at 4°C. The resin was washed three times with 40 volumes of extraction buffer, and the bound proteins were eluted by boiling in 3 volumes of sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer. The samples were Western blotted and probed with antibodies specific to the TAP tag, anti-acetylated histone H3, anti-histone H3, or anti-acetyl-lysine.

Table 2.3 Antibodies used in this thesis.

Antibody	Company	Catalog number
Rabbit Immunoglobulin G (IgG)	Chemicon	PP64
α-TAP tag	Sigma-Aldrich Co.	P2026
α-histone H3	Millipore	06-599
α-H3	Abcam, Inc.	ab1791
α-acetyl-lysine	Abcam, Inc.	ab409
Goat α-rabbit (680)	Licor	926-32210

Antibody	Company	Catalog number
Goat α-mouse (800)	Licor	926-32221
Goat α-rabbit (680)	Licor	926-32210
α-yeast histone H3	GenScript	Rabbit polyclonal - raised to yeast specific antigen CKDIKLARRLRGERS
α-Hho1	Abcam, Inc.	ab71833
α-histone H4	Abcam, Inc.	ab31830
α-HA	Roche	High affinity 3F10 clone 11867423001

2.3 Synthetic Dosage Lethality Screen

The synthetic genetic array (SGA) starting strain Y7092 (*MATα can1Δ::STE2pr-Sp-his5 lypΔ1 his3Δ1 leu2Δ0 met15Δ ura3Δ0*) was transformed with p*GAL.HHO1*. The resulting query strain was mated to the *MAT***a** deletion mutant array. SGA methodology, previously described for a plasmid-based synthetic dosage resistance screen (Martin et al., 2006), was used with the following modifications: (i) medium lacking uracil was used to maintain the plasmid, and (ii) hits were scored against strains containing *pGAL.HHO1* grown on galactose using the "Baloney" program, which was developed by Dr. Barry Young and Dr. Chris Loewen. The screen was performed in triplicate and all hits were confirmed using PCR confirmation of the deletion strain, and traditional transformation and dilution plating.

2.4 Quantitative Western Blot Analysis

Whole cell extracts were analyzed by SDS PAGE and western blotting with antibodies listed in Table 2.2-1 followed by fluorescence detection and quantification using the Licor Odyssey System. Standard deviation was calculated from results produced by at least three biological replicates.

2.5 Chromatin Immunoprecipitation and Quantitative PCR (ChIP-qPCR)

Chromatin immunoprecipitation (ChIP) was performed as previously described, with a few modifications (Nelson et al. 2006). Briefly, cells were grown in 50 mL of YPD to mid-log phase and cross-linked with 1% (v/v) formaldehyde for 30 min at room temperature. The reaction was stopped with 125 mM glycine for 15 min and cells were washed twice with PBS. Samples were sonicated (Biorupter, Diagenode, high output for 6 x 30 on/off) to obtain an average sheared DNA fragment length of 500 bp. The samples were cleared at a 10,000 rpm for 10 minutes, and the pellet was discarded. Antibodies used for ChIP are listed in Table 2.2-1. Antibodies or IgG were added and incubated with the whole cell extract overnight. Magnetic Protein G Dynabeads (Invitrogen) were added and incubated with the sample for 30 mins. After reversal of crosslinking and DNA purification, immunoprecitated and input DNA were amplified using an MJ Research Opticon Monitor 3 Thermal Cycler using primer pairs designed to amplify the 5' end of the indicated genes Table 2.5-1. Each PCR reaction consisted of 13.5 µl ddH2O, PCR buffer (20 mM Tris-Cl, 10 mM KCl, 10 mM (NH4)2SO4, 2 mM MgSO4 and 0.1% TX-100), 0.2 mM dNTPs, 1.6 mM of each primer, and 1 µl of a 1:1000 dilution of SYBR green. PCRs went through a program of 94°C for 2 min followed by 40 cycles of 94°C 30 s, 57°C 1 min, 72°C 1 min using the Opticon Monitor 3 (MJ Research). Average IP values for each sample were normalized to average input values (%IP) and compared to a mock (gene deleted) or IgG control control.

Table 2.4 Primers used in this thesis.

Name	Number	Sequence 5'-3'
COX10 s+142	636	CGGAATCATGGCGGGAAAC
COX10 a+335	637	GGAAGTTGTGTGCTTGCATCG
CDC8 s+33	936	GGATTGGATAGGACTGGTAAAACC
CDC8 a+210	937	CGAAAACAAGAGGTGAATTGCCTG
IPK1 s+33	1033	CTGATTGATTATGGGGATCCTACG
IPK1 a+199	1034	CGACGTCAATCAGATACATCG

2.6 Chromatin Association Assay

The Chromatin Association Assay was performed as previously described with a few modifications (Liang and Stillman 1997). After incubation in pre-spheroplast buffer (100mM Tris pH9.4, 10mM DTT) cells were incubated in spheroplast buffer (50mM KPO4 pH 7.5, 0.6M Sorbitol, 0.5 mM PMSF) with 10mg/mL Zymolyase 20T for 30min at 30°C. Spheroplasts were prepared as previously described (Liang and Stillman, 1997) and lysed with 1% TX-100. WCE was saved and the remaining fraction was separated into supernatant and chromatin pellet through centrifugation in lysis-X buffer (lysis buffer + 1% TX-100). The chromatin pellet was resuspended in lysis-X buffer, and adjusted to 4mM NaOH.

2.7 Chromatin Immunoprecipitation for Sequencing (ChIP-Seq)

Chromatin Immunoprecipitation was done as described in Maltby et al., 2012, with a few modifications. Briefly, cells were grown in 1L of YPD to mid-log phase and cross-linked with 1% formaldehyde for 15 mins at 30°C. The cross-linking reaction was stopped with 125 mM liquid glycine and cells were washed twice with cold PBS. Cells were resuspended in lysis buffer

(50 mM Hepes-KOH [pH 7.5], 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate) and flash frozen in liquid nitrogen and ground in a coffee grinder with dry ice for 10 x 3 mins on/off to lyse. Samples were thawed, normalized by protein content, and sonicated (Biorupter, Diagenode, Sparta NJ, high output for 30 x 30 sec on/off) to obtain an average DNA fragment length of 200 to 400 bp. The lysate was cleared at 10,000 rpm for 10 minutes, and the supernatant was retained for the whole cell extract. Magnetic Protein-G Dynabeads (Invitrogen) were added and incubated with the whole cell extract for one hour, then removed. Antibodies were added (15.0 ul of the α-Hho1 antibody, Table 2.2-1) and incubated with the whole cell extract overnight. Magnetic Protein-G Dynabeads (Invitrogen) were added and incubated with the sample for 30 mins. After reversal of crosslinking and DNA purification, immunoprecitated DNA was visualized for library preparation.

2.8 Library Preparation, Illumina Sequencing and Data Analysis

The library construction protocol was performed as described in Maltby et al., 2012. Library construction for the Illumina platform was performed using a custom procedure for paired-end sequencing. Briefly, 2–10 ng of ChIP material was end-repaired and A-tailed before being ligated to TruSeq PE adaptors. In between each reaction, the material was purified using phenol:chloroform:isoamyl alcohol extraction followed by ethanol precipitation. The resulting material was then amplified in the Phusion HF master mix (New England Biolabs) using TruSeq PE PCR primer 1.0 and custom indexed multiplexing primers [5′ AAGCAGAAGACGGCATACGAGATNNNNNNGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC 3′, where “NNNNNN” corresponds to unique hexamer barcodes]. PCR amplification

was performed as follows: denaturation at 98°C for 60 s; eight cycles of (98°C, 30 s; 65°C, 30 s; 72°C, 30 s), and a final extension at 72°C for 5 min. Amplified libraries were purified using 0.8 (vol) Agencourt AMPure XP solid phase reversible immobilization paramagnetic beads and eluted in 10 mM Tris·HCl pH 8.5. An aliquot of each library was run on an Agilent High Sensitivity chip to check the size distribution and molarity of the PCR products. Equimolar amounts of indexed, amplified libraries were pooled, and fragments in the 200–600 bp size range were selected by excision on an 8% (wt/vol) Novex TBE PAGE gel (Invitrogen). An aliquot (1 μL) of the library pool was run on an Agilent High Sensitivity chip to confirm proper size selection and measure DNA concentration.

The pooled libraries were diluted to 15 nM and their concentration was confirmed using the Quant-iT dsDNA HS assay kit and Qubit fluorometer (Invitrogen). Libraries were sequenced on the Illumina HiSeq platform at the UBC Biodiversity Research Centre NextGen Sequencing Facility. Clusters were generated on the cBOT (HiSeq2000) and paired-end 100 nucleotide reads generated using v3 sequencing reagents on the HiSeq2000 (SBS) platform. The hexamer barcode was sequenced using the following primer [5′ GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT 3′]. Image analysis, base-calling, and error calibration were performed using Casava 1.8.2 (Illumina). Reads were aligned to the *S. cerevisiae* genome using BWA (Li and Durbin, 2009). The peaks were visualized using SeqMonk (v0.21.0: http://www.bioinformatics.babraham.ac.uk/projects/seqmonk/). The midpoints of total counts were aligned around various genomic features, and normalized for the average counts across the genome. Midpoints were generated using bedtools. Data was smoothed in Seqmonk by 10 base pair windows before being graphed using Microsoft Excel. The k-means clustering analysis was done using the “Galaxy/Cistrome” platform with the feature strand taken

into account (Liu et al., 2011; Giardine et al., 2005). The graph of the Hho1 occupancy over genes and binned by transcription rate was done using the program ChIP-Py (programmed by Karimi, M., unpublished).

Chapter 3: Acetylation of Rsc4 by the HAT Gcn5 Is Essential in the Absence of Histone H3 Acetylation

Histone acetylation plays an important role in chromatin as both a binding motif and a mechanism through which histone:DNA interactions are destabilized. Histone acetylation has been shown to occur on all of the core histones, and acetylation of lysines on the H3 and H4 N-terminal tails is highly correlated with transcriptional activity (Alfrey et al., 1964; Sterner and Berger, 2000; Li et al., 2007; Bannister and Kouzarides, 2011). Histone acetylation is catalyzed by histone acetyltransferases; and in yeast the only essential histone acetyltransferase is the H4, H2A, and H2A.Z targeting Esa1, which is part of the NuA4 and picNuA4 HAT-complexes (Smith et al., 1998; Clarke et al., 1999; Boudreault et al., 2003).

Genetic analysis of the other HATs has shown that the combined deletion of two other HAT genes *GCN5* and *SAS3* also results in an inviable strain of yeast (Howe et al., 2001). Gcn5 and Sas3 are the catalytic subunits for the HAT-complexes SAGA/SLIK/SALSA and NuA3, respectively, which acetylate the histone H3 N-terminal tail (Grant et al., 1997; Reifsnyder et al., 1996; Pray-Grant et al., 2002, Sterner et al., 2002; John et al., 2000). However, unlike the H4 tail, where mutation of the four acetylatable lysines results in cell death (Megee et al., 1990), the H3 tail is dispensable for viability (Ling et al., 1996; Morgan et al., 1991).

The above data suggested to us that the inviability of the *gcn5Δsas3Δ* double mutant was due to loss of acetylation at a HAT-target protein other than histone H3. In addition to acetylating histones, Gcn5 has been shown to acetylate Rsc4K25, which is an essential component of the RSC chromatin-remodeling complex (VanDemark et al., 2007). Acetylated

Rsc4K25 has been shown, *in vitro*, to bind to one of the two bromodomains in the Rsc4 protein, and it was, also, shown that this interaction antagonized the binding of the second Rsc4 bromodomain to acetylated H3K14 (VanDemark et al., 2007). Mutation of Rsc4K25 to an alanine, however, resulted in only minor phenotypes leaving the importance of this acetylation in question.

3.1 The *gcn5*Δ *sas3*Δ Synthetic Lethality is Not Due to Loss of Histone H3 Acetylation by Gcn5p

We previously demonstrated that strains with deletions of *GCN5* and *SAS3* are inviable (Howe et al., 2001). A temperature-sensitive *gcn5*Δ*sas3C357Y/P375A* strain fails to recover after growth at a nonpermissive temperature, indicating that disruption of both *GCN5* and *SAS3* results in lethality (Howe et al., 2001; data not shown). Although plasmids expressing wild-type HATs rescue the viability of a *gcn5*Δ *sas3*Δ strain, plasmids expressing Gcn5 and Sas3 with substitutions of conserved amino acids within the acetyl coenzyme-A binding domains do not (Figure 3.1-1A). While this confirms that the synthetic lethal phenotype is a result of loss of the acetyltransferase activities of these proteins, a major paradox is that while *GCN5* or *SAS3* is essential for viability, the histone H3 tail, which is the major target of these enzymes, is not (Ling et al., 1996, Morgan et al., 1991). Both Gcn5 and Sas3 have been shown to acetylate histone H3 in vivo (Howe et al., 2001; Kou et al., 1998; Rosaleny et al., 2007, Zhang et al., 1998); however, whether the *gcn5*Δ *sas3*Δ inviability is due to loss of acetylation of a substrate other than histone H3 has not been explored.

To determine whether this phenotype is due to loss of histone H3 acetylation by either Gcn5 or Sas3, we sought to determine whether mutation of target lysines within histone H3

could recapitulate the *gcn5*Δ *sas3*Δ synthetic lethality in either a *gcn5*Δ or *sas3*Δ strain. To this end, we generated wild type, *gcn5*Δ, and *sas3*Δ strains that expressed the sole copy of the histone H3 gene from a *URA3* plasmid. We next introduced *TRP1*-based plasmids expressing histone H3 with arginine substitutions of acetylatable lysines and examined the synthetic phenotypes on 5-fluoroorotic acid (5-FOA). Simultaneous mutation of lysines 9, 14, 18, 23, and 27 to arginines caused a noticeable growth defect in a wild-type strain but resulted in lethality in a *gcn5*Δ strain (Figure 3.1-1B). This phenotype can be recapitulated by mutation of lysines 14 and 23 alone (data not shown), which are the sites targeted by the NuA3 complex (Howe et al., 2001). These data are consistent with the fact that in the absence of *GCN5*, the acetylation of histone H3 by Sas3 is essential. In contrast to the synthetic phenotypes observed upon mutation of histone H3 in a *gcn5*Δ strain, deletion of *SAS3* in the K9,14,18,23,27R mutant did not result in any additional phenotype (Figure 3.1-1B). In addition to lysines 9, 14, 18, 23, and 27, Gcn5 has recently been shown to acetylate lysine 36 of histone H3 (Morris et al., 2007). However, deletion of *SAS3* in a strain with concomitant mutations of lysines 9, 14, 18, 23, 27, and 36 to arginines did not result in loss of viability of this mutant. The fact that we were unable to phenocopy the *gcn5*Δ *sas3*Δ synthetic lethality by mutating all of the known Gcn5-targeted sites on histone H3 in a *sas3*Δ background suggests that Gcn5 is required for a function other than acetylating the N-terminal tail of histone H3. It is loss of this acetylation that is lethal in the absence of histone H3 acetylation.

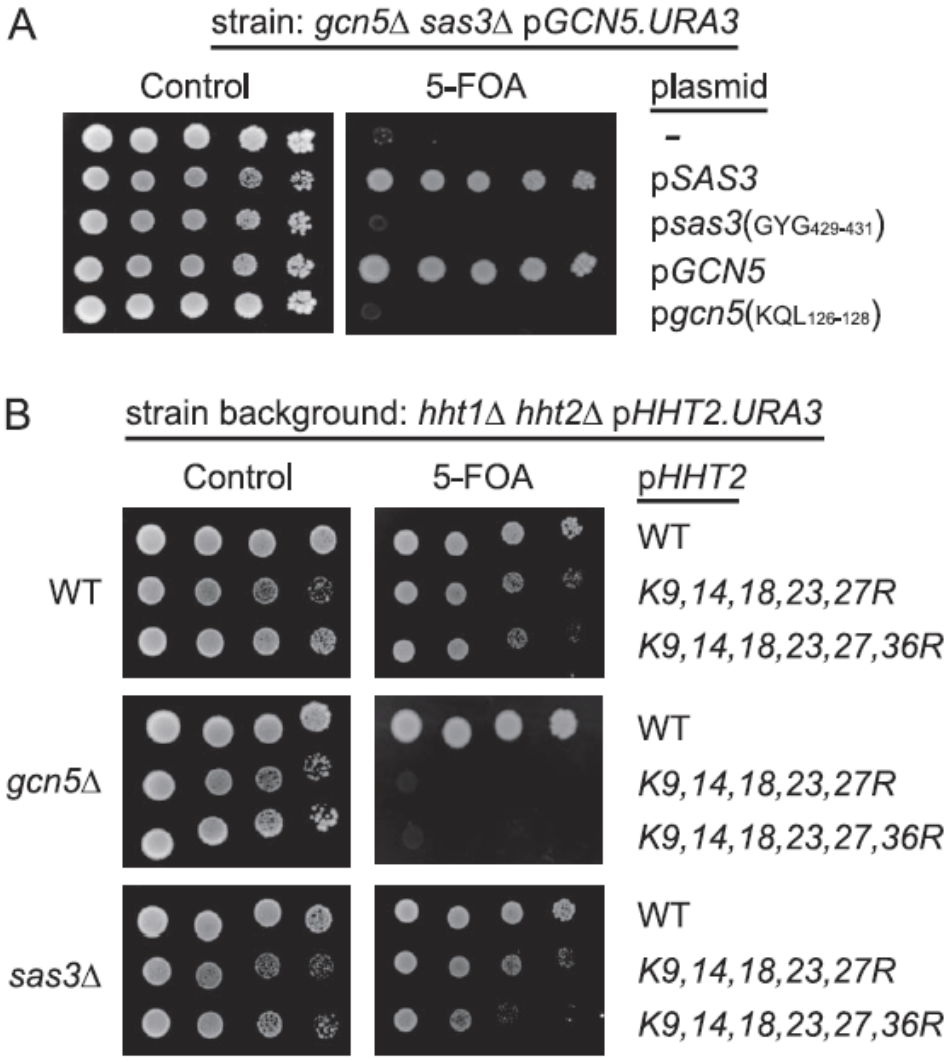


Figure 3.1. The *gcn5Δ sas3Δ* synthetic lethality is not due to loss of histone H3 acetylation by Gcn5. (A) and (B) Ten-fold serial dilutions of the indicated strains with the specified plasmids were plated on synthetic complete media without (Control) and with 5-FOA and incubated at 30°C for 3 days for WT and *sas3Δ* strains and 4 days for *gcn5Δ* strains. GYG: *sas3* with a triple alanine substitution of amino acids $GYG_{429-431}$; KQL, *gcn5* with a triple alanine substitution of amino acids $KQL_{126-128}$.

3.2 Gcn5 Has a HAT Complex-independent Function

Our phenotype analysis suggests that Gcn5 acetylates a substrate other than the histone H3 tail. In addition to histone H3, Gcn5 has been shown to acetylate lysines 11 and 16 of histone H2B in vivo (Suka et al., 2001). Additionally, the possibility that Gcn5 is acetylating unidentified sites within any of the core histones cannot be excluded. To determine whether the *gcn5Δ sas3Δ* phenotype is due to loss of Gcn5's ability to acetylate histones, we sought to determine whether any of the Gcn5-dependent HAT complexes are essential in a *sas3Δ* strain. Gcn5 is the catalytic subunit of at least three different HAT complexes, including SAGA, SLIK/SALSA, and ADA (Eberharter et al., 1999; Grant et al., 1997; Pray-Grant et al., 2002; Sterner et al., 2002). In addition to Gcn5, these HATs also share Ada2, and experimental evidence suggests that Ada2 is required for Gcn5 HAT activity (Balasubramanian et al., 2001; Syntichaki and Thireos, 1998). Furthermore, phenotypes associated with deletions of *GCN5* are indistinguishable from those associated with deletions of *ADA2* (Georgakopoulos et al., 1995). Thus, both *in vitro* and *in vivo* evidence supports the fact that Ada2 is required for the function of Gcn5 as a HAT. However, despite the requirement of Ada2 for Gcn5 HAT activity, *ada2Δ sas3Δ* strains are viable (Figure 3.2-1A) (Howe et al., 2001).

In contrast, deletion of *NTO1*, a gene encoding a structural component of NuA3, is lethal in a *gcn5Δ* strain (Figure 3.2-1A). These data indicate that although the *gcn5Δ sas3Δ* synthetic lethality is due to loss of NuA3 in a *gcn5Δ* strain, the Gcn5-dependent HAT complexes are dispensable in a *sas3Δ* strain. To further confirm that the *gcn5Δ sas3Δ* synthetic lethality is not due to loss of any of the Gcn5-dependent HATs, we sought to determine whether a mutation in Gcn5 that disrupts the incorporation of this protein into a HAT complex is lethal in a *sas3Δ* strain. It has been previously demonstrated that although the first 261 amino acids of Gcn5 are

sufficient for histone HAT activity in vitro, additional residues located carboxyl-terminal to this HAT domain are required for the incorporation of this protein into HAT complexes (Candau et al., 1997). We sought to determine whether a mutant version of Gcn5, lacking the HAT interaction domain, could rescue the *gcn5Δsas3Δ* synthetic lethality. Figure 3.2-1B demonstrates that a *TRP1* plasmid expressing $Gcn5_{1\text{-}261}$ rescues the growth of a *gcn5Δ sas3Δ* p*GCN5*.*URA3* strain on 5-FOA, further confirming that the *gcn5Δ sas3Δ* synthetic lethality is not due to loss of any of the Gcn5-dependent HAT complexes. This strain does show a growth defect compared to an isogenic strain expressing full-length Gcn5.

To determine whether this is due to decreased stability of truncated Gcn5, we fused full-length and truncated Gcn5 to carboxyl-terminal triple hemagglutinin (HA) tags and examined levels of Gcn5 in whole-cell extracts (WCE) by Western blotting. Figure 3.2-1C shows that Gcn5(1-261) is significantly less abundant than full-length Gcn5, suggesting that the growth defect shown in Figure 3.2-1B is due to lower levels of Gcn5. The fact that the HAT interaction domain is not required to rescue the *gcn5Δ sas3Δ* synthetic phenotype and that Gcn5 must be incorporated into a HAT complex to acetylate nucleosomal histones (Balasubramanian et al., 2002; Grant et al., 1997) suggests that the *gcn5Δ sas3Δ* synthetic lethality is a result of a failure of Gcn5 to acetylate a nonhistone substrate in a strain lacking histone H3 acetylation.

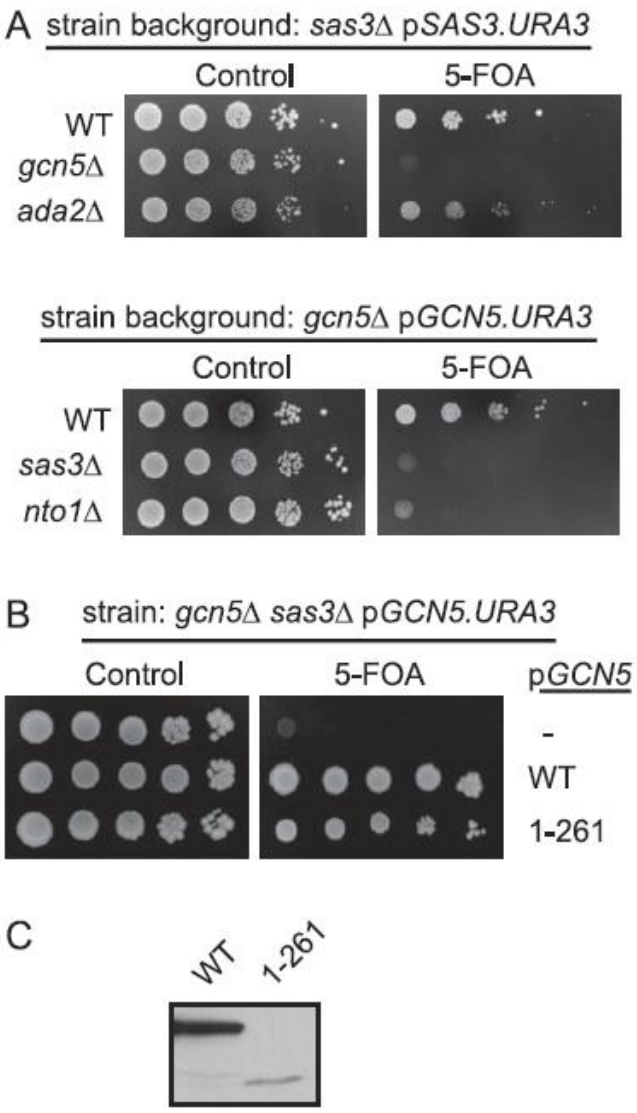


Figure 3.2. The *gcn5Δ sas3Δ* synthetic lethality is not due to loss of the known Gcn5-dependent HAT complexes. (A) The indicated strains were plated in 10-fold serial dilutions on synthetic complete media without (Control) and with 5-FOA. (B) Strains transformed with vector alone (−), or plasmids expressing either full-length Gcn5 (WT) or Gcn5p1-261 (1-261) were plated on synthetic complete media without (Control) and with 5-FOA and incubated at 30°C for 3 days. (C) WCE prepared from cell expressing triple HA-tagged full-length (WT) and C-terminal truncated (1-261) Gcn5 were blotted and probed for the HA tag.

3.3 The Acetylation of Rsc4p and That of Histone H3 are Redundant

Gcn5 has been shown to acetylate numerous proteins in yeast in addition to the canonical core histones, including Rsc4, Sin1, and Htz1 (Babiarz et al., 2006; Millar et al., 2006; Pollard and Peterson, 1997; VanDemark et al., 2007). Whether acetylation of these proteins requires the Gcn5-dependent HAT complexes has never been tested. To determine whether the *gcn5*Δ *sas3*Δ phenotype is due to a failure to acetylate Rsc4 in a *sas3Δ* strain, we sought to determine whether we could recapitulate the *gcn5Δ sas3Δ* synthetic lethality by mutating the Rsc4 acetylation site (lysine 25) in a strain lacking histone H3 acetylation. Since histone H3 is acetylated by both Sas3 and multiple Gcn5-dependent HATs, we disrupted H3 acetylation by concomitant deletions of *SAS3*and *ADA2*.

We generated *rsc4Δ, rsc4Δ sas3Δ, rsc4Δ ada2Δ,* and *rsc4Δsas3Δ ada2Δ* strains that expressed *RSC4* from a *URA3*-based plasmid. A plasmid shuffle experiment was performed using plasmids expressing wild type Rsc4 (WT), and Rsc4 with an alanine substitution of lysine 25 (K25A). Figure 3.3-1A demonstrates that while *rsc4K25A* mutants are viable in wild-type, *sas3Δ,* and *ada2Δ* backgrounds, mutation of Rsc4K25 in an *ada2Δ sas3Δ* strain is lethal. The phenocopy of the *gcn5Δ sas3Δ* synthetic lethality by mutation of Rsc4K25 to alanine in a mutant lacking histone H3-specific HATs confirms that this phenotype is due to redundancy in acetylation of histone H3 and Rsc4K25. As a further confirmation, we tested whether mutation of Rsc4K25 results in lethality in a strain with point mutations of the acetylatable lysines within the H3 tail. Figure 3.3-1B shows that while *rsc4K25A HHT2* and *rsc4K25A hht2K14R* strains are viable, simultaneous mutation of histone H3K9, 14, 18, and 23 to arginines is lethal in a strain lacking Rsc4K25, further confirming the redundant function of these residues in maintaining cell viability.

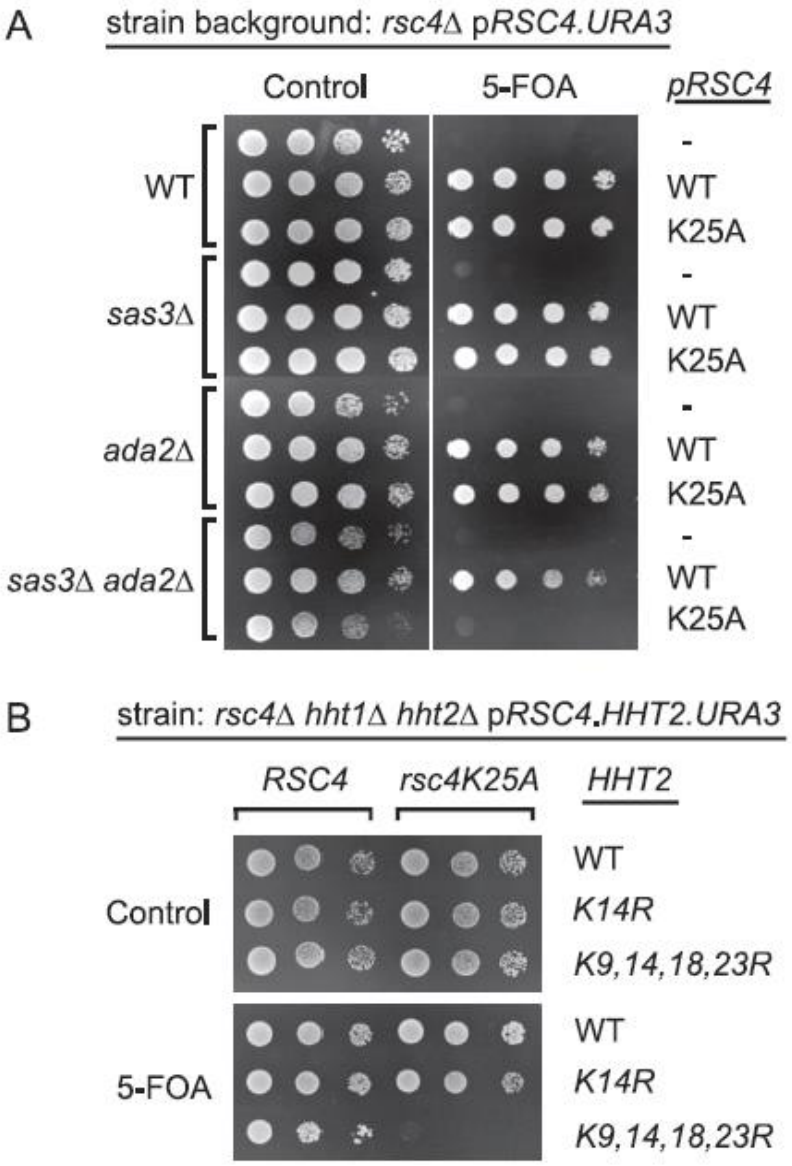


Figure 3.3. The *gcn5Δ sas3Δ* synthetic lethality is due to redundancy between acetylation of histone H3 and Rsc4. Ten-fold serial dilutions of the indicated strains transformed with the specified plasmids were plated on synthetic complete media without (Control) and with 5-FOA and incubated at 30°C for 3 days.

3.4 Distinct Proteins are Required for Rsc4 and H3 Acetylation by Gcn5

The fact that *ada2Δ sas3Δ* strains are viable (Figure 3.2-1A) (Howe et al., 2001) suggests that *ADA2* is not required for acetylation of Rsc4. This represents the first example of a Gcn5 function that is independent of the known HAT complexes. To verify that acetylation of Rsc4 is independent of the Gcn5-containing HAT complexes, we tested whether we could detect Rsc4 acetylation in an *ada2Δ* strain. To this end, we purified RSC from a Rsc2TAP strain using calmodulin affinity purification and subjected the coprecipitating proteins (RSC) to Western blot analysis with anti-TAP (α-Rsc2) and anti-acetyl-lysine (α-acLys) antibodies. Figure 3.4-1A shows that, as observed by others, purified RSC contains an acetylated protein that comigrates with Rsc4. Mutation of lysine 25 of Rsc4 results in loss of this signal (Figure 3.4-1B), demonstrating that the acetylated-lysine signal is indeed from Rsc4K25. As shown by others, acetylation of Rsc4 is disrupted by deletion of *GCN5* (Figure 3.4-1A, compare α-acLys signal in lanes 1 and 2) (VanDemark et al., 2007). Interestingly, deletion of *ADA2* has only a minimal effect on the levels of acetylated Rsc4 (lane 3), suggesting that acetylation of Rsc4 is independent of any of the known Gcn5-dependent HAT complexes.

As a control we performed Western blot analysis of WCE from each strain using an anti-acetyl H3 antibody (α-acH3) to verify that deletion of *ADA2* resulted in the same loss of histone H3 acetylation seen in a *gcn5*Δ strain (Figure 3.4-1A, compare lanes 1, 2, and 3). As a final confirmation that the acetylation of Rsc4 is independent of the Gcn5 HAT complexes, we examined the levels of Rsc4 acetylation in a strain lacking the HAT interaction domain of Gcn5. As shown in Figure 3.4-1C, Rsc4 is still acetylated in strains expressing Gcn5(1-261). The level of acetylation is severely reduced compared to strains expressing full-length Gcn5 consistent with the fact that there is less Gcn5 in these cells (see Figure 3.2-1C). These results confirm a

novel function for Gcn5 that is independent of the accessory proteins found in the SAGA, ADA, and SLIK/SALSA HAT complexes.

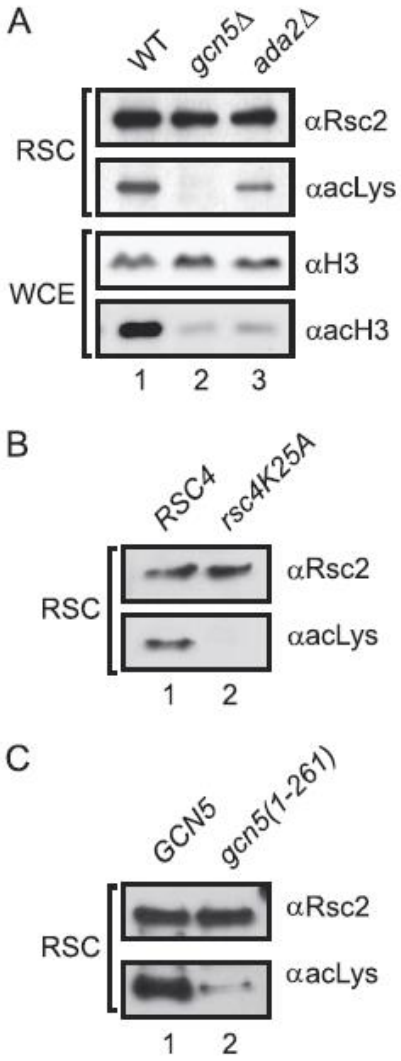


Figure 3.4. The acetylation of Rsc4p by Gcn5p does not require any of the Gcn5p-dependent HAT complexes. WCE and calmodulin affinity-purified RSC (RSC) from the specified strains were blotted and probed with the antibodies indicated.

3.5 Histone Acetylation is Important for Neutralizing the Positive Charge of the Histone H3 tail

The results in Figure 3.3-1B suggest that Rsc4K25 acetylation has a role that is independent of regulating the Rsc4 bromodomain2-H3K14ac interaction. Rsc4 acetylation, therefore, has multiple roles in RSC function and, based on what we know about Rsc4 acetylation alone, it is difficult to speculate as to why the loss of histone H3 and Rsc4 acetylation results in loss of viability. Fortunately, unlike the recently discovered Rsc4 acetylation, histone acetylation has been the focus of intense study for many decades. To date, histone H3 acetylation has been proposed to function in two, non-mutually exclusive manners: to act as a molecular "tag" for the recruitment of chromatin-modifying complexes (Yang, 2004) and to directly alter chromatin structure by weakening histone-DNA contacts (Ferreira et al., 2007; Toth et al., 2006).

We hypothesized that if the loss of H3 acetylation is disrupting the binding of a chromatin-modifying complex to the H3 tail, then mutation of the acetylatable lysines to glutamine should recapitulate the phenotypes of arginine substitutions and be lethal in a *rsc4K25A* mutant. In contrast, if histone H3 acetylation is required to weaken histone-DNA contacts, then substitution of acetylatable lysines with uncharged glutamines should be tolerated in a strain lacking Rsc4 acetylation Figure 3.5-1A shows that unlike a *rsc4K25A* strain with arginine substitutions of lysines 9, 14, 18, and 23 of histone H3, strains with glutamine substitutions are viable. These results are reminiscent of observations made by Zhang (Zhang et al., 1998), which showed that, while simultaneous mutation of several sites on histones H3 and H4 to arginine in a *gcn5Δ* background results in lethality, mutation of the same sites to glutamine bypasses the need for *GCN5* for transcriptional activation by Gal4-VP16. These data strongly suggest that the *rsc4K25A hht2K9,14,18,23R* inviability, and hence the *gcn5*Δ *sas3*Δ synthetic lethality, is due to a failure to neutralize the positive charge on the histone H3 tail. This charge

neutralization is required to weaken histone-DNA contacts, which is essential when RSC function is impaired due to loss of Rsc4 acetylation.

The direct effects of histone acetylation on chromatin structure have been the focus of intense study. The majority of these studies have examined the impact of simultaneous acetylation of all four core histones, although two studies have directly examined the effect of histone H3 acetylation alone. First, chemical acetylation of histone H3 has been shown to result in a transient unwrapping of DNA from the octamer, as shown by measuring distances between the linker DNA ends using FRET analysis (Toth et al., 2006). Second, tetra-acetylation of histone H3 using a peptide ligation strategy results in a twofold increase in the rate of intrinsic mono-nucleosome sliding in vitro (Ferreira et al., 2007). Thus, histone H3 acetylation may weaken histone-DNA contacts resulting in both enhanced nucleosome "breathing" and increased octamer mobility. To confirm that it is the loss of these events that results in lethality in a *rsc4K25A* strain, we sought to determine whether we could bypass the requirement for *GCN5* and *SAS3* by deleting a gene that inhibits both nucleosome breathing and octamer mobility.

Linker histones associate with the linker DNA that extends between nucleosomal core particles and are essential for condensation of nucleosome arrays into the 30-nm fibers. Incorporation of histone H1 into a nucleosome causes the linker DNA to contact as a "stem" at the nucleosome edge (Bednar et al., 1998; Toth et al., 2006) and restricts passive nucleosome movement (Millar et al., 2006; Morgan et al., 1991). *S. cerevisiae* encodes a single linker histone, Hho1, which, unlike linker histones in higher eukaryotes, has two globular domains. However, Hho1 binds nucleosomes in vitro (Patterton et al., 1998), is expressed during S phase coordinately with the core histones (Spellman et al., 1998), and colocalizes with the four core

histones in vivo (Zanton and Pugh, 2006). Moreover, regions of the yeast genome with high Hho1p levels tended to be underacetylated at lysines 9 and 14 of histone H3 (Zanton and Pugh, 2006), and thus, an intriguing hypothesis is that, in addition to weakening histone-DNA contacts, histone H3 acetylation may also prevent the binding of histone H1 to chromatin.

To determine whether loss of *HHO1* rescues growth of a *gcn5Δ sas3Δ* mutant, we generated a *gcn5Δ sas3Δ hho1Δ* strain that expressed *SAS3* from a *URA3*-based plasmid. This strain failed to grow on 5-FOA (data not shown); however, when using a conditional *gcn5Δ sas3Δ* mutant that expressed a temperature-sensitive version of Sas3 (TS), we found that deletion of *HHO1* could rescue growth of this strain at the nonpermissive temperature (Figure 3.5-1B). When taken together with the fact that Rsc4K25 acetylation is essential in strains that fail to neutralize the positively charged lysines within the H3 tail, these results suggest that histone H3 acetylation is required to disrupt histone-DNA contacts, and loss of this activity is lethal in strains with impaired RSC function. We envision a model whereby both RSC and histone acetylation function to destabilize chromatin structure, and concomitant deletions of *GCN5* and *SAS3* hinder both events resulting in loss of viability.

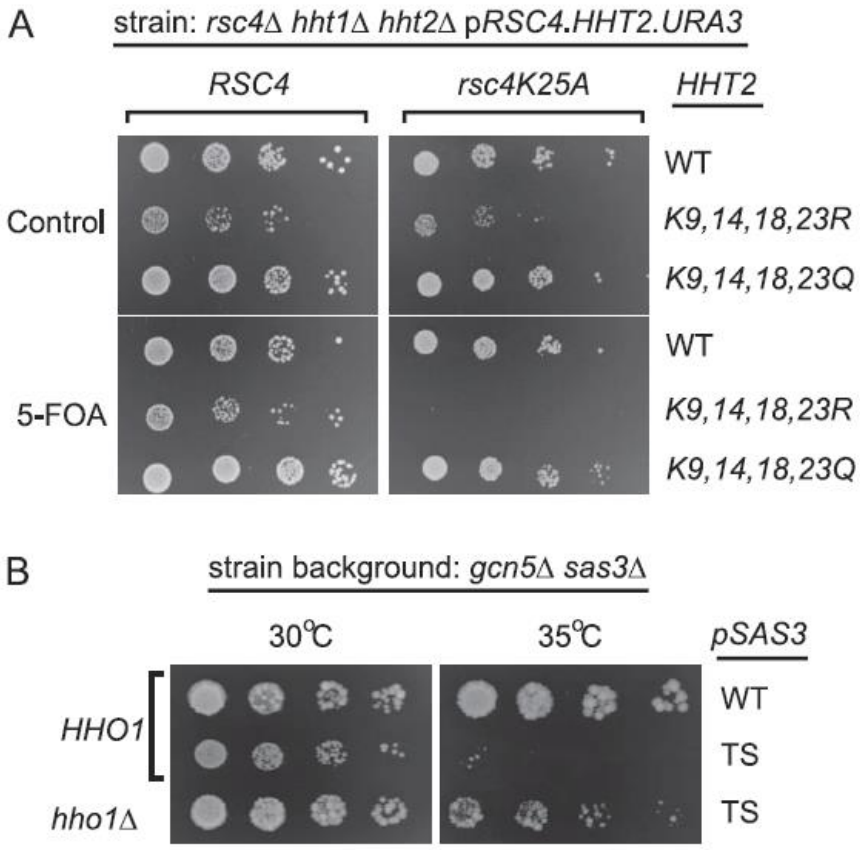


Figure 3.5. Histone acetylation is important for neutralizing the positive charge of the histone H3 tail. (A) Strains carrying the indicated mutations within Rsc4 and/or histone H3 were plated on synthetic complete media without (Control) and with 5-FOA and incubated at 30°C for 2 days. (B) *gcn5Δsas3Δ* strains with the indicated plasmids were plated in ten-fold serial dilutions on YPD and incubated at 30°C and 35°C for 3 days. WT, a wild type SAS3; TS, temperature-sensitive Sas3C357YP375A.

3.6 Discussion and Conclusions

In *S. cerevisiae*, the RSC-complex is an important member of the SWI/SNF family of chromatin remodelers and it has been shown to have roles in transcriptional activation, kinetochore function and cohesion association, and double-strand break repair (Cairns et al.,

1996; Saha et al., 2006; Soutourina et al., 2006; Huang et al., 2004; Shim et al., 2005; Shim et al., 2007; Liang et al., 2007). The synthetic phenotype observed upon loss of Rsc4 and histone H3 acetylation is surprising when one considers the proposed function of Rsc4 acetylation. Previous data suggests that within Rsc4, Rsc4K25ac binds to bromodomain1 (BD1), inhibiting the interaction of H3K14ac with bromodomain2 (BD2) (VanDemark et al., 2007). Considering the opposing roles of H3K14ac and Rsc4K25ac in the binding and release of RSC from chromatin, respectively, clearly mutation of Rsc4K25 should not result in an enhanced phenotype in a strain with a mutation of H3K14. Instead, these results suggest an additional function for Rsc4 acetylation that is independent of regulating the bromodomain 2-H3K14ac interaction. The impact of Rsc4 acetylation on RSC function could be at the level of complex integrity, remodeling activity, or DNA or histone binding. The data presented in this study underscore the need for further study into the function of Rsc4p acetylation, which obviously has a role in RSC that is essential in the absence of histone H3 acetylation.

Work done by another lab, since the publication of this, showed that the acetylation of Rsc4K25 has no impact on RSC's ability to bind acetylated nucleosomes. Rather they presented evidence that this acetylation was important for resistance to DNA damage (Charles et al., 2011). This group showed that the mutant *rsc4K25R* has synthetic interactions with genes coding for proteins in the DNA-damage repair pathway, and suggested that the function of the Rsc4K25ac:Rsc4BD1 interaction protects the acetyl-group from removal. Further, they suggested that the acetylation of Rsc4K25 acts as a switch that regulates RSC function in transcription and DNA-damage response (Charles et al., 2011).

Our findings in this chapter, also, show that the neutralization of the charged lysine residues on histone H3 is important when the acetylation of Rsc4 is compromised through

mutation of Rsc4K25. Previous studies looking at the impact of histone H3 acetylation on nucleosome stability showed that acetylation of H3 results in decreased binding of the linker histone to nucleosomes, which would allow for more passive nucleosome movement (Millar et al., 2006; Morgan et al., 1991; Mistelli et al., 2000). In order to investigate the role of acetylation in regulating the yeast linker histone, we deleted Hho1, and show that this deletion alleviates the synthetic sickness of a conditional *gcn5Δsas3Δ* mutant strain that expressed a temperature-sensitive version of Sas3. This confirms that acetylation of the core histones, particularly of histone H3, is a mechanism through which the cell regulates binding of the linker histone.

Chapter 4. The Loss of the Histone Variant Htz1 Leads to the Increased Binding of Hho1 to Nucleosomes

The linker histone, H1, is associated with higher order chromatin structure. It has previously been shown that removal of H1 is required for the decondensation of chromatin to allow access to DNA (Robinson et al., 2008). In multicellular eukaryotes, the general paradigm is that there is, on average, one linker histone per nucleosome. In yeast, the linker histone, called Hho1, is present in sub-stoichiometric levels compared to nucleosomes: either at a ratio of 1:37 or 1:4 (Freidkin and Katcoff, 2001; Downs et al., 2003). The *S. cerevisiae* linker histone, which is called Hho1, is a non-canonical linker histone that has two globular domains instead of the canonical tripartite histone structure (Kasinsky et al., 2001, Ushinski et al., 1997; Landsman, 1996). It has been suggested that the yeast linker histone binds to two nucleosomes simultaneously, which could partially account for the low numbers of linker histone in the yeast cell; however, *in vitro* and *in vivo* experiments have shown that Hho1 appears to interact with nucleosomes at a 1:1 ratio through only a single globular domain, similar to the canonical linker histones (Ono et al., 2003; Ali et al., 2004; Ali and Thomas, 2004; Schafer et al., 2005; Patterton et al., 1998; Yu et al., 2009).

Among the questions still to be answered regarding the yeast linker histone, is the question of how Hho1 binding is regulated in the cell. Human H1 variants are known to reside for less time at nucleosomes that are highly acetylated (Misteli et al., 2000; Raghuram et al., 2010), and *in vitro* studies assaying chicken erythrocyte linker histone binding to human nucleosomes demonstrate that linker histones do not bind well to H2A.Z nucleosomes (Thakar et

al., 2009). In yeast, H2A.Z (Htz1) is found at the promoters of 65% of genes (Guillemette et al., 2005; Li et al., 2005; Raisner et al., 2005; Zhang et al., 2005), and deletion of *HTZ1* affects numerous cellular processes, including galactose induction and chromosome segregation (Halley et al., 2009; Krogan et al., 2004).

4.1 The Histone H2A Variant, Htz1, is Required for Resistance to *HHO1* Overexpression

The linker histone, H1, is associated with higher order chromatin structure. It has previously been shown that removal of H1 is required for the decondensation of chromatin to allow access to DNA (Robinson et al., 2008). In multicellular eukaryotes, the general paradigm is that there is, on average, one linker histone per nucleosome. In yeast, the linker histone, called Hho1, is present in sub-stoichiometric levels compared to nucleosomes: with ratios of 1:37 and 1:4 reported (Freidkin and Katcoff, 2001; Downs et al., 2003). However, the yeast genome has a high proportion of transcribed genes to DNA and very few regions of heterochromatin, compared with other eukaryotes. Embryonic stem cells, also, have low ratios of linker histone to nucleosomes, and have chromatin that is "open" and low in heterochromatin (Fan et al., 2005, Gaspar-Maia et al., 2012). This suggests that the amount of linker histone is inversely correlated to the relative openness of chromatin. In yeast and embryonic stem cells, low levels of linker histone would keep chromatin in a generally decondensed state, and would only act on chromatin at a local level to help fold specific areas of the genome (Freidkin and Katcoff, 2001).

We were interested in determining the effect of increasing the expression of *HHO1* to over-produce the linker histone, which I will refer to from here on as *HHO1* overexpression (*HHO1*-OE). To study the effects of *HHO1*-OE, *in vivo*, we created a plasmid whereby *HHO1*

was put under the control of the *GAL1* promoter on a *URA3* plasmid and introduced this plasmid into our wild type yeast strain (Table 2.1-2). Comparison of the growth differences between the *HHO1*-OE wild type and the same strain carrying a vector control, clearly showed that overexpression caused a slow growth phenotype on galactose media (Figure 4.1-1)

In order to identify proteins that were involved in regulating the interaction of the linker histone with chromatin and to determine the genetic pathways underlying the slow growth phenotype, we performed a variation of the yeast synthetic genetic array (SGA) assay. SGA is a high-throughput screening method in which a strain carrying a mutation of interest is crossed with the yeast library of non-essential gene mutants (i.e. each strain is null for one non-essential gene) and then replica-pinned on a series of selective media to generate haploid double mutants (Tong et al., 2001). At the end of the screen, the fitness of the individual strains is assayed by colony size, and a fitness score is generated. Two gene deletions are considered to be synthetically lethal if they are not by themselves lethal, but are inviable as a double mutant. An example of a synthetically lethal interaction is seen in the simultaneous deletion of the genes *SAS3* and *GCN5* (Howe et al., 2001), which was discussed in Chapter 3. Besides lethality, three additional phenotypes can be observed in an SGA assay: 1) no change in phenotype; 2) suppression, where the second mutation counteracts the effect of the original mutation making the strain more fit; and 3) synthetic enhancement, or synthetic sickness, where the second mutation exacerbates the severity of the first (Guarente 1993).

The SGA assay has been adapted to look at dosage interactions, and to analyze how the overexpression of a gene, which in a wild type strain is non-lethal, changes the phenotype of a cell when combined with a single gene deletion (Measday et al., 2005; Tong and Boone, 2006). Synthetic dosage lethality (SDL) occurs when the combination of overexpression and mutation

results in the death of the cell. The same genetic interactions that can occur in double mutants, as described above, can occur in dosage screens. For example, when the deletion of a gene suppresses the phenotype caused by overexpression of another gene, this is called synthetic dosage resistance (SDR). Conversely, deletions that exacerbate the overexpression phenotype are said to create a synthetic dosage sickness (SDS).

In our case, we generated a library of single mutant yeast that over-produced Hho1, and we were interested in looking at the effects of mutation in the context of an increased dose of linker histone in the cell. Due to the fact that cells overexpressing *HHO1* were sick on galactose containing media (Figure 4.1-1), we assayed the deviation in colony size from the *HHO1*-OE wild type and considered anything that grew either greater than 120% or less than 60% a mutant that caused a synthetic dosage interaction (SDI). An analysis of the SDI hits using published gene ontology (GO) and protein network data, via the Cytoscape plug-ins ClueGO and CluePedia (Bindea G et al., 2009; Bindea G et al., 2013), showed a number of genes that encode proteins important for various chromatin functions, such as chromatin remodeling and sister chromatid segregation (Figure 4.1-2).

Some of the mutants that displayed SDR were genes encoding histone deacetylases. It has been shown that incubating live cells with deacetylase inhibitors to create highly acetylated core histones increases the mobility of linker histones in the nucleus, suggesting that histone acetylation negatively regulates linker histone binding (Misteli et al., 2000). The identification of HDAC mutants as SDR hits suggested to us that the chromatin-associated SDIs identified in our overexpression screen reflected changes in the interaction of the linker histone with chromatin. Another suppressor mutant was *jhd2Δ*, which is the demethylase that targets H3K4. H3K4 methylation, like histone acetylation, is correlated with active transcription and has been

proposed to recruit the histone acetyltransferase NuA3 (Martin et al., 2006; Taverna et al., 2006). The fact that the deletion of these genes, which encode for proteins that may result in more open chromatin structures, suppressed the slow growth phenotype of *HHO1*-OE suggested that an increased amount of linker histone was binding to chromatin in the *HHO1*-OE mutant. It also suggested that an increase in PTMs associated with active transcription on nucleosomes disrupted Hho1 binding, resulting in a suppressed phenotype in the double mutant.

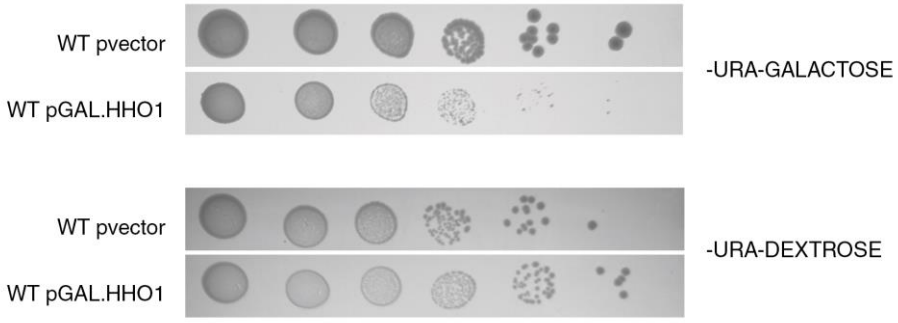


Figure 4.1. The overexpression of the yeast linker histone Hho1 caused a growth defect. Ten-fold serial dilutions of the wild type strain containing the indicated plasmid were plated on minimal media lacking uracil with dextrose or minimal media lacking uracil with galactose for 2 days 30˚C.

Table 4.1 HAT-related genes from the *HHO1* overexpression SDI screen. Genes in this table were identified as suppressing or exacerbating the phenotype of a strain over-producing the linker protein, Hho1. To determine whether there was an SDI, we compared the colony size of the final *HHO1*-OE single mutant to the colony size of the *HHO1*-OE wild type on the final screen plates and arrived at the number in the column "Ratio". In the screen, three replicate colonies were pinned, and only double mutants with 2 or more colonies on the final plates were considered. The "Ratio.SD" is the standard deviation in size between the ratios of the three colonies. Only gene deletions that caused cells to grow to a ratio of >120% or <60% were determined to be hits.

Gene	Complex	ORF	Ratio	Ratio.SD
EAF3	NuA4	YPR023C	0.6313457	0.06467175
EAF7	NuA4	YNL136W	0.4983014	0.03767828
SPT8	SAGA	YLR055C	1.7220038	0.12531999
YAF9	NuA4	YNL107W	0.1171745	0.06829281

Table 4.2 Chromatin remodeler-related genes from the *HHO1* overexpression SDI screen. Genes in this table were identified as suppressing or exacerbating the phenotype of a strain over-producing the linker protein, Hho1. To determine whether there was an SDI, we compared the colony size of the final *HHO1*-OE single mutant to the colony size of the *HHO1*-OE wild type on the final screen plates and arrived at the number in the column "Ratio". In the screen, three replicate colonies were pinned, and only double mutants with 2 or more colonies on the final plates were considered. The "Ratio.SD" is the standard deviation in size between the ratios of the three colonies. Only gene deletions that caused cells to grow to a ratio of >120% or <60% were determined to be hits.

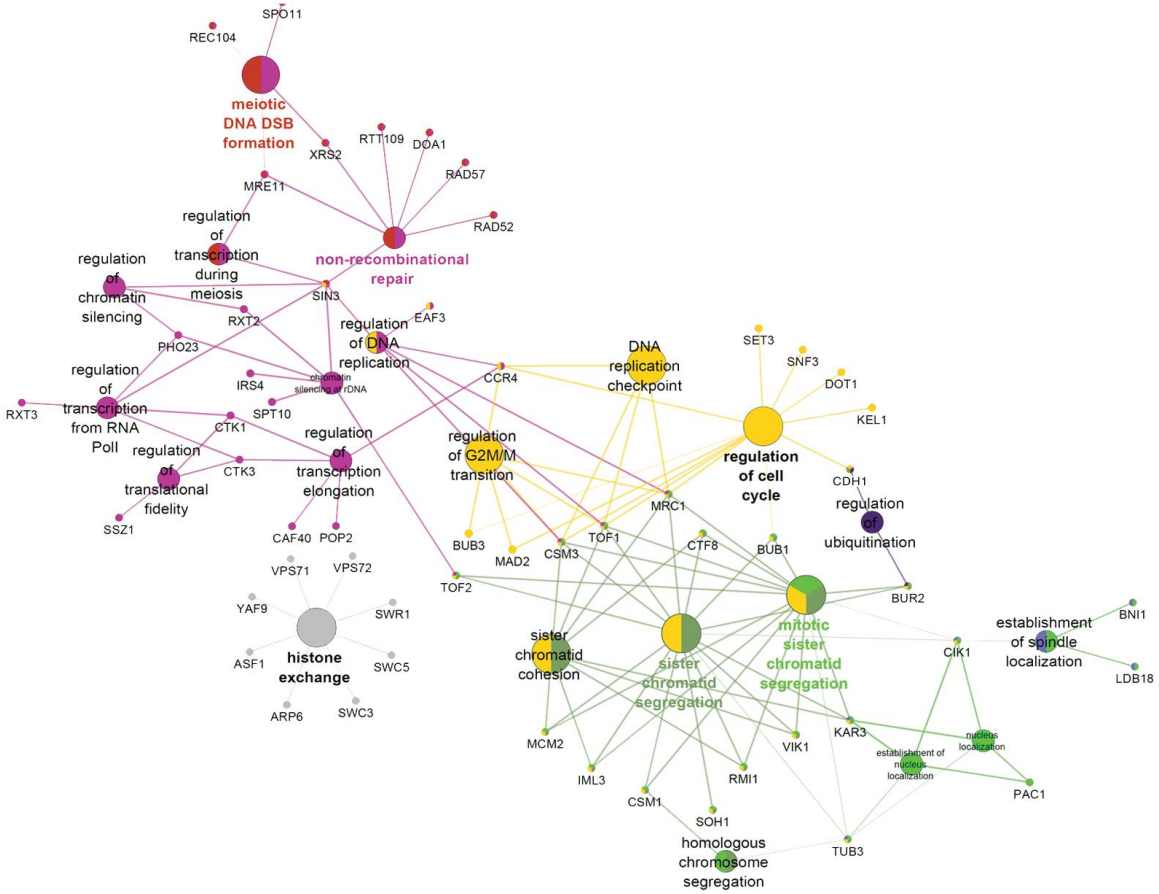
Gene	Complex	ORF	Ratio	Ratio.SD
ARP6	SWR1C	YLR085C	0.464379	0.09341205
IES1	INO80	YFL013C	1.3575841	0.03959985
SWC3	SWR1C	YAL011W	0.4435618	0.03337219
SWC5	SWR1C	YBR231C	0.5623044	0.12044777
SWR1	SWR1C	YDR334W	0.6364994	0.0328933
VPS71	SWR1C	YML041C	0.3687342	0.2674061
VPS72	SWR1C	YDR485C	0.5651589	0.18138979
YAF9	SWR1C	YNL107W	0.1171745	0.06829281
HTZ1		YOL012C	0.217963	0.084433

The chromatin remodeling complex SWR1-C was also identified by GO analysis of our SDI data (Table 4.1-2). SDI hits that exacerbated the *HHO1*-OE phenotype were significantly enriched for components of the SWR1-C protein complex, represented by seven of the nine genes (Table 4.1-2). SWR1-C is the chromatin remodeler involved in deposition of the histone H2A variant Htz1 (Kobor et al., 2004; Krogan et al., 2003; Mizuguchi et al., 2004). *HTZ1* was

also one of the phenotype-exacerbating SDI hits. Htz1 is involved in many cellular processes such as gene activation, chromosome segregation, and regulation of silent chromatin; and in many other eukaryotes H2A.Z is essential (Zlatanova and Thakar, 2008).

To confirm that both *HTZ1* and the genes that code for SWR1-C complex proteins were true hits from the array, we confirmed that the colonies noted as having those deletions were in fact correct. We took the original mutant strains from the yeast deletion collection and transformed them with the plasmid overexpressing *HHO1* to recreate the yeast strains from the final stage of the SGA screen. We confirmed that overproduction of Hho1 in yeast lacking either Htz1 or Swr1 had an exacerbated phenotype compared to *HHO1*-OE in the wild type strain (Figure 4.1-3). In total, there were 98 genes identified in our SDI data that have been previously shown to interact with *HTZ1*, either physically or genetically as curated on BioGRID[3.2], which led us to hypothesize that the proper incorporation of the histone variant Htz1 is required for resistance to *HHO1* overexpression.

A


SPO11
REC104
meiotic DNA DSB formation
XRS2
RTT109
DOA1
RAD57
MRE11
RAD52
regulation of transcription during meiosis
non-recombinational repair
regulation of chromatin silencing
SIN3
EAF3
RXT2
PHO23
regulation of DNA replication
IRS4
chromatin silencing at rDNA
CCR4
regulation of transcription from RNA Poll
RXT3
SPT10
CTK1
regulation of translational fidelity
CTK3
SSZ1
regulation of transcription elongation
CAF40
POP2
DNA replication checkpoint
SET3
SNF3
DOT1
KEL1
regulation of G2M/M transition
regulation of cell cycle
CDH1
regulation of ubiquitination
MRC1
BUB3
MAD2
CSM3
TOF1
CTF8
BUB1
BUR2
TOF2
VPS71
VPS72
YAF9
SWR1
ASF1
histone exchange
SWC5
ARP6
SWC3
sister chromatid cohesion
sister chromatid segregation
mitotic sister chromatid segregation
establishment of spindle localization
BNI1
LDB18
CIK1
MCM2
IML3
CSM1
RMI1
VIK1
KAR3
nucleus localization
establishment of nucleus localization
PAC1
SOH1
homologous chromosome segregation
TUB3

B

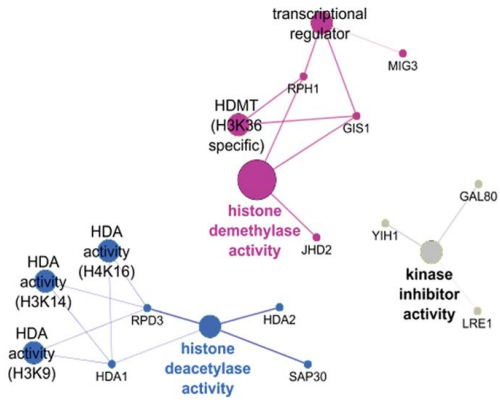

transcriptional regulator
MIG3
RPH1
HDMT (H3K36 specific)
GIS1
histone demethylase activity
JHD2
GAL80
YIH1
kinase inhibitor activity
LRE1
HDA activity (H4K16)
HDA activity (H3K14)
HDA activity (H3K9)
RPD3
HDA2
HDA1
histone deacetylase activity
SAP30

Figure 4.2. ClueGO visualization of genes that code for chromatin-associated proteins the deletion of which caused a synthetic dosage interaction with *HHO1* overexpression. Genes that were identified as having a SDI with *HHO1*-OE were analyzed by the gene ontology analysis program ClueGO, which is a plug-in for the network visualization program Cytoscape, and mapped using the Molecular Function tool (Bindea et al., 2009; 2013). The network stringency was set to medium and the GO term connection restriction was set to 0.3. (A) Genes that caused an exacerbation of the *HHO1*-OE phenotype (SDS). (B) Genes that caused a repression of the *HHO1*-OE phenotype (SDR).

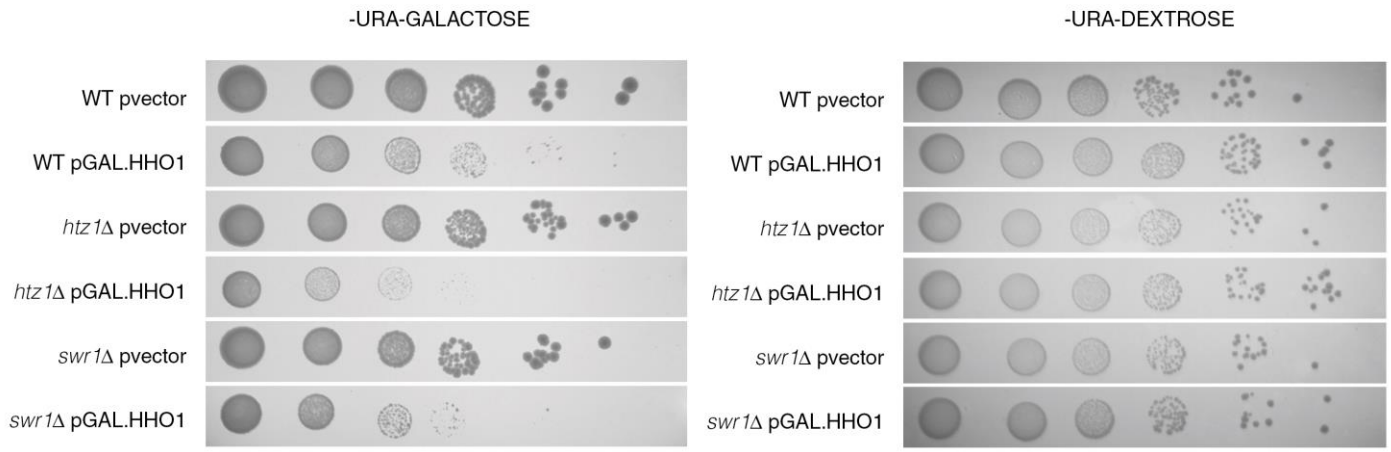


Figure 4.3. Loss of Htz1 deposition exacerbated the phenotype caused by overexpressing *HHO1*. Ten-fold serial dilutions of the indicated strain and plasmid were plated on synthetic media lacking uracil with dextrose or synthetic media lacking uracil with galactose, for 2 days 30℃.

Htz1 is found at the promoters of 65% of genes (Guillemette, et al., 2005; Li et al., 2005; Raisner et al., 2005; Zhang et al., 2005), and yeast strains with a deletion of *HTZ1* have many different phenotypes, including a defect in the kinetics of galactose induction (Wang et al., 2011;

Osada et al., 2008; Krogan et al., 2004; Meneghini et al., 2003; Halley, 2009). Our data suggested that a loss of Htz1 in chromatin exacerbated the slow growth phenotype caused by the overexpression of the gene *HHO1*. We imagine two possible explainations for these observations. The first is that the over-production of Hho1 causes more of the linker histone to be bound to chromatin and the presence of Htz1 negatively regulates this binding; and the second is that Htz1 influences the amount of Hho1 produced when overexpressed, and in an *htz1Δ* strain more Hho1 is over-produced than in a wild type strain.

To assess the amount of Hho1 in both the WT and the *htz1Δ* strains when *HHO1* was overexpressed from the *GAL1*-promoter, we assayed the amount of total cellular linker histone by quantitative western blot (Figure 4.1-4). As mentioned above, loss of Htz1 affects the kinetics of galactose induction. Consequently, there could have been a difference in the amount of Htz1 between the two strains. We assayed the amount of total cellular linker histone by quantative western blot, to determine if the bulk amount of linker histone was significantly different between the two strains. Statistical analysis showed that there was not a significant difference, using a two-tailed t-test, in the amount of either endogenous or over-produced Hho1 between the two strains.

Most importantly, we saw that both the WT and the *htz1Δ* strain expressed endogenous Hho1 in equal amounts and that in galactose the linker histone was over-produced in both strains compared to the endogenous amounts (Figure 4.1-4, compare pvector lanes). This showed that overexpression of *HHO1* did, in fact, cause an increased amount of cellular Hho1 which could make its way onto chromatin. Therefore, we hypothesized that the wild type strain was better able to cope with the increased amount of linker histone by keeping it off chromatin in an Htz1-dependent manner.

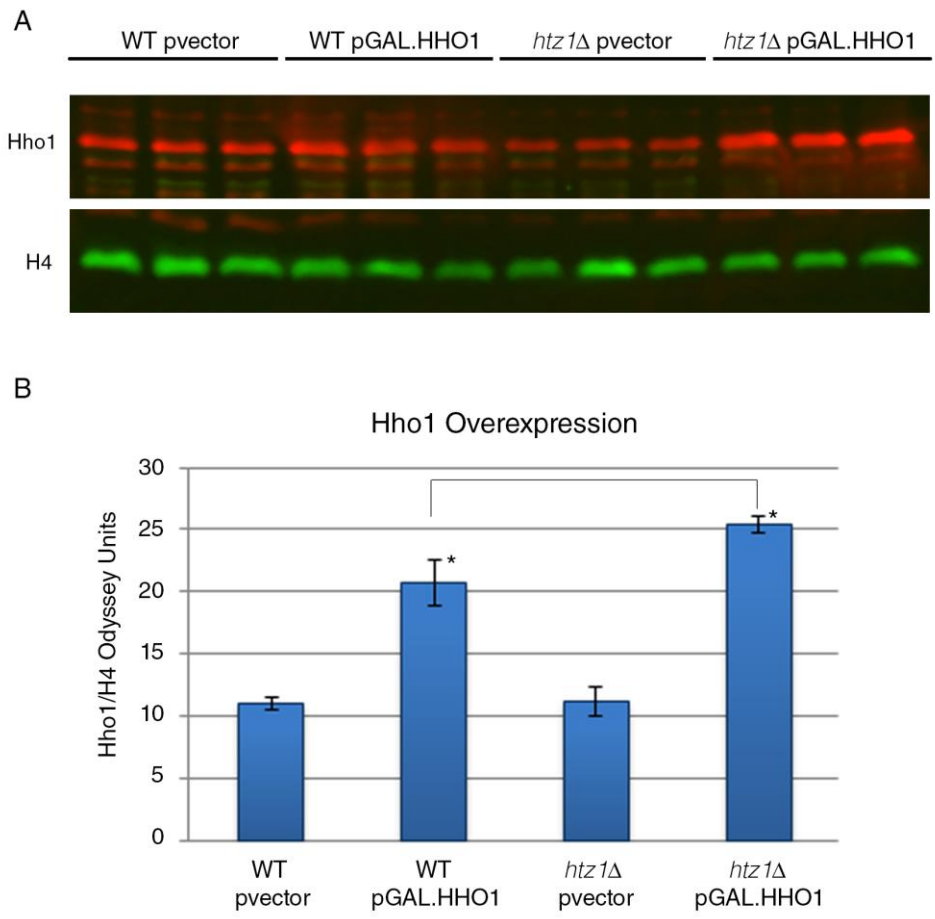


Figure 4.4. Deletion of *HTZ1* did not cause a large change in the amount of either endogenous or over-produced Hho1 protein in the cell. (A) Representative western blot of the total cellular amount of galactose-induced Hho1 and endogenous H4 from whole cell extracts. Three biological replicates were prepared for each sample. (B) Quantified western blots representing the Odyssey signal from (A), shown as the average of α-Hho1/α-H4. Error bars represent the standard error between the samples; two-tailed t-test *p=0.258.

4.2 Hho1 is Enriched at *IPK1* and *CDC8* in a Strain Lacking Htz1.

Our genetic data suggested to us that the presence of Htz1 in a nucleosome physically interferes with linker histone binding. Previously, it has been found using HeLa derived nucleosomes that the H2A C-terminal tail is required for efficient binding of the linker histone, and may mediate the H1-nucleosome interaction (Vogler et al., 2010). In yeast, the C-terminal domain of Htz1 shares less than 40% of amino acids identity with H2A, therefore, the differences in this region could result in Htz1 inhibiting Hho1 binding to a Htz1-containing nucleosome. Additionally, *in vitro* studies on reconstituted human histones showed that nucleosomes containing H2A.Z did not bind to chicken erythrocyte H1 similarly to canonical or H3.3 containing nucleosomes (Thakar et al., 2009). Interestingly, a hybrid Htz1 protein where the last 20 amino acids were replaced with the same region of H2A exhibits the same phenotype as wild type Htz1 on drug supplemented media (Wang et al., 2011). This suggests that the last 20 amino acids may not be the only region in Htz1 preventing Hho1 from binding.

If Htz1 interfered with chromatosome formation, we would expect more linker histone bound to nucleosomal DNA in an *htz1Δ* mutant than in the WT. To test whether Hho1 interacts differently with nucleosomes containing H2A or Htz1 in an *in vivo* context, we performed ChIP-qPCR (Chromatin immunoprecipitation followed by quantitative PCR) in both a wild type and an *htz1Δ* mutant, using antibodies specific for Hho1, or *S. cerevisiae* H3 (Figure 4.2-1). ChIP-qPCR is an assay that measures the amount of a protein bound to a specific locus in the genome by cross-linking proteins to DNA, using formaldehyde, and then assessing the amount of DNA that is immunoprecipitated along with the protein of interest by quantitative PCR. Each copy of the DNA fragment being tested by qPCR is assumed to have been bound by the immunoprecipitated protein. Since antibodies are used to immunoprecipitate the proteins of

interest, ChIP-qPCR is a technique that is particularly reliant on having highly specific antibodies.

IPK1 and *CDC8* were chosen as candidate genes to assay linker histone binding because genome-wide studies have shown that they have a relatively high enrichment for Htz1 (Venters et al., 2011). Additionally, the same study found that they have a small amount of Hho1 present at both loci. This was particularly important for our ChIP-qPCR assay since we wanted to control against potential factors precluding Hho1 binding at our loci of interest. Examination of the amount of linker histone bound to those same regions, normalized to the input, showed that there was a modest increase in Hho1 binding in the absence of Htz1 (Figure 4.2-2B). Our data also showed a commensurate decrease of H3 in the mutant, at the 5'ends of two genes tested, *IPK1* and *CDC8,* suggesting that *htz1Δ* mutant yeast have fewer nucleosomes near the transcription start site (TSS) (Figure 4.2-3A). Since Hho1 is not known to bind to non-nucleosomal DNA in chromatin, we calculated the enrichment of Hho1 per nucleosome, by normalizing the values for the Hho1-ChIP to the H3-ChIP, and showed that there was a significant increase in the number of nucleosomes bound by the linker histone in the mutant strain (p_{CDC8}=0.0016 and p_{IPK1}=0.0019) (Figure 4.2-3B). Our results, therefore, suggest that the loss of Htz1 at the 5' end of these two genes results in more nucleosomes bound by Hho1, but fewer nucleosomes overall.

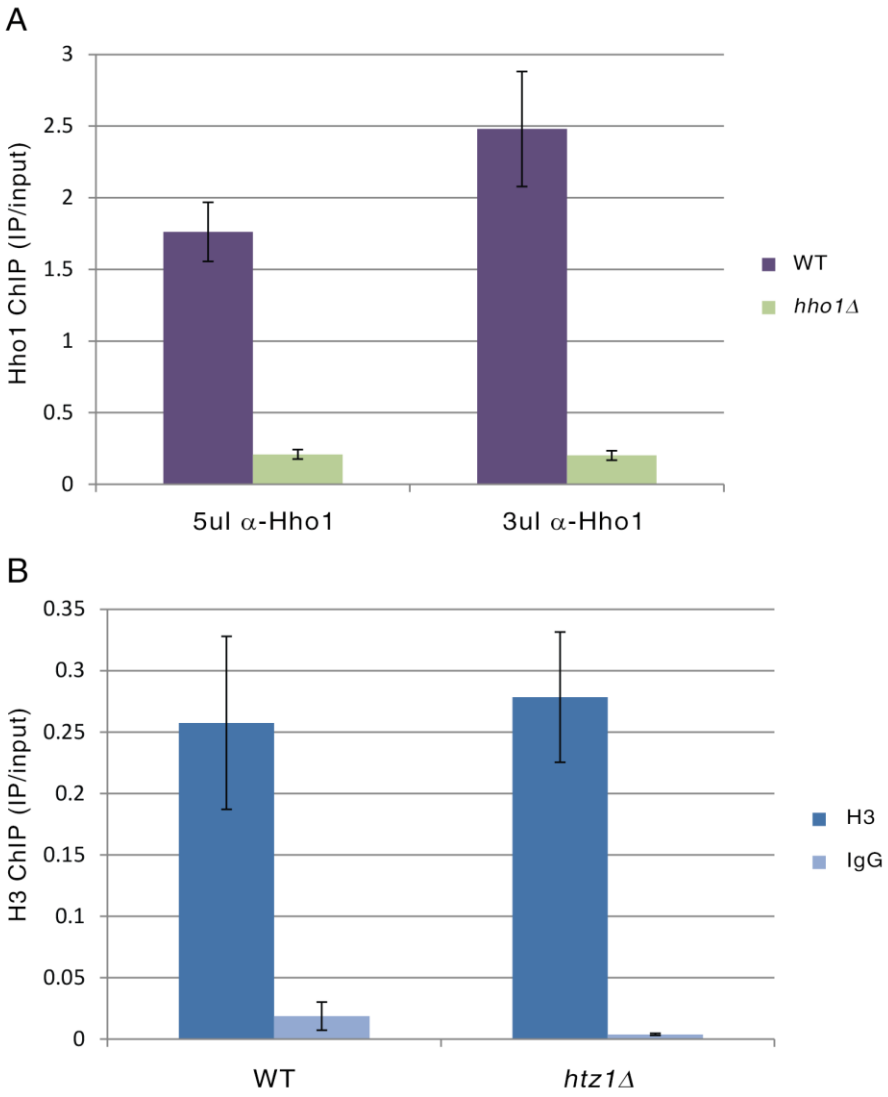


Figure 4.5. The α-Hho1 and α-H3 antibodies were suitable for use in ChIP. (A) Hho1 ChIP-qPCR at the *IPK1* locus using varying amounts of the α-Hho1 antibody in a WT (purple) or a *HHO1* knockout strain (green). (B) H3 ChIP-qPCR at the *COX10* locus for H3, using an equivalent amount of IgG as a control for the immunoprecipitation. Error bars for both (A) and (B) are the standard error of the mean.

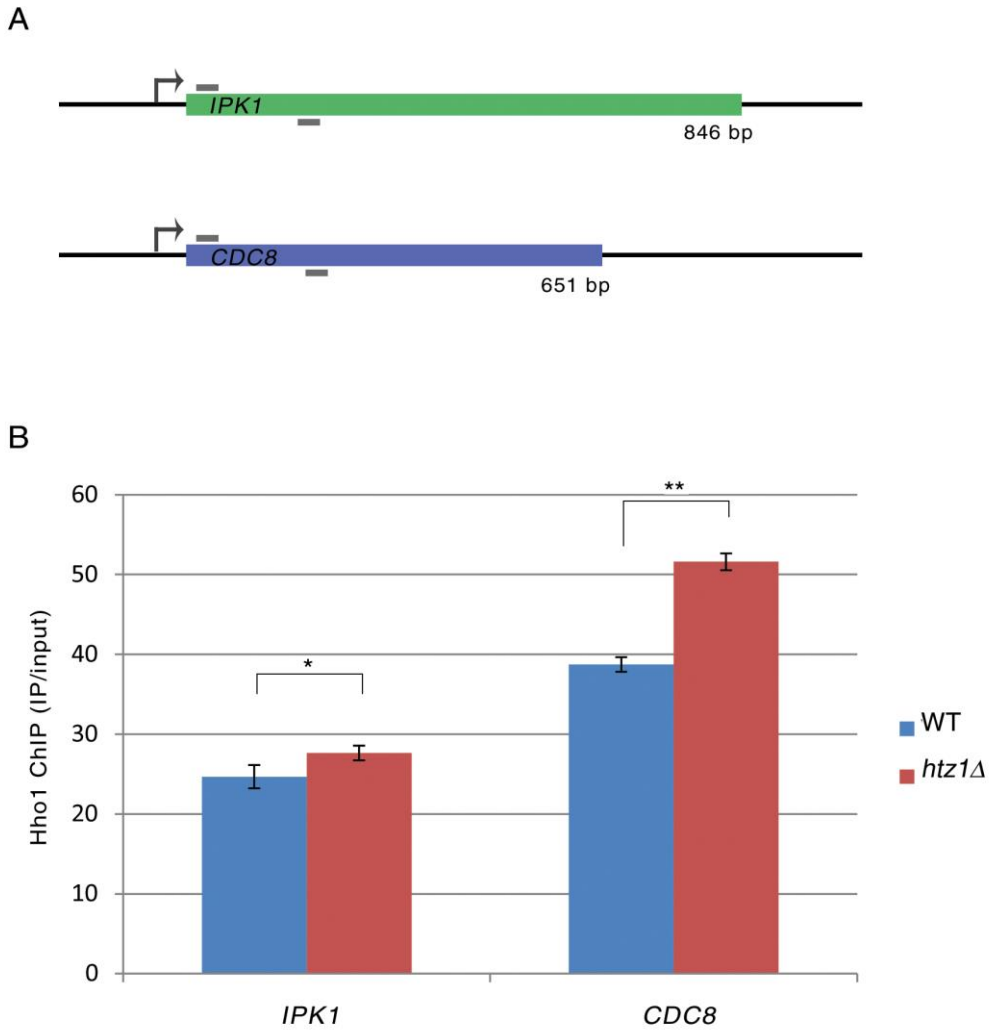


Figure 4.6. Hho1 binding is increased at the 5' end of genes in an *htz1Δ* strain. (A) Schematic of the primer locations on *IPK1* and *CDC8*. The genes are depicted by the labeled coloured bars, and the primers are represented as grey bars with the forward primer above the gene and the reverse primer below. (B) Hho1 ChIP-qPCR at the 5' region of the indicated genes in either a WT or an *htz1Δ* strain normalized to the input. The error bars represent the standard error of the mean, and p-values for WT compared with *htz1Δ* for *CDC8* and *IPK1* are p=0.06 and $p=3.09\times10^{-7}$, respectively.

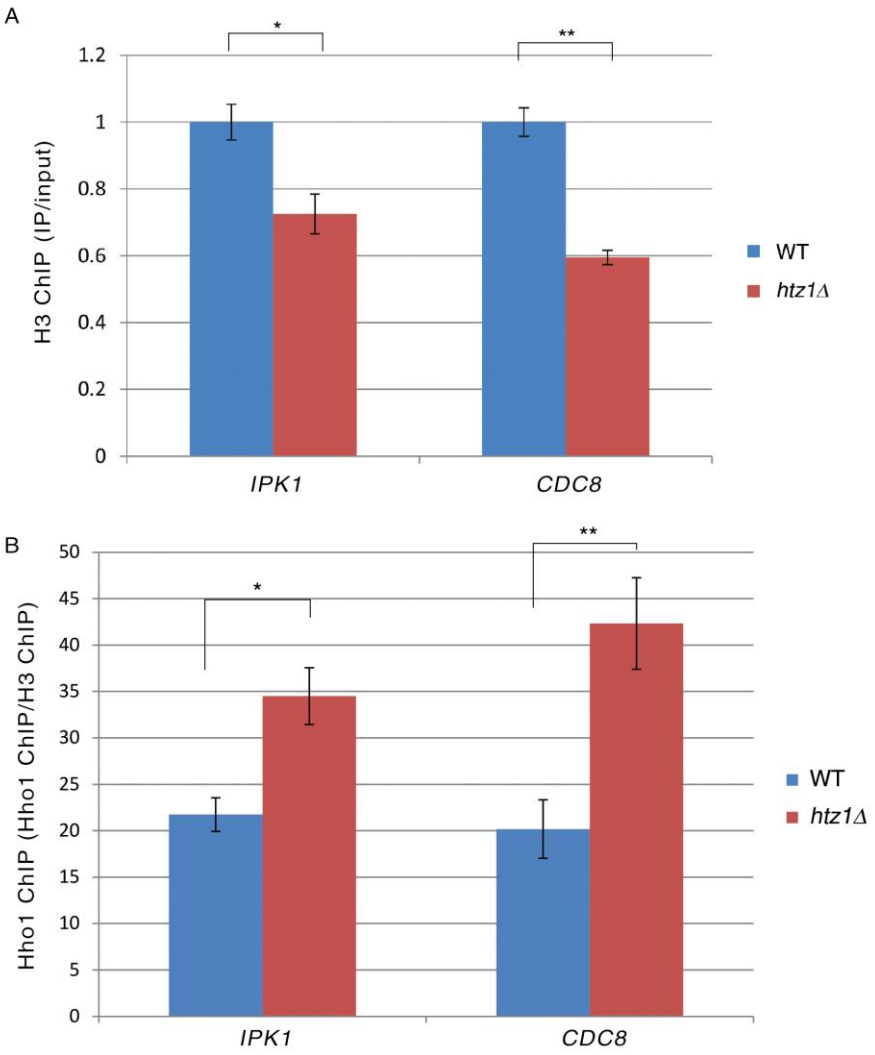


Figure 4.7. Loss of histone variant Htz1 results in increased Hho1 binding to nucleosomes at the 5' end of select genes. (A) H3 ChIP-qPCR for the indicated genes in either a WT or *htz1Δ* strain**.** Levels of H3 are shown relative to input, normalized to the WT amount at each locus. The error bars represent the standard error of the mean, and the p-values for a one-tailed t-test are *p=0.0008, and **p=5.01×10^{-5}. (B) Hho1 ChIP-qPCR at the 5' region of the indicated genes in either a WT or an *htz1Δ* strain normalized to the value from the H3-ChIPs at the same genes shown in Figure #R2.2-2. The error bars represent the standard error of the mean, and p-values for WT compared with *htz1Δ* for *IPK1* and *CDC8* are *p=0.0019 and **p=0.0016, respectively.

4.3. Hho1Does Not Protect Two Nucleosome-repeat Lengths of DNA, and is ~2-fold Enriched on Chromatin in an *htz1Δ* Strain

To test whether Hho1 enrichment in the absence of Htz1 was a global phenomenon, we performed ChIP-Seq with the α-Hho1 antibody in the wild type and *htz1Δ* strains. ChIP-Seq is a variation on the locus-specific ChIP-qPCR, in which all of the DNA bound to the protein of interest is sequenced using next generation sequencing technology. These sequenced fragments are then mapped to regions of the genome and a density map is generated that represents the number of times a specific base pair is identified during the sequencing. Using the final density map of aligned sequences, genomic regions can be identified that have a higher density of reads than they would by chance. These high density areas represent regions of protein binding to the genome (Furey, 2012).

Due to the PCR amplification during the library preparation for sequencing, the amount of DNA pulled down during the ChIP must be normalized between samples before the the sequenced reads can be assessed quantitatively. Many research groups use statistical tools, such as MAnorm, to allow them to compare binding enrichment across different ChIP-Seq samples (Shao et al., 2012). Since our lab performed both the preparation and analysis, to normalize the final sequenced data between the wild type and the *htz1Δ* strain, we decided to supplement both the ChIP and input DNA with two artificial fragments of DNA. In all of our analysis we made the assumption that these artificial fragments would be purified and amplified exactly like the input and ChIP DNA. These internal controls allowed us to normalize the amount of DNA across all of our samples and quantitate the difference in DNA bound to Hho1 between the wild type and *htz1Δ* mutant samples. After the libraries were prepared by PCR amplification, they were pair-end sequenced on the Illumina HiSeq 2000 platform at the UBC Biodiversity Research

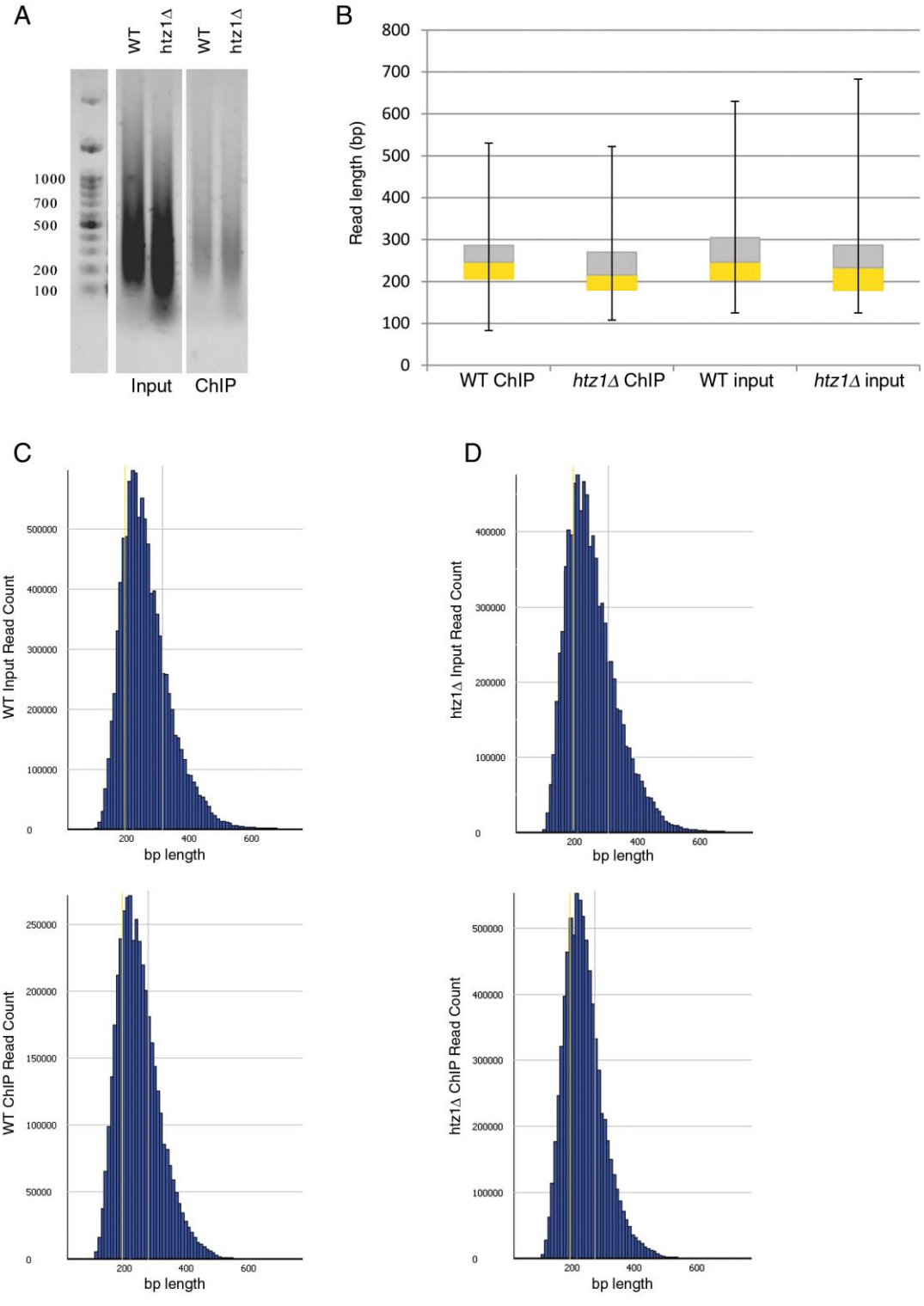
Center. These raw reads were mapped to the Saccer3 genome assembly (*Saccharomyces cerevisiae* S288c assembly from the Saccharomyces Genome Database (GCA_000126055.2)), and to our synthetic DNA genome assemblies (Table 4.3-1). Assuming the ideal case, in which samples that were processed identically produced equivalent total reads for the synthetic DNA, we were able to determine the relative amounts of DNA (by read count) in our original, pre-processed samples. Using this method, we calculated that there was 1.8 times the amount of DNA pulled down in the mutant strain, relative to the WT strain during the ChIP step, taking into account the difference in input DNA. This was consistent with our ChIP-qPCR results, which showed that there was 1.5-2 times the amount of linker histone bound to the 5' ends of *IPK1*and *CDC8* in the *htz1Δ* mutant.

Table 4.3 Total paired reads after sequencing. The raw number of total paired sequence reads generated from the Illumina HiSeq 2000, and the total number of reads that were mapped to the Saccer3 genome assembly and to our artificial DNA fragments. The ChIP reads were normalized to the artificial fragments.

	WT α-Hho1	*htz1 Δ* α-Hho1	WT Input	*htz1 Δ* Input
# Total Paired Reads	8510155	15612759	20471816	17036433
# Paired Reads Mapped to Saccer3	8494726	15599994	20471766	17036397
# Paired Reads Mapped to Normalized1	1720	1443	5	4
# Paired Reads Mapped to Normalized2	13709	11322	45	32
Mapped/Normalized1	4938.8	10810.8	4094353.2	4259099.3
Mapped/Normalized2	619.6	1377.8	454928.1	532387.4

When preparing our library samples we cut out a large band that represented DNA fragments sized between approximately 200-600 bp (including the library adaptors), so that we could determine whether Hho1 bound to one or two nucleosomes. Since our reads were paired, we could use the mapped reads to determine the length of all of the DNA fragments that were

sequenced, and we could graph the distribution of those reads (Figure 4.3-1). In both the input and immunoprecipitated DNA fragments the mid-point of the read-length histogram was at a fragment length of ~245 bp. For the ChIP samples the fragments in the second and third quartiles were between the lengths of 200 and 280 bp, whereas in the input samples the fragments in the second and third quartiles were between the lengths of 200 and 300 bp (Figure 4.3-1B and C). If Hho1 preferentially interacts with di-nucleosomes, we would expect the DNA in the Hho1-ChIPs to be enriched for DNA equivalent to two nucleosome repeat lengths, which in yeast would be ~334 bp. Fragments larger than 360 bp were 5% less represented in the ChIP fraction compared with the input. Therefore, our data supported the finding of Yu et al. that showed that Hho1 only bound to a single nucleosome (Yu et al., 2009).


A
WT
htz1Δ
WT
htz1Δ
1000
700
500
200
100
Input
ChIP
B
Read length (bp)
800
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600
500
400
300
200
100
0
WT ChIP
htz1Δ ChIP
WT input
htz1Δ input
C
WT Input Read Count
bp length
WT ChIP Read Count
bp length
D
htz1Δ Input Read Count
bp length
htz1Δ ChIP Read Count
bp length

Figure 4.8. Distribution of ChIP-Seq paired-end reads. (A) Agarose gel image of input and ChIP, uncrosslinked DNA from ChIP-Seq experiment, before library preparation. (B) Box plots depicting the number of mapped reads per fragment size from the ChIP-Seq mapped read dataset summarized in Table #R2.3-1. (C) WT and (D) *htz1Δ* histograms of (B). The visualization was created using the program SeqMonk. The yellow bar delineates the start first quartile and the grey bar delineates the end of the third quartile.

4.4 The Linker Histone Binds to Nucleosomes and is Minorly Enriched at Genes with a Low Transcription Rate.

Differing chromatin states of DNA in the genome is a factor that can bias fragmentation by sonication. A previous study, looking at DNA fragmentation at the *HMR* locus in either a WT or *sir1Δsir2Δ* strain showed that there was consistently less shearing in the WT strain (Ozaydin and Rine, 2010). Further, sequencing of input DNA showed that heterochromatic regions, such as telomeres, are subject to systemic under-representation in ChIP-Seq experiments due to size selection and sequencing bias (Teytelman et al., 2009). Since deletion of *HTZ1* may have resulted in different chromatin structure, we wanted to check to make sure that this deletion did not bias the shearing of DNA in our ChIP-Seq samples. Analysis of the read coverage around annotated genes (-1000 to +1000 bp around the start codon) for both mapped input samples showed that the fragmentation of the two samples had equal genomic coverage, as their enrichment at any loci is highly correlated: R=0.987 (Figure 4.4-1A) Therefore, we could be reasonably certain that both samples were comparable to each other, despite the slight differences in the fragmentation of the wild type and mutant samples.

The nucleosome free region or nucleosome depleted region (NFR/NDR), which is found between the -1 and +1 positioned nucleosomes in yeast, sits just before the transcriptional start

site (TSS) of a gene. The NFR, as its name implies, is a region that is generally nucleosome depleted across all corresponding regions in the yeast genome, and is on average ~200 bp upstream of the start codon (Lee et al., 2007). We mapped the midpoints of all reads to the genome and plotted the normalized cumulative read count relative to the start codon of all of the yeast genes. In our input samples, we had an enriched read coverage slightly upstream of the +1 nucleosome, over the -1 nucleosome and over the NFR compared to either up- or downstream regions (Figure 4.4-1B). The DNA in this region, at the majority of genes, is also highly sensitive to cleavage by the enzyme DNase1, which indicates that the DNA is unprotected by bound proteins (Hesselberth et al., 2009). These regions were highly represented in our input DNA presumably because of the increased sonication shearing that occurs at unprotected DNA, and our size selection of DNA fragments between 100 and 400 bp for sequencing. The asymmetry of the input fragment abundance over the +1 nucleosome compared to the -1 nucleosome, suggested that the +1 nucleosome was more abundant and better positioned, which led to less fragmentation.

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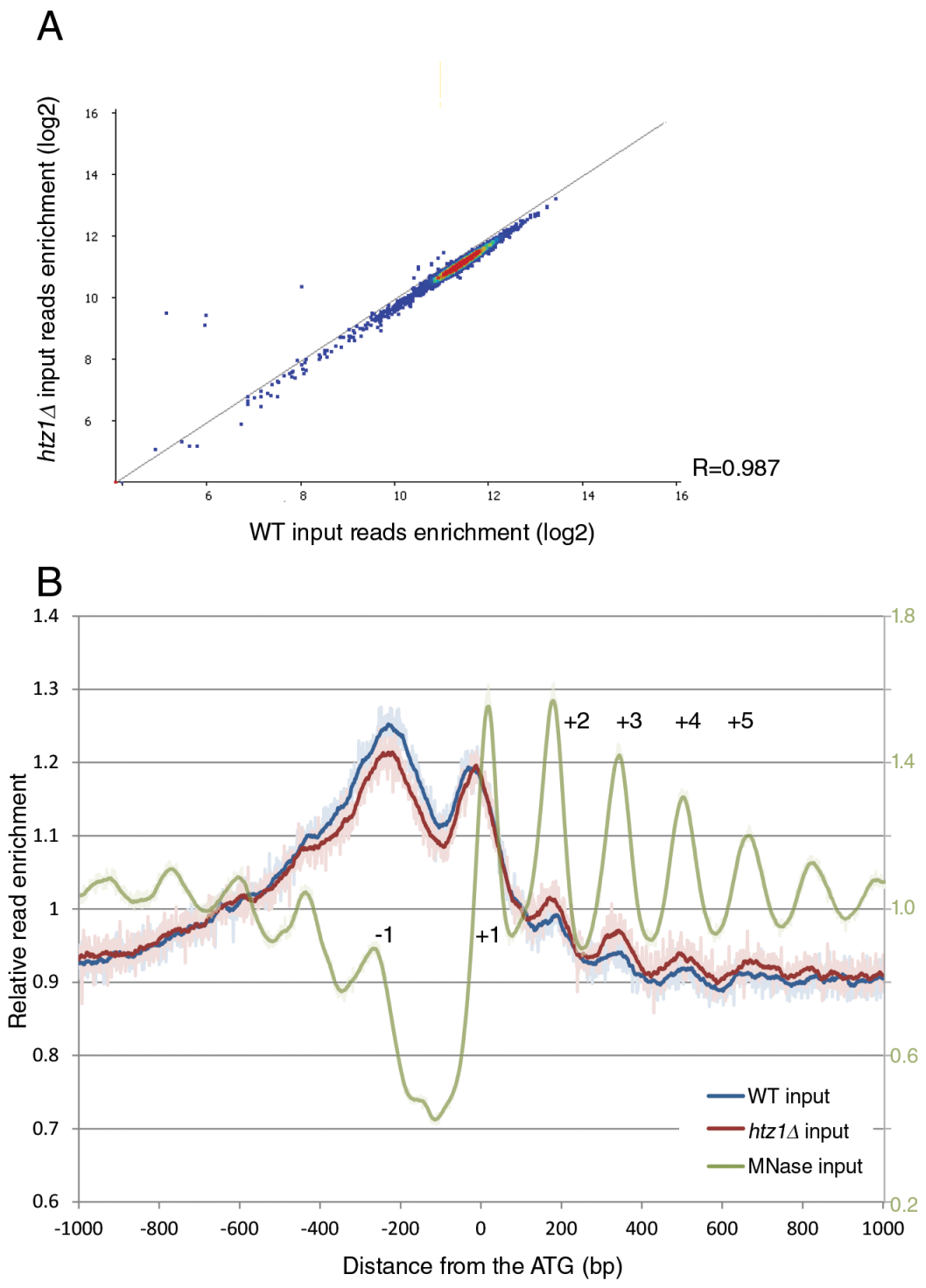
A
htz1Δ input reads enrichment (log2)
WT input reads enrichment (log2)
R=0.987
B
Relative read enrichment
Distance from the ATG (bp)
-1
+1
+2
+3
+4
+5
WT input
htz1Δ input
MNase input

Figure 4.9. The read enrichment of the input samples from both the WT and *htz1Δ* mutant were highly correlated and had increased read coverage over the -1 nucleosome, the NFR and the +1 nucleosomes. (A) Scatter plot of the read count enrichment over all positions in the genome for both the WT and the *htz1Δ* mutant ChIP-Seq input samples, created using the program SeqMonk. The Pearson's correlation coefficient was R=0.987. (B) Midpoints of sequenced DNA reads from the WT and *htz1Δ* input samples for the Hho1-ChIPs. Both sets of reads were normalized to the average read count across the depicted region (1000 bp around the start codon). The dark blue and red lines represent the smoothed data, and the light blue and pink shadows represent the raw data. Midpoints of sequenced MNase input reads from a previous ChIP-Seq experiment (Maltby et al., 2012), in which MNase was used to digest the chromatin down into mono-nucleosome sized fragments. The positions of the positioned nucleosomes are indicated on the graphs.

After sonication of our input samples, α-Hho1 antibodies were added to both the WT and the *htz1Δ* mutant whole cell extracts (WCE) to immunoprecipitate chromatin associated with the linker histone. As mentioned above, analysis of the sequenced ChIP read coverage confirmed that there was 1.8 times the amount of DNA pulled-down with the linker histone in the *htz1Δ* mutant. We were interested in determining whether the pattern of Hho1 binding in both the wild type and *htz1Δ* strains show a different binding pattern from a previous study which used a 3xFLAG-tagged Hho1 protein (Bryant et al., 2012). However, this is not completely unexpected since there were a number of differences between the Bryant et al. ChIP-Seq conditions and ours. Particularly notable differences are the 3xFLAG-tagged Hho1 construct, which could protect a larger DNA fragment than the native linker histone during the formaldehyde crosslinking step, and use of diploid yeast. Since both datasets were analysed using the midpoints of the sequence fragment, a difference in the average DNA fragment length would create a different binding pattern.

When the midpoints of our ChIP-Seq reads were graphed across an average gene for both the WT and *htz1Δ* mutant samples, it was clear that Hho1 was enriched over nucleosomal DNA (Figure 4.4-2A). This suggested that the linker histone bound to the DNA at the entrance and exit of the nucleosome, and protected a fairly symmetrical amount of DNA around the nucleosome. In both the WT and *htz1Δ* mutant ChIPs there was a clear enrichment over the +1 and +2 nucleosomes, and a strong depletion before the +1 nucleosome in the NFR (Figure 4.4-2). These profiles were similar, but not identical to the data from the previous study, for the reasons mentioned above (Bryant et al., 2012). However, our data confirmed that Hho1 was bound to nucleosomes *in vivo*, and showed that all nucleosomes, within a population, had the ability to bind to linker histones.

The deletion of *HTZ1* clearly affected the uniformity of binding of Hho1 to chromatin. The *htz1Δ* mutant ChIP average gene profile showed greater occupancy of the linker histone over the ORF, a stronger anti-correlation between Hho1 binding and transcription rate, and a more regular binding pattern regardless of transcription rate (Figure 4.4-2, compare B and C). Nuclesomes are restricted to certain positions on the genome by a number of barriers: DNA sequence, adjacent proteins, and chromatin remodeler activity. The well positioned +1 nucleosome, which is enriched for Htz1, is the barrier against which all of the other nucleosomes across a gene are positioned, and the rest are all positioned according to statistical packing principles (Kornberg, 1981; Mavrich et al., 2008). As the nucleosomes get farther away from the +1 nucleosome barrier they are subject to positional decay, whereby their position becomes increasingly uncertain (Kornberg and Stryer, 1988). This suggested that the loss of Htz1, at the +1 nucleosome, may have affected the positioning of the nucleosomes in the promoters and ORFs of genes.

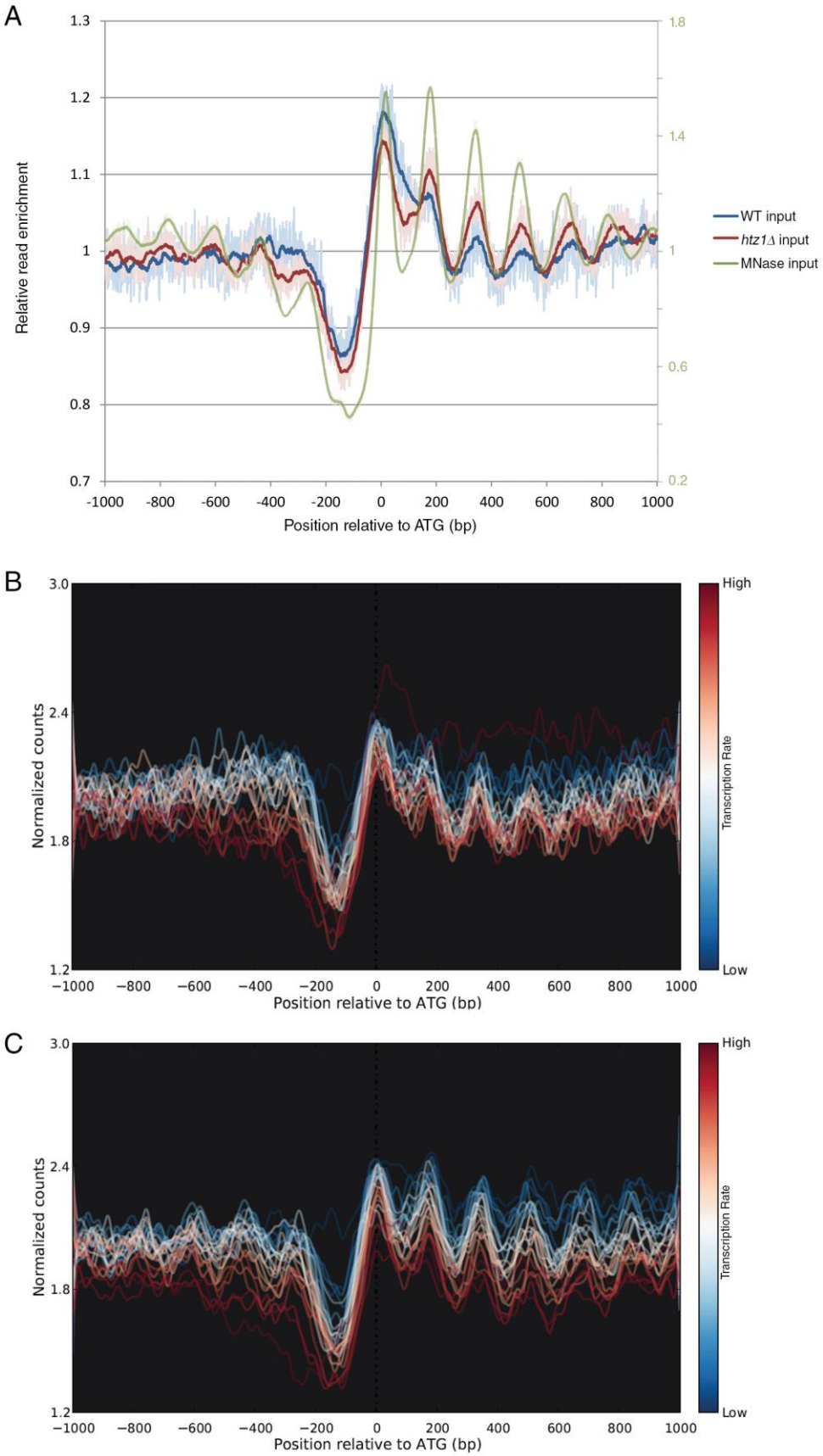
A
Relative read enrichment
1.3
1.2
1.1
1
0.9
0.8
0.7
1.8
1.4
1
0.6
0.2
-1000 -800 -600 -400 -200 0 200 400 600 800 1000
Position relative to ATG (bp)
WT input
htz1Δ input
MNase input
B
Normalized counts
3.0
2.4
1.8
1.2
−1000 −800 −600 −400 −200 0 200 400 600 800 1000
Position relative to ATG (bp)
High
Transcription Rate
Low
C
Normalized counts
3.0
2.4
1.8
1.2
−1000 −800 −600 −400 −200 0 200 400 600 800 1000
Position relative to ATG (bp)
High
Transcription Rate
Low

Figure 4.10. Hho1 binds to nucleosomes at the linker DNA and is absent from the 5'NFR. (A) Sequenced read midpoints from the Hho1 ChIP from either the WT or *htz1Δ* mutant and sequenced input reads from a previously analysed, MNase-digested sample (Maltby et al., 2012). The reads were normalized to the average read count over all genes. The dark blue and red lines represent the smoothed data, and the light blue and pink shadows represent the raw data. (B) Reads from the Hho1 ChIP-Seq depicted in (A) grouped into bins of 50 genes, by transcription rate using the data from Miller et al., 2011, and depicted using the program ChIP-Py (programmed by Karimi, M., unpublished). (C) Reads from the *htz1Δ* Hho1 ChIP-Seq depected in (A) grouped into bins of 50 genes, by transcription rate using the data from Miller et al., 2011, and depicted using the program ChIP-Py

4.5 Hho1 Binding is Enriched at the Promoters of Genes that Would Normally be Associated with Htz1 Nucleosomes, in the *htz1Δ* Mutant Strain.

The presence of Htz1 is a common feature of the +1 nucleosome (Albert et al., 2007; Maverich et al., 2008; Zilbermann et al., 2008). Considering this, the result in Figure 4.4-2 did not support our hypothesis, that Htz1 negatively regulates Hho1 binding, because loss of Htz1 actually resulted in a net decrease in Hho1 at the +1 nucleosomes genome-wide. However, not all genes have Htz1 in the +1 nucleosome. To examine the effects of Htz1 loss on Hho1 binding at only those nucleosomes that contained Htz1, we aligned the sequenced reads of a Htz1 ChIP-Seq data set (Watanabe et al., 2013) by their TATA-elements and clustered all yeast genes into four bins based on the pattern of Htz1 occupancy (Figure 4.5-2D). We then examined Hho1 occupancy in WT and htz1 strains for each cluster. Data from others in our lab showed that

MNase digested nucleosomes showed a more consistent pattern when the midpoints of the sequenced reads were plotted relative to the TATA and TATA-like elements, compared to either the start codon or the transcriptional start site (data not shown). Therefore, for the rest of our analysis, we chose to use the TATA and TATA-like elements as the genomic reference feature for our gene plots.

Separating the normalized reads into the four clusters of genes showed that Hho1 binding in the WT strain was fairly consistent between the clusters at the +1 nucleosome, despite the difference in nucleosome occupancy (Figure 4.5-1A and C). In an *htz1Δ* mutant, however, differences in the profile of Hho1 occupancy more closely resemble that of nucleosome occupancy (compare figures 4.5-2C and B) suggesting that the presence of Htz1 altered the ability of nucleosomes to bind Hho1.

Observation of the differences in binding pattern at the +1 and -1 nucleosomes in Cluster #1 versus Cluster #3 suggested that changes in nucleosome occupancy were a function of the absence of Htz1. In Cluster #1 and Cluster #3 there are Htz1-containing nucleosomes in the wild type strain at the +1 nucleosomes, and in both of these clusters there was an increase in Hho1 binding in the *htz1Δ* strain (Figure 4.5-1B and D). The -1 nucleosome in Cluster #3, however, was not an Htz1-containing nucleosome and there is no increase in Hho1 binding at that nucleosome in the mutant strain (Figure 4.5-1B and D). Importantly, both Cluster #1 and Cluster #3 showed equivalent binding of Hho1 in the WT Hho1-ChIP at the +1 and -1 nucleosomes (Figure 4.5-1A). This clear enrichment of linker histone binding only to +1 or -1 nucleosomes that in the wild type strain contained Htz1, provided evidence that the presence of Htz1 in a nucleosome directly regulates Hho1 binding. Therefore, this data suggests that Htz1 in a

nucleosome interferes with Hho1 binding, *in vivo*, which supports the *in vitro* data which showed the same, using human nucleosomes (Thakar et al., 2009).

Surprisingly, loss of Htz1 affected linker histone binding to nucleosomes downstream of the +1 nucleosome more than binding to the +1 nucleosome itself. Comparison of the normalized sequence reads from the WT Hho1-ChIP and the *htz1Δ* Hho1-ChIP for Clusters #1-3 showed that there was more Hho1 bound to downstream nucleosomes in the mutant (Figure 4.5-2 A-C). Cluster 4, which has little promoter Htz1 in the WT, showed that there the same or fewer Hho1 bound nucleosomes in the mutant (Figure 4.5-2D). This suggested that loss of Htz1 from the +1 nucleosome did not just cause an increase in binding of the linker histone at the Htz1-nucleosome in the *htz1Δ* mutant strain. As mentioned above, this could be due to a decrease in the positional decay for the downstream nucleosomes on the gene, which could allow more Hho1 to bind to those nucleosomes.

Deletion of *HTZ1* results in a change of expression in ~600 genes with a confidence of p=0.001, with approximately half being down-regulated (Morillo-Huesca et al., 2010; Meneghini et al., 2003). To confirm that changes in transcription rate did not affect linker histone binding, we analyzed the binding of Hho1 at the *htz1Δ* mutant up- and down-regulated genes. We saw that genes with transcription defects in an *HTZ1* mutant showed similar increases in Hho1 occupancy as genes that showed no change in expression (Figure 4.5-3 compare A and B). In contrast, genes that were upregulated in an *HTZ1* mutant showed similar levels as Hho1 as wild type. This likely reflects the fact that the increased transcription counteracted the effects of loss of Htz1 on Hho1 binding (Figure 4.5-3C). These data suggested, therefore, that the increase in Hho1 binding between the WT and *htz1Δ* ChIPs was not caused by changes in gene activity.

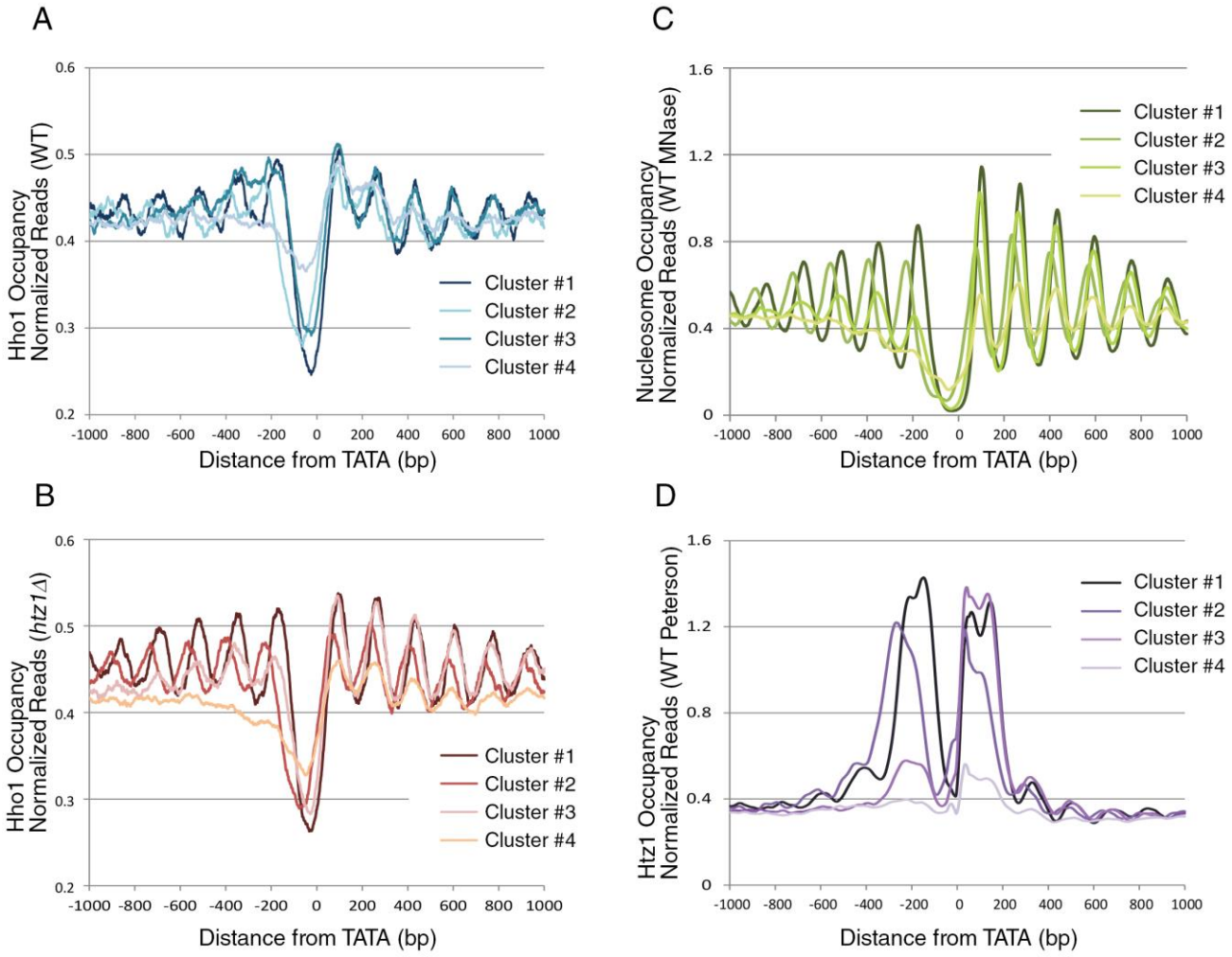

Figure 4.11. Hho1 is enriched in an *htz1Δ* mutant at nucleosomes that had Htz1 in the WT. (A) Sequenced reads from a WT Hho1-ChIP, subsetted by Htz1 Cluster, and normalized by total read coverage and gene count per Cluster. Aligned to the TATA-elements. (B) Sequenced reads from the *htz1Δ* Hho1-ChIP and normalized and graphed same as (A). (C) Sequenced reads from the WT MNase input normalized and graphed same as (A). (D) Sequenced reads from the WT Htz1-ChIP normalized and graphed same as (A).

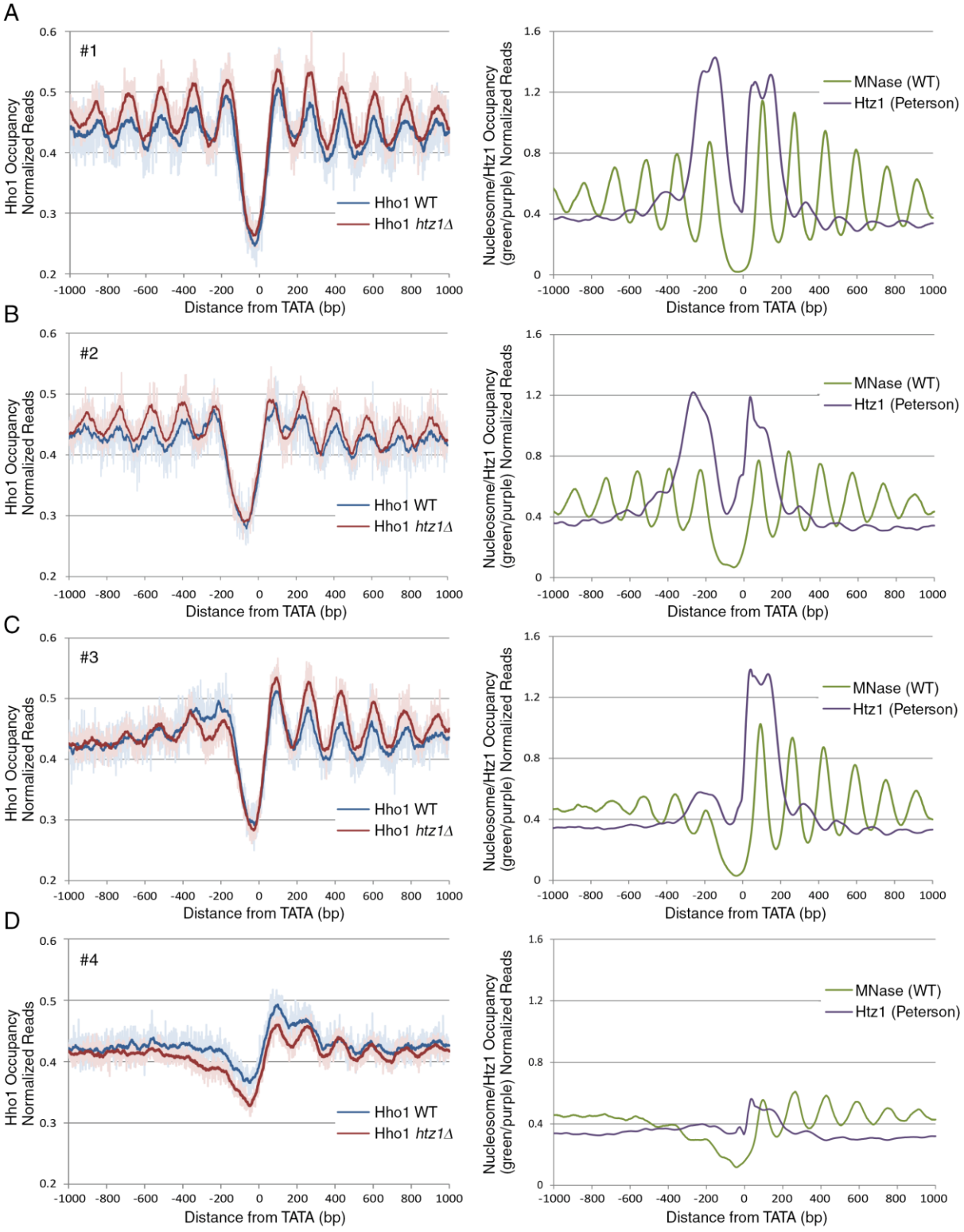
A
#1
Hho1 Occupancy Normalized Reads
Hho1 WT
Hho1 htz1Δ
Distance from TATA (bp)
Nucleosome/Htz1 Occupancy (green/purple) Normalized Reads
MNase (WT)
Htz1 (Peterson)
B
#2
C
#3
D
#4

Figure 4.12. Hho1 binding is increased downstream of the +1 nucleosome, in the *htz1Δ* mutant, at genes that had Htz1 in the WT. Sequenced reads from the WT Hho1-ChIP, *htz1Δ* Hho1-ChIP, WT MNase input, and WT Htz1-ChIP were normalized to the total read coverage, and to each other. Reads were aligned to the TATA-elements. The dark blue and red lines represent the smoothed data, and the light blue and pink shadows represent the raw data. (A) Htz1 Cluster #1. (B) Htz1 Cluster #2. (C) Htz1 Cluster #3. (D) Htz1 Cluster #4.

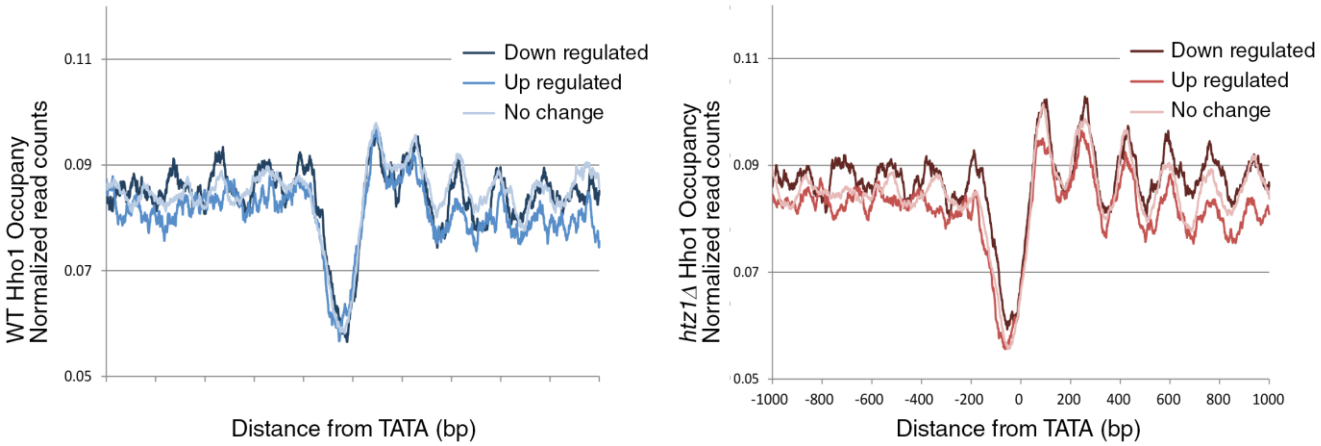

Figure 4.13. Changes in Hho1 occupancy in an *htz1Δ* mutant cannot be explained by altered gene activity. Sequenced reads from the WT and *htz1Δ* ChIPs at genes that were up-regulated, down-regulated, or unchanged upon deletion of *HTZ1* (expression data from Morillo-Huesca et al., 2010), normalized to the total read count and aligned to the TATA-element.

4.6 Discussion and Conclusions

The cell has evolved numerous processes to mobilize the linker histone away from the +1 nucleosome. One pathway to linker histone displacement is through the phosphorylation of H1 by the kinase Cdk2/CyclinA, which happens upon hormone-dependent transcriptional activation (Vicent et al., 2011; Bhattacharjee et al., 2001; Koop et al., 2003; Lever et al., 2000). Another is through the acetylation of histones, which has been implicated in increasing the mobility of

linker histones (Misteli et al., 2000). The extent to which these two mechanisms contribute to displacing H1 from the +1 nucleosome at any given promoter is unknown.

In this chapter, I presented evidence that Htz1 incorporation is a third mechanism for mobilizing the linker histone away from the +1 nucleosome. Using ChIP-Seq, I showed that in the *htz1Δ* strain there was an increase in Hho1 binding to +1 and -1 nucleosomes that in the wild type strain contained Htz1. Further, I showed that this enrichment was not caused by a change in transcription. This data recapitulates previous *in vitro* finding that showed chicken erythrocyte linker histones were less able to bind to purified H2A.Z-containing nucleosomes than to purified canonical nucleosomes (Thakar et al., 2009). Further, these results are congruent with our *HHO1*-OE screen data, which showed that loss of *HTZ1* or genes coding for proteins involved in Htz1 deposition in combination of *HHO1*-OE exacerbated the sick phenotype seen in the *HHO1*-OE wild type strain.

The hypothesis that Htz1 is important for preventing Hho1 binding to the +1 nucleosome to maintain proper access to chromatin fits with data that shows that the histone variant Htz1 is important for normal induction kinetics of the *GAL*-genes (Halley et al., 2010). *In vitro* studies looking into the unwrapping dynamics of nucleosomes shows that increasing the number of histone:DNA contacts that must be broken to expose a DNA sequence decreases the frequency of spontaneous DNA sequence exposure by minutes (Tims et al., 2011). This suggests that increased binding of the linker histone to nucleosomes, particularly promoter nucleosomes, could decrease the access of DNA-binding activator proteins to nucleosome-occluded DNA sequences.

Htz1's role in preventing Hho1 binding to the +1 nucleosome, and subsequently to the downstream nucleosomes can also help describe chromatin compaction in sporulation. Western blot and ChIP-Seq data, show that the amount of Hho1 bound to chromatin 12 hours into

sporulation is approximately five times greater than during exponential growth (Bryant et al., 2012). Although the protein levels of Htz1 during meiosis are not known, analysis of the expression of *HTZ1* during meiosis show the level of *HTZ1* expression decreases upon the start of sporulation, reaching the lowest levels at hour 15 (Klutstein et al., 2010). In contrast, expression of *HHO1* gradually increases throughout sporulation (Klustein et al., 2010). The rate of Htz1 removal from nucleosomes during this time period is unclear; however, this suggests that the loss of Htz1 from meiotic chromatin may be an important step to allow linker histone binding to chromatin.

At an averaged gene, the increase in linker histone binding at the -1/+1 nucleosomes and the downstream nucleosomes is subtle. However, given the low Hho1 to nucleosome ratio in a yeast cell, reported either as 1:4 or 1:37, this enrichment is striking (Freidkin and Katcoff, 2001; Downs et al., 2003). A question that is raised by the increase of linker histone binding in the *htz1Δ* mutant strain is how the cell chaperones and manages the nuclear pool of Hho1 in the wild type strain. We showed by quantitative western blot that the two strains had similar total cellular levels of endogenous Hho1 (Figure 4.1-4, compare pvector lanes). Combined with our ChIP-Seq data, this means that at any given time in a wild type strain at least half of the total available Hho1 is not associated with chromatin. Therefore, in a wild type strain there has to be a mechanism that manages free nuclear linker histones. The specific pathways for Hho1 deposition and displacement remain a topic for further study.

Chapter 5. Human-yeast Hybrid H3 Histones Rescue the Loss of Endogenous Yeast H3

In human cells, most of the DNA is associated with nucleosomes containing two copies of either H3.1, H3.2, or H3.3. Human H3.1 and H3.2 are extremely similar. They differ in only one amino acid, and they are both expressed during S-phase and are incorporated into chromatin in a replication-dependent manner (Kaufman et al., 1995). The variant H3.3, in contrast, is constitutively expressed throughout the cell cycle, is primarily incorporated into transcriptionally active regions in chromatin, and is deposited into chromatin by the variant-specific histone chaperones HIRA or DAXX/ARTX (Tagami et al., 2004; Goldberg et al., 2010).

H3.3 is found enriched near the TSS of transcriptionally active chromatin, and in the gene body where it shows an increasing gradient of abundance increasing from 5' to 3' over the transcribed region of the gene (Ahmad and Henikoff, 2002; Goldberg et al., 2010, Tamura et al., 2009, Jin et al., 2011). Additionally, nucleosomes containing H3.3 have been shown to be less stable than canonical nucleosomes (Jin and Felsenfeld, 2007). These data, have led to the suggestion that H3.3 is important for creating open nucleosome structures that contribute to the transcriptionally permissive chromatin structure. However, studies done in *Drosophila* have shown that animals with only one of dH3.3 or dH3.2 were normal, excepting defects in gametogenesis (Hödl and Basler 2006; Hödl and Basler 2012). This suggested that the differences between H3.3 and H3.1 are not relevant to the histones' function in nucleosomes during transcription or replication.

5.1 Human Histone H3 Variants do Not Substitute for Yeast H3

Human H3.1 and H3.2 are extremely similar, differing in only one amino acid (Figure 5.1-1A); they are both expressed during S-phase and are incorporated into chromatin in a replication-dependent manner (Kaufman et al., 1995). In HeLa cells, hH3.1 is the most abundant of the three proteins, and is 3.5 times and 7 times more abundant than hH3.2 and hH3.3, respectively (Tachiwana et al., 2011). hH3.3 is constitutively expressed throughout the cell cycle, and is primarily incorporated into transcriptionally active regions in chromatin by the variant-specific histone chaperone HIRA. hH3.3 differs from hH3.1 by 5 amino acids, mainly in the globular domain (Figure 5.1-1B) (Tagami et al., 2004; Ahmad and Henikoff 2002). Human H3.3 has traditionally been considered to be yeast-like because of the sequence conservation at the phosphorylation site, H3S31, and in the globular domain, at H3I89 and H3G90 and its association with euchromatin (Malik and Henikoff, 2003; Postberg et al., 2010).

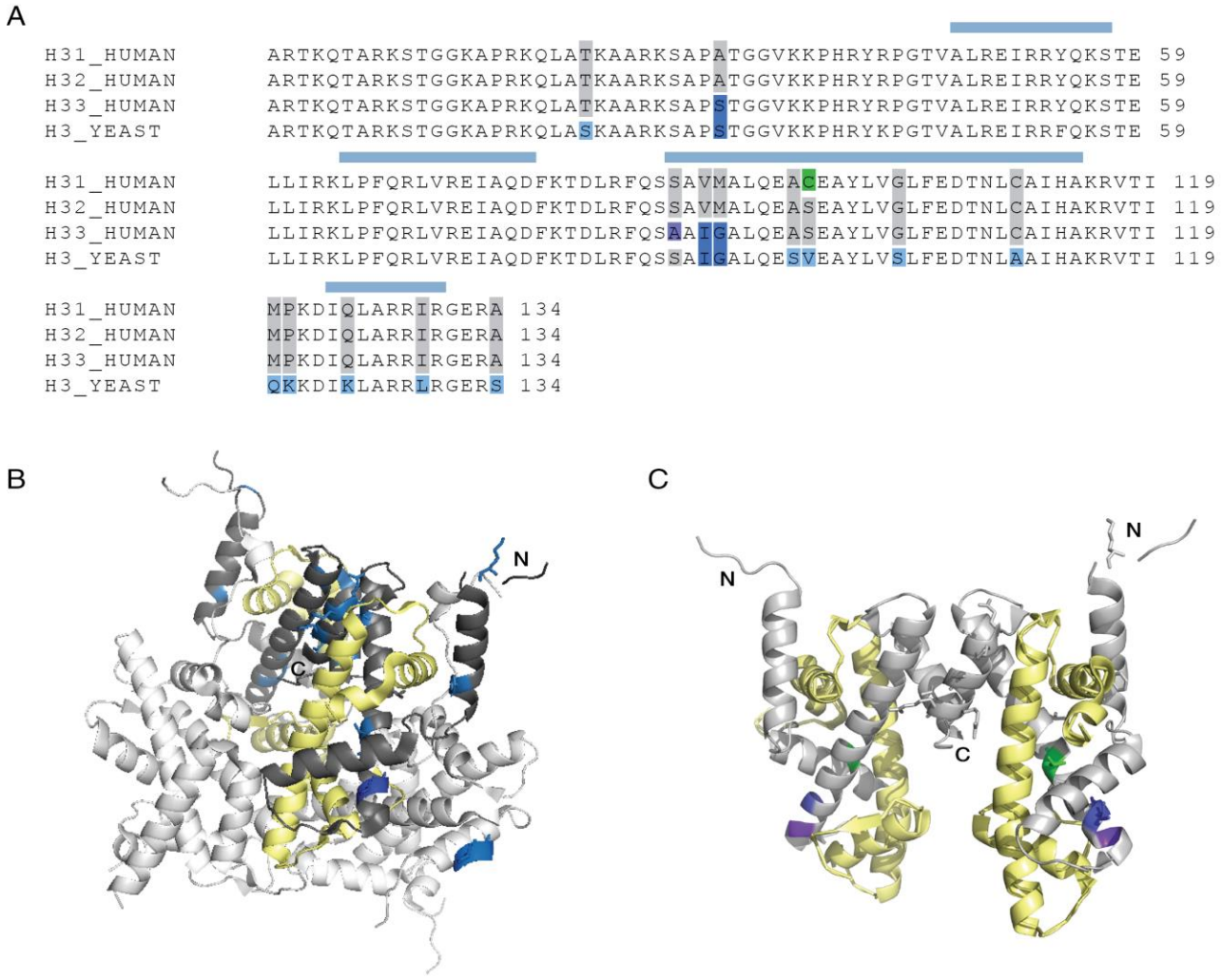

Figure 5.1. The amino acid sequence of the yeast histone H3 is similar to the three non-centromeric major human H3 variants. (A) Amino acid sequence of yeast and human H3s. The green highlighted cysteine is the only difference between histones H3.1 and H3.2. The dark blue highlighted amino acids are the similarities between yeast H3 and human H3.3. The light blue highlighted amino acids are the differences between yeast H3 and the human H3 variants. The purple highlighted amino acid is the amino acid variation in H3.3 that is not shared with the other histones. The blue bars above the sequence represent the alpha helices in the crystal structure. (B) Pymol structure of the histone ocatmer based upon the crystal structure from yeast (Protein Database ID: 1ID3; White et al., 2001). The dark grey represents the two copies of H3, with the differences in sequence between the yeast histone and human histones coloured

according to (A). Histone H4s are represented in yellow. (C) Pymol structure of the (H3:H4)2 tetramer, with the H3s coloured grey and the H4s coloured yellow. Amino acids different between the human variants H3.1 and H3.3 are coloured as in (A).

Previous studies have shown that hH3.3 is found enriched near the TSS within genes of transcriptionally active chromatin. It is also enriched in the gene body where it shows an increasing gradient of abundance from 5' to 3' over the transcribed region of the gene (Ahmad and Henikoff, 2002; Goldberg et al., 2010, Tamura et al., 2009, Jin et al., 2011). As transcription proceeds, the octamers in the open reading frame of a gene are evicted by chromatin remodelers and the actively transcribing RNA Pol II, and the canonical histone octamers are replaced with the variant containing octamers (Kireeva et al., 2002; Lorch et al., 2006; Phelan et al., 2000; Bowman et al., 2003, Goldberg et al., 2010; Jin et al., 2011) At the TSS, H3.3 is rarely found in a nucleosome without the histone variant H2A.Z (Jin et al., 2011). *In vitro* salt-dependent dissociation assays show that together these two histones create a particularly unstable nucleosome (Jin and Felsenfeld, 2007). Nucleosomes containing H3.3 and canonical H2A are more stable than H3.3:H2A.Z nucleosomes, but less stable than a fully canonical nucleosome (Jin and Felsenfeld, 2007). This suggests that the amino acid differences between H3.3 and H3.1/2 are sufficient to alter the protein:protein or protein:DNA interactions of the octamer and may contribute to the chromatin structure of transcribed genes (Figure 5.1-1).

Since the yeast genome is considered to be, in general, more transcriptionally active than the human genome, we posited that human H3.3 could substitute for yeast H3. To test whether human H3 could substitute for yeast H3, we created yeast-codon optimized gene fragments for the variants hH3.1 and hH3.3, cloned them into a yeast expression vector under the control of the endogenous yeast H3 promoter and terminator, and introduced them into yeast. The yeast strain

that we used for all of our human H3 experiments, which I will refer to as wild type-shuffle (WT-S), was derived from our wild type S228C strain. Since both copies of yeast H3 and H4 are expressed off of divergent promoters, we deleted both copies of endogenous H3 and H4. Therefore, our strain carries a copy of *HHT2* and *HHF2* on a *URA3* plasmid to maintain viability (i.e. *hht2.hhf2::KANMX6 hht1.hhf1::HIS3*, p*HHT2.HHF2.URA3*). Due to the genotype of WT-S, all of our H3-plasmids also carry the gene *HHF2*, under the control of its endogenous promoter; however, for clarity this designation will be omitted from future plasmid nomenclature.

Despite being the "yeast-like" histone variant, hH3.3 did not rescue the loss of yeast H3; similarly, neither did hH3.1 (Figure 5.1-2A). Due to the high degree of similarity between yeast H3 and the human variants, we were surprised that neither histone fully or partially rescued loss of yeast H3, and we wanted to confirm that the lack of rescue was not because of expression problems with the human histones. Since our α-H3 antibody recognizes both human and yeast H3, we generated a variation of our WT-S strain that carried a truncated version of yH3 that lacked the N-terminal tail (yH3Δ3-29) in lieu of the *HHT2-URA3* plasmid. This allowed us to differentiate between yeast and human H3 because the truncated version of yH3 runs visibly further than the wild type by SDS-PAGE. Western blot analysis of whole cell extracts prepared from WT-S yeast with yH3Δ3-29 and either hH3.1 or hH3.3 showed that the human histones were not being expressed (Figure 5.1-2B). This suggested to us that one or more of the amino acid differences between human and yeast H3 was responsible for inhibiting human histone H3 expression or promoting protein degradation.

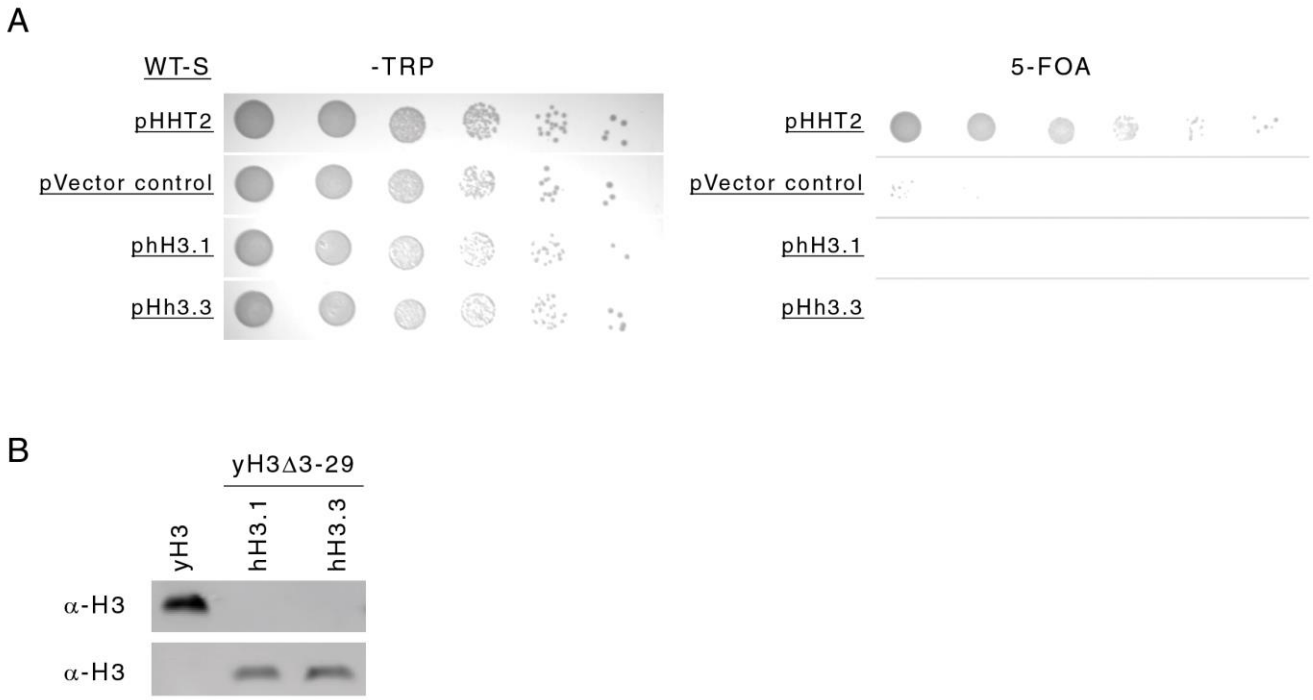

Figure 5.2. Neither human H3.1 nor H3.3 can substitute for yeast H3. (A) Ten-fold serial dilutions of the strain WT-S transformed with the indicated plasmid were plated on synthetic media lacking tryptophan (-TRP) or synthetic complete media supplemented with 5-FOA and grown for 2 days at 30˚C. (B) Western blot of yeast whole cell extracts prepared from the strain WT-S with either a plasmid copy of WT yH3, or plasmid copies of both the truncated yH3 and a human H3 variant.

5.2 The Yeast C-terminal Domain is Essential for Proper Histone H3 Function in Yeast Chromatin.

The differences between the yeast and human H3s occur throughout the entire protein. To determine which amino acids prevented the hH3s from substituting for yH3, we created hybrid yeast-human histones, where we divided the histone protein into three different domains and

substituted each of the three human domains for its analogous yeast H3 domain (Figure 5.2-1). Shuffle in the strain WT-S of plasmids that had N-terminal domains that were human instead of yeast, resulted in viable yeast that grew very similarly to yeast with yH3 (pH3.A and D-E) (Figure 5.2-2). This was not surprising since there is only one difference between yH3 and hH3.3 in the N-terminal region, and two between yH3 and hH3.1.

The shuffle of plasmids that had the middle domain of yeast histone H3 switched to the human sequence also grew similarly to the wild type (pH3.A and F-G) (Figure 5.2-1B). This result was surprising because the amino acids that distinguish H3.1 from H3.3 in the globular domain of the histone H3 protein have been shown to be important for binding to histone chaperones and deposition patterns in chromatin (Goldberg et al., 2010; Tagami et al., 2004). However, since yeast only have a single version of H3, it is possible that the yeast chaperones are not as specific in their ability to bind histone proteins as the human chaperones. Interestingly, changing the C-terminal domain (pH3.H/I) from yeast to human caused a severe growth defect (Figure 5.2-2B). Although this domain is conserved in most of the human H3 variants, it is significantly different from yeast H3. The crystal structure of the octamer shows that the main interactions in the C-terminal region of histone H3 are between the two copies of H3. Since the regions in the histone that interact with the differing amino acids are conserved between yeast and human version of H3 this suggested that the growth defect is not due to disruption of H3:H3 contacts, but may impact H3:H4 interactions (Luger et al., 1997).

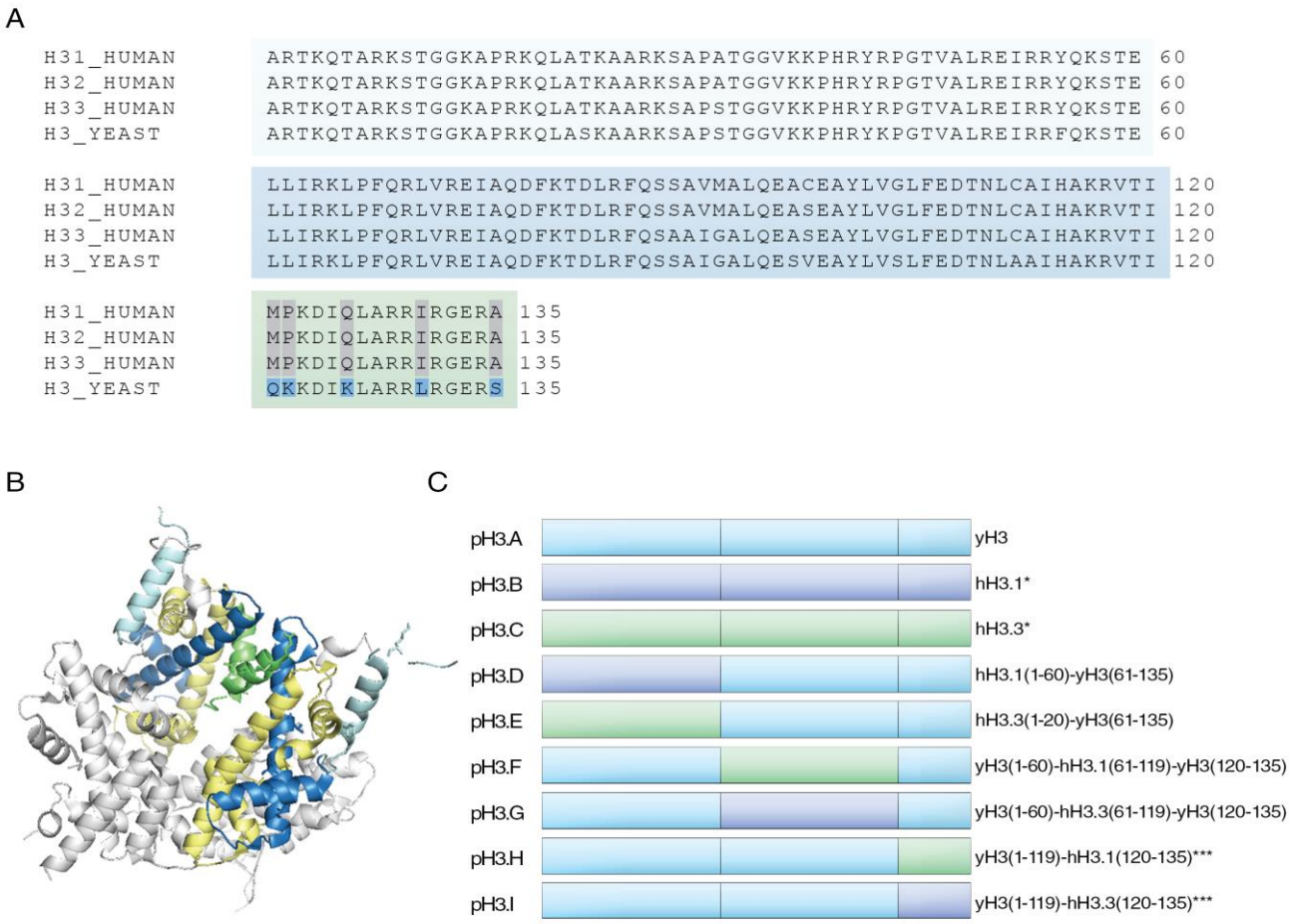

Figure 5.3. The three different domains chosen for the yeast-human hybrid histones. (A) Teal: N-terminal domain (amino acids 1-60). Blue: the middle-globular domain (amino acids 61-119). Green: the C-terminal domain (amino acids 120-135). The five amino acid differences between human and yeast H3 within the C-terminal domain are highlighted, and are the same between the human H3 variants. (B) Pymol structure of the octamer based upon the crystal structure from yeast (Protein Database ID: 1ID3; White et al., 2001) Both copies of histone H3 are coloured according to (A), with a significant portion of the N-terminal domain missing due to crystallization limitations. Histone H4s are represented in yellow. (C) Schematic of histone H3 hybrid proteins used in this study. The symbol * indicates hybrids that failed to rescue loss of yH3 and *** indicates hybrids that had a growth defect when present as the only source of H3. Note that pH3.H and pH3.I are identical in sequence, and will subsequently be referred to as pH3.H/I.

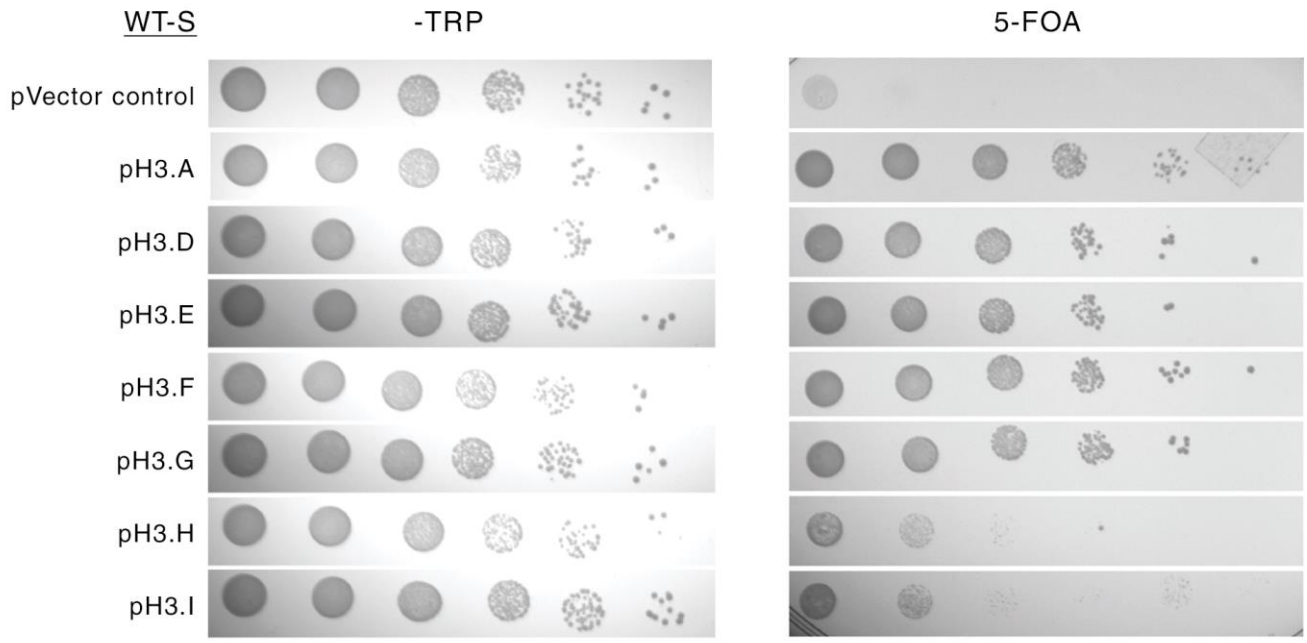

Figure 5.4. The C-terminal domain of yeast H3 is important for its normal function. Ten-fold serial dilutions of the strain WT-S transformed with the indicated plasmid were plated on synthetic medial lacking tryptophan (-TRP) or synthetic complete media supplemented with 5-FOA and grown for 2 days at 30˚C.

To determine whether this growth defect was due to an inability to express the necessary amount of H3, we grew cells from the 5-FOA media and prepared whole cell extracts from each of the hybrid-containing strains. The bulk amount of H3 in each strain was assayed by an α-H3 western blot, and we found that all six hybrid strains expressed an equal amount of histone H3 (Figure 5.2-3A). We then wanted to know if the changes in the C-terminal domain affected the incorporation of the hybrid pH3.H/I into chromatin. We performed a chromatin association assay, whereby we fractionated the contents of detergent-lysed yeast cells into two fractions: a chromatin pellet and a non-chromatin supernatant. Surprisingly, comparison of the chromatin

association of the hybrid histones to the wild type yeast control showed no apparent difference in the level of incorporation into chromatin; that is, all of the H3 histone was found in the chromatin pellet (Figure 5.2-3B). Thus, it appeared that the growth defect, resulting from the substitution of the C-terminal domain from yH3 to hH3, was due to an interaction independent of nucleosome assembly and chromatin deposition.

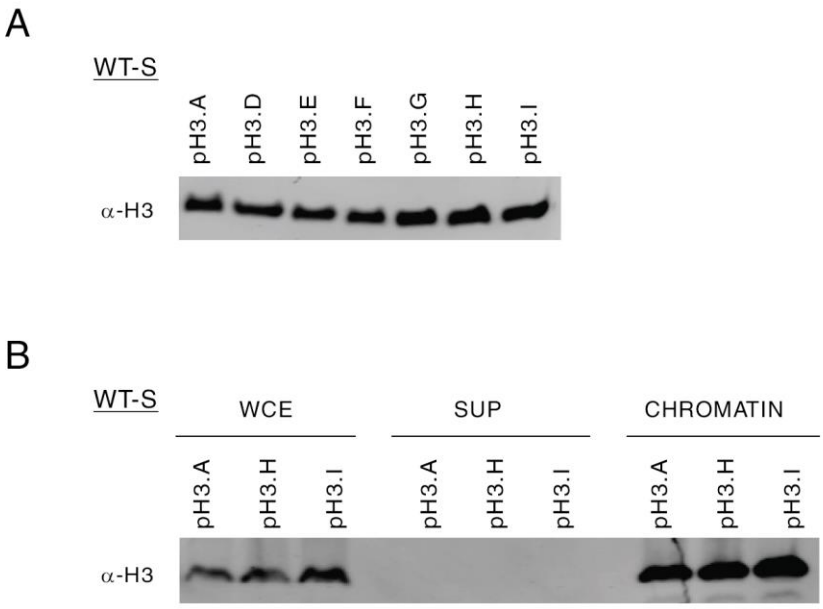

Figure 5.5. Human-yeast histone hybrids that are the sole copy of H3 in a cell are expressed in yeast and associated with the chromatin fraction. (A) An α-H3 western blot of yeast whole cell extracts prepared from WT-S with the indicated plasmid after growth on 5-FOA, and normalized by cell pellet weight. (B) Chromatin association assay of WT-S containing pH3.A, pH3.H, or pH3.I. The different lanes were normalized across the fractions by the original optical density of the yeast cultures. The SUP fraction is all of the supernatant not associated with the chromatin pellet, and the CHROMATIN fraction is the chromatin pellet that was resuspended in lysis buffer.

The C-terminal sequence of yeast H3 and the human H3s have five differing amino acids: H3Q120M, H3K121P, H3K125Q, H3L130I, and H3S134A (Figure 5.2-4B). This domain has been shown to be important for the interaction between the H3/H4 heterodimer and the histone chaperone Asf1, which is non-essential in (Figure 5.2-4C) yeast (Antczak et al., 2006; English et al., 2006; Agez et al., 2007, Lin et al., 2010). In yeast, Asf1 interacts with a number of residues including H3L130, which is H3I130 in both human variants. Mutation of H3L130 to an alanine has been shown to be lethal in two different yeast strains, S288C and GRF167, as has mutation of H3Q120 to a glutamic acid (Dai et al., 2008). In order to determine whether any of the yeast to human substitutions caused the slow growth phenotype, we created single point mutants in yeast H3 and tested their function as the sole copy of H3 in the cell (Figure 5.2-4). Interestingly, all of the single mutants showed WT phenotypes, even the substitution of H3K121 to proline.

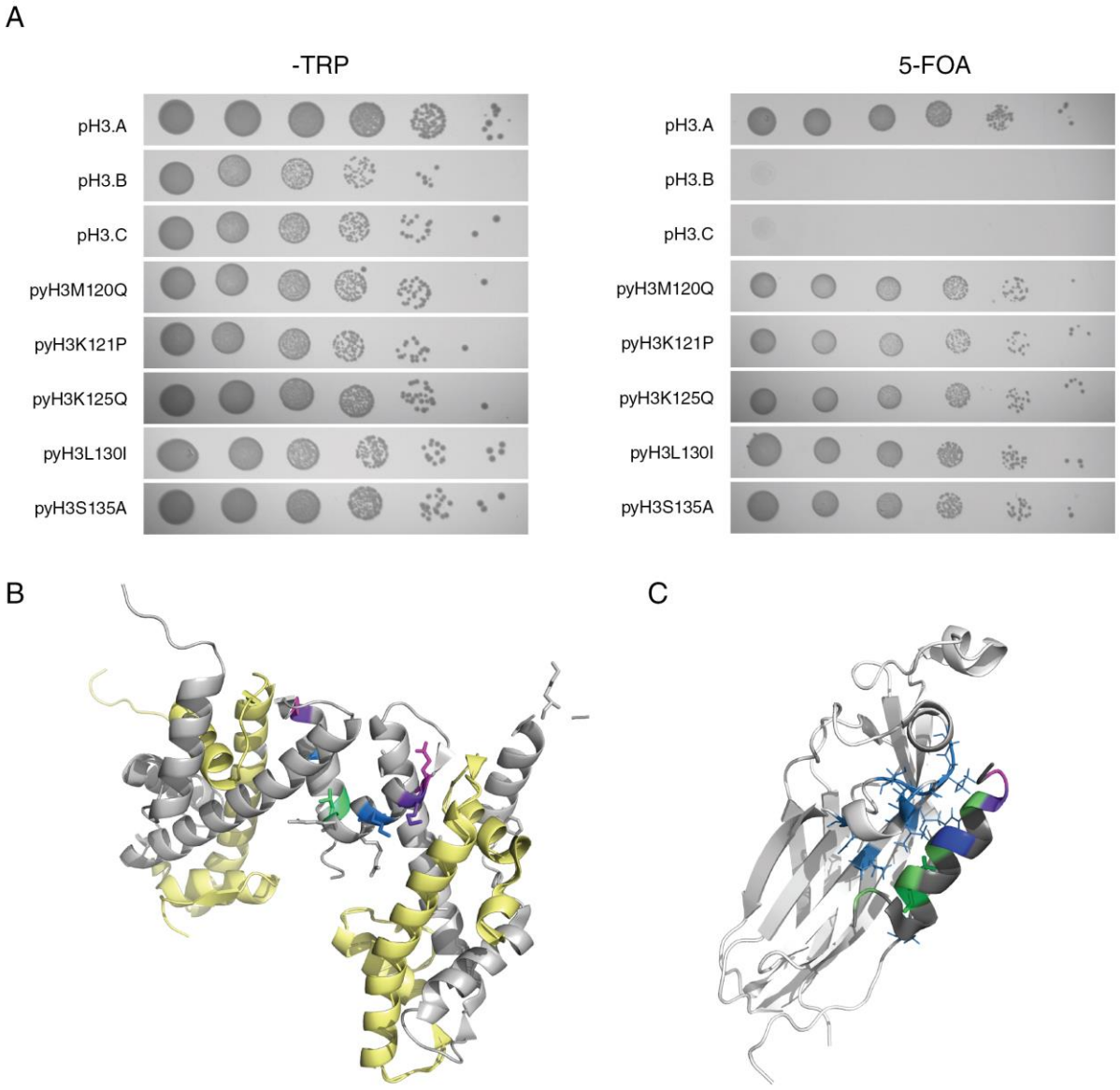

Figure 5.6. Individual amino acid substitutions in the C-terminal of H3 do not cause a growth defect. (A) Ten-fold serial dilutions WT-S transformed with the indicated plasmids were plated on synthetic media lacking tryptophan or synthetic complete media supplemented with 5-FOA and grown for 2 days at 30˚C. (B) Pymol structure of the $(H3:H4)_2$ tetramer (Protein Database ID: 1ID3; White et al., 2001). The two H3 histones are coloured grey, and the amino acids H3Q120 are coloured pink, H3K121 are coloured purple, H3K125 are coloured blue, and H3K130 are coloured green. The histones H4 are coloured yellow. (C) Pymol structure of the C-terminal tail of histone H3 modeled with the structure of Asf1 (Protein Database ID: 2IIJ,

Antczak et al., 2006). The histone H3 C-terminal tail is coloured grey and with the amino acid residues that are different between the human and yeast sequence coloured as in (B). H3 C-terminal tail amino acids that make contacts with Asf1 are coloured in green. Asf1 is coloured white with the amino acids that interact with the H3 C-terminal tail coloured in blue and modeled with lines.

As a further attempt to recapitulate the growth defect when the sequence for the human H3 C-terminal domain was substituted for the yeast sequence, we created a series of double mutants. Data from our collaborator, Dr. Chris Nelson at the University of Victoria, showed that a triple mutant of yH3Q120M,K121P,K125Q caused a growth defect similar to that of the replacement of the entire yeast H3 C-terminal domain sequence with the human C-terminal domain sequence (personal communication, unpublished). Therefore, we focused our efforts on the double mutant combinations of yH3K120M,K121P; yH3K120M,K125;, yH3K121P,K125Q (Figure 5.2-5B). Dilution plating of the yeast strain WT-S transformed with the double mutants did not reveal any significant growth defects (Figure 5.2-5A). Therefore, it appeared that the slow growth phenotype caused by replacement of the yeast H3 C-terminal domain with the sequence from the human H3 C-terminal domain was mediated by the combination of changes to the three amino acids M120, K121, and K125 (Compare Figure 5.2-5 and Figure 5.2-2). We imagine two possible explainations that reconcile the growth defect and chromatin association assay data. The first is that the differences in the C-terminal domain of the hybrid-histone result in an abberantly stable nucleosome; and the second is that the changes in the C-terminal tail result in a defect in histone:histone chaperone interactions, which prevents proper nucleosome removal during transcription.

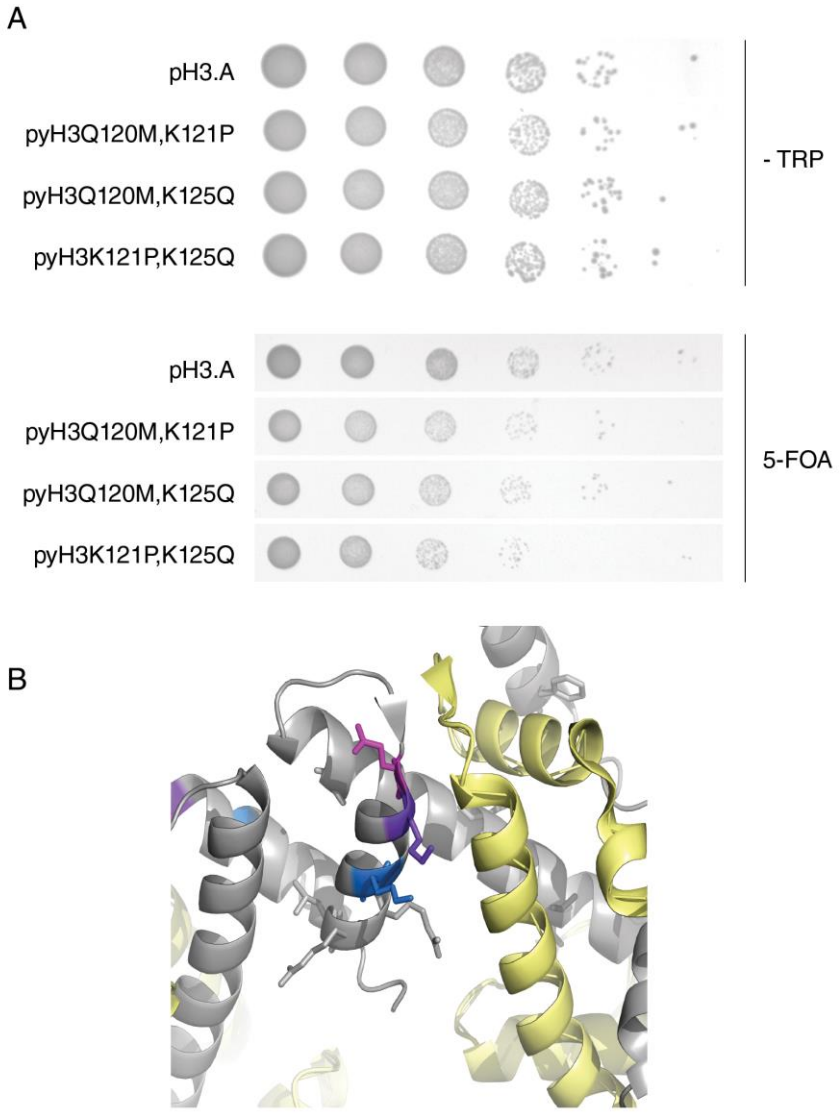

Figure 5.7. The yeast C-terminal tail double mutant strains do not exhibit a striking growth defect. (A) Ten-fold serial dilutions of WT-S transformed with the indicated plasmids were plated on -TRP media or synthetic complete media supplemented with 5-FOA and grown for 2 days at 30˚C. (B) Pymol structure of an H3:H4 tetramer, plus the second copy of H3 (PDB ID: 1ID3; White et al., 2001). The two H3 molcules are coloured grey, and H4 is coloured white. H3M120 is coloured pink, H3K121 is coloured purple, and K2K125 is coloured blue.

5.3 Yeast Containing the hH3.1-hybrid Grew Similarly to Yeast Containing the hH3.3-hybrid Under Different Nutrient Conditions.

Yeast cells expressing histone H3s with either a human N-terminal tail, or a human globular domain grew very similarly to the yeast wild type in an overnight culture, and expressed similar levels of H3, irrespectively of whether these regions had originated from hH3.1 or hH3.3 (Figure 5.2-3A). In order to verify that substitution of yeast H3 sequences with human H3 sequences outside of the C-terminal domain could fully rescue loss of yeast H3, we created two additional hybrid plasmids where the N-terminal and globular domains of H3 were human and the C-terminal domain was yeast (Figure 5.3-1A). Yeast strains with either pH3.J or pH3.K as the only source of H3 grew identically to the wild type strain on plates containing synthetic complete media supplemented with 5-FOA (Figure 5.3-1B). These results paralleled studies done in *Drosophila* showing that transcription occurred normally in animals that had only one of dH3.3 or dH3.2 (Hödl and Basler 2006; Hödl and Basler 2012). This suggested to us that hH3.1 and hH3.3 would be structurally and functionally interchangeable during transcription.

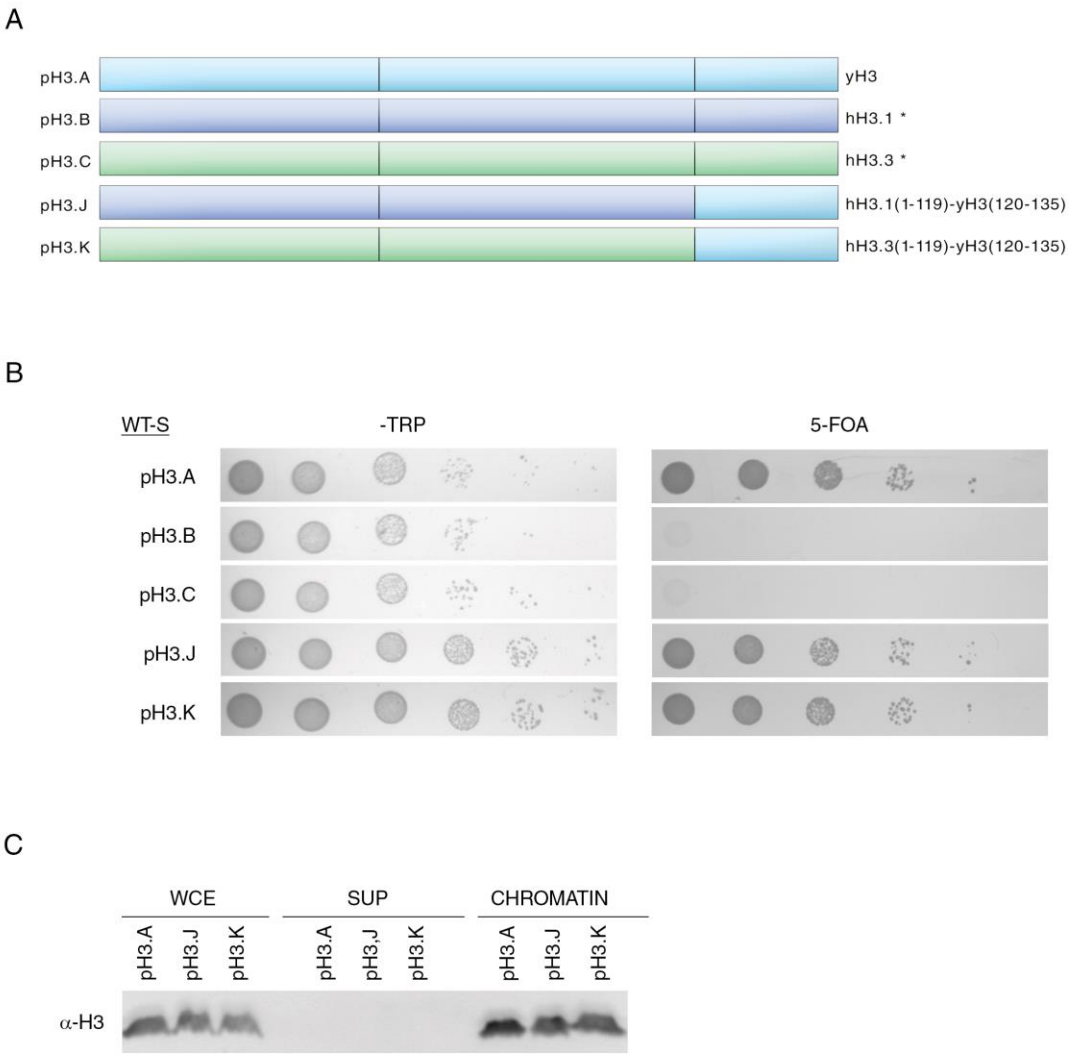

Figure 5.8. Yeast with only human histone H3.1 or H3.3, with a yeast C-terminal region, are viable. (A) Schematic of histone H3 hybrid proteins. All of the human proteins have been codon optimized for yeast expression, and each hybrid is on a plasmid being expressed off of the endogenous yH3 promoter. * indicates strains that were inviable. (B) Ten-fold serial dilutions of WT-S transformed with the indicated plasmid were plated on synthetic medial lacking tryptophan (-TRP) or synthetic complete media supplemented with 5-FOA and grown for 2 days at 30̊C. (C) Chromatin association assay of WT-S containing pH3.A, pH3.J, or pH3.K. The different lanes were normalized across the fractions by the original optical density of the yeast cultures. The SUP fraction is all of the supernatant not associated with the chromatin pellet, and the CHROMATIN fraction is the chromatin pellet that was resuspended in lysis buffer.

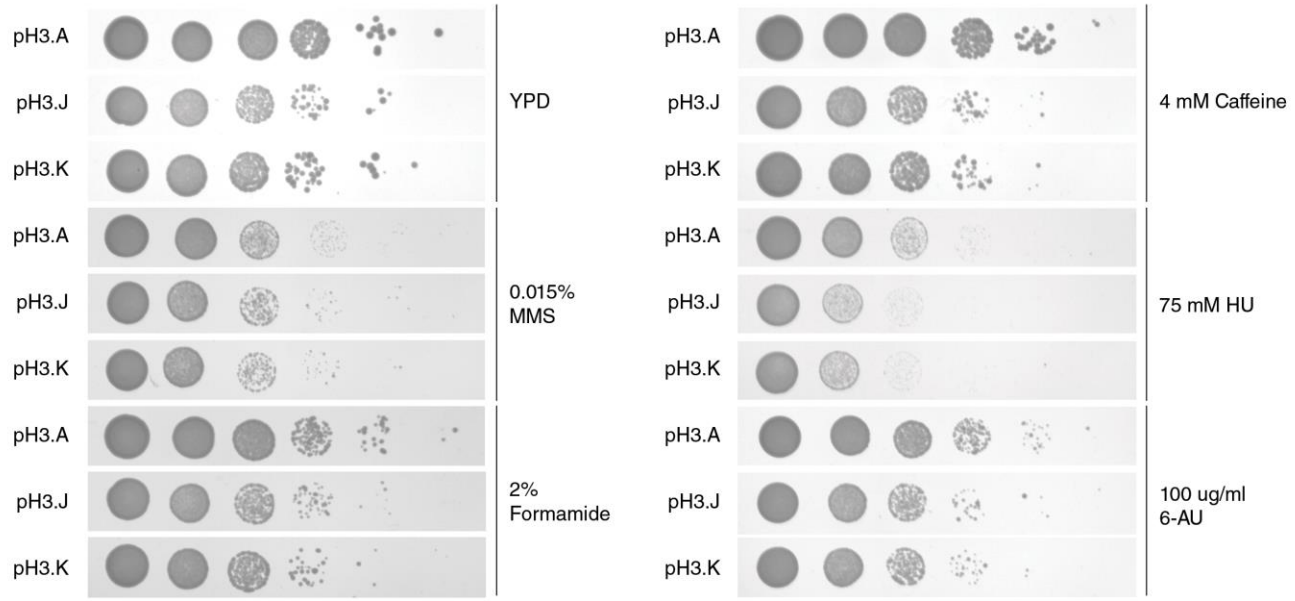

Figure 5.9. Human-hybrid histones could compensate for loss of yeast H3 when plated on drug supplemented media. Ten-fold serial dilutions WT-S with the indicated plasmid shuffled in as the sole source of H3 were plated on YPD media supplemented with the indicated chemical and grown for 2 days at 30̊ C.

Hybrid histones from plasmid pH3.J and K were expressed in yeast at similar levels to the yeast wild-type, and were completely associated with the chromatin fraction (Figure 5.3-1C). When challenged with drug supplemented media, yeast containing pH3.J or pH3.K grew similarly to the WT strain (Figure 5.3-2). Growth on solid media cannot measure subtle growth variations, such as lags in growth or yeast cell survival. Therefore, we performed a growth rate assay in liquid media. Using a Tecan Infinite M200 Pro, we monitored the growth of a small

culture of cells for 24 hours grown in rich media supplemented with different carbon sources: dextrose, galactose, and sucrose (Figure 5.3-3).

The different carbon source conditions in the growth assay resulted in three very different patterns of yeast growth. In YPD, yeast strains transformed with any of the three H3 plasmids (pH3.A, pH3.J, or pH3.K) grew equally well during the exponential growth phase, however, the strains with the human-hybrid histone H3s reached a lower saturating OD than the yeast WT (Figure 5.3-3A). This suggested that the yeast strains transformed with the hybrid-histones did not survive as well as the strain with the yeast wild-type histones.

In galactose media (YPG), both strains transformed with the human-hybrids grew more slowly than the yeast wild-type, which suggested that the strains containing the human-hybrids had trouble inducing the genes necessary for galactose metabolism. Interestingly, the strain transformed with pH3.K (hH3.3) lagged noticeably behind the strain transformed with pH3.J; however, once both strains were out of the lag-phase, they appeared to grow at an equal rate. Another finding from the growth assays was that the yeast strain transformed with pH3.K was slightly better at metabolizing sucrose than either the strain transformed with the yeast wild-type or pH3.J (Figure 5.3-3C).

Sucrose is the major carbon source for industrial bioethanol production by *S. cerevisiae*. Sucrose metabolism occurs both extracellularly and intercellularly through the hydrolysis of sucrose into glucose and fructose by the invertases encoded by *SUC2*, which has two different start codons (Carlson and Botstein, 1982; Carlson et al., 1983; Gascon et al., 1968). The secreted form of *SUC2* is derepressed upon the transition of yeast from glucose to sucrose. Since the strain transformed with the wild-type barely grew in the sucrose containing media, it appeared that there was a strain defect in sucrose metabolism. The slight growth in the strain transformed

with the hH3.3-hybrid histone suggests that this hybrid may be able to partially overcome this defect.

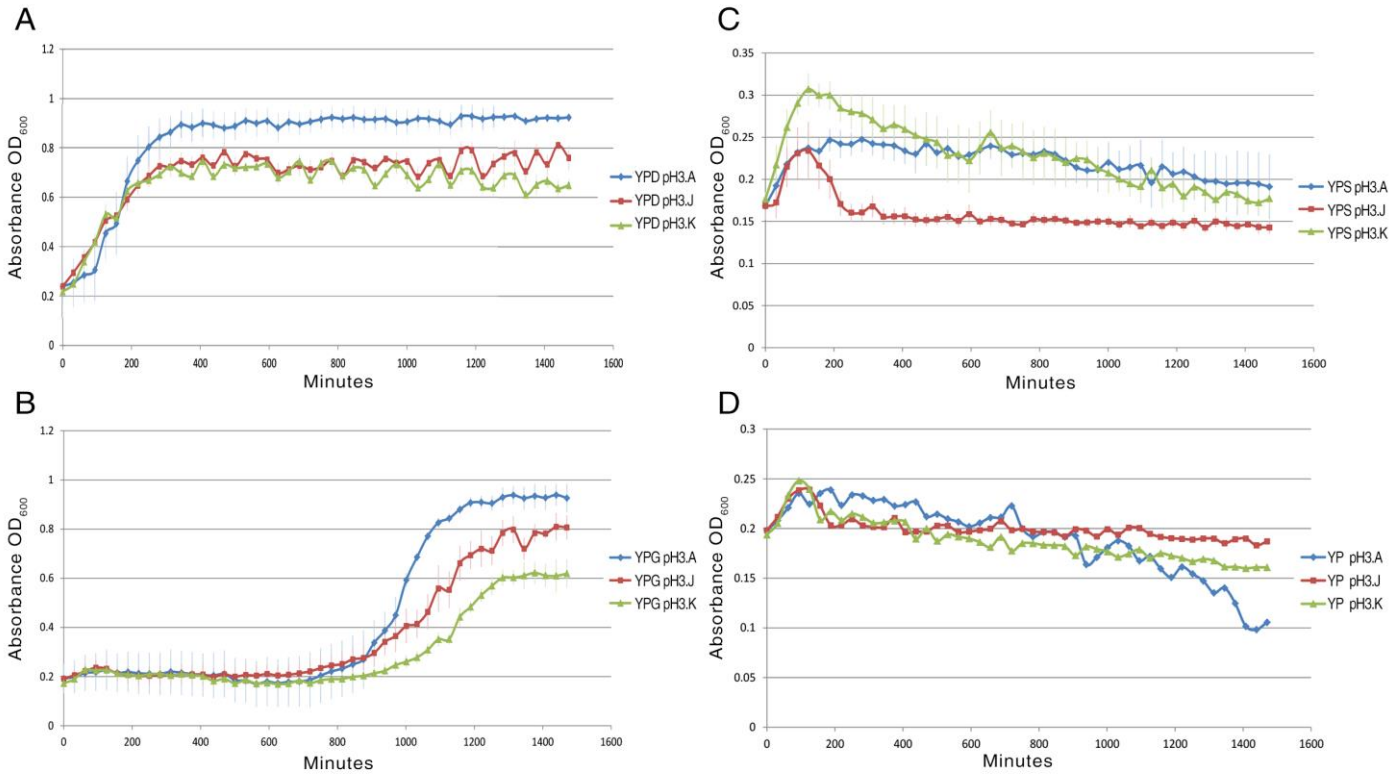

Figure 5.10. Yeast expressing the human-hybrid histones show growth defects in liquid culture. Triplicate cultures of yeast cells containing yeast H3, pH3.J (hH3.1), or pH3.K (hH3.3), as the only source of H3, were grown in 100 ul of liquid media and incubated at 30℃ for 24 hours. The cultures were agitated for 5 minutes every 30 minutes before an optical density reading was taken at 600 nm. The error bars on the graphs represent the standard deviation between the triplicate readings. (A) rich media with dextrose, (B) rich media with galactose, (C) rich media with sucrose, (D) control media, rich media with no sugar.

5.4 Discussion and Conclusion

The two human histone H3 variants, H3.1 and H3.3, are very similar and differ in only four amino acids. In the N-terminal tail domain it is at amino acid 30, which is an alanine in H3.1 and a serine in H3.3; and in the globular domain the differences are at amino acids 87, 89, and 90, which are a serine, a valine and a methionine in H3.1 and an alanine, an isoleucine and a glycine in H3.3. Due to the different deposition mechanisms that assemble either histone variant into a nucleosome, and the strong correlation of H3.3 with active promoters (Ahmad and Henikoff, 2002; Schwartz and Ahmad, 2005), it has been proposed that H3.3 has an important role in the regulation of transcription. Previous studies in *Drosophila* have demonstrated the flies that lack the variant dH3.3, and only have the canonical dH3.2, are viable (Hodl and Basler, 2009). The same group, also, demonstrated that flies that had dH3.2 as the only source of histone H3 were similarly viable (Hodl and Basler, 2012). However, other groups have shown that the H3.3 nucleosome is more easily displaced than a H3.1 nucleosome, particularly in combination with H2A.Z (Jin et al., 2009; Jin and Felsenfeld, 2007). Therefore, it remained unclear whether the differences between canonical and variant histone H3s went beyond different expression timing and different interactions with histone chaperones.

In this chapter, I showed that a yeast strain containing either the human H3.1- hybrid or H3.3-hybrid (pH3.J and pH3.K, respectively), which have the yeast N-terminal and globular domain sequences replaced by the appropriate human sequences, can compensate for the loss of yeast H3 under a variety of growth conditions. However, there were subtle growth defects in the strains that were transformed with the human-hybrid histones, particularly with regards to their ability to metabolize galactose, confirming that H3.3-containing octamers do have a different effect on nucleosome structure, *in vivo*.

Derepression of the genes that control the galactose metabolism pathway requires significant changes in chromatin structure at the *GAL* genes. These changes include disassociation of the transcriptional co-repressor complex Ssn6-Tup1, which recruits histone deacetylases (Keleher et al., 1992; Treitel and Carlson, 1995; Wu et al., 2001; Davie et al., 2003; Malave and Dent, 2006), and the subsequent recruitment of the histone acetyltransferase SAGA and the general transcription factors (Wu et al., 1996; Xie et al., 2000; Hidalgo et al., 2001; Jeong et al., 2001; Larcshan and Winston, 2001; Klein et al, 2003). The lag in growth of the strain transformed with the H3.3-hybrid was surprising because previous studies in T-cells have shown that hH3.3 is deposited and hH3.1 is lost at genes following induction (Sutcliffe et al., 2009) The T-cell data suggested that hH3.3 deposition by HIRA or DAXX/ATRX is an important pathway in gene induction (Tagami et al., 2004; Goldberg et al., 2010). Yeast has a homolog of HIRA, called HIR, but not DAXX/ATRX (Green et al., 2005). Therefore, we expect that deposition of the hybrid occurs efficiently.

Another pathway that regulates the induction of the *GAL* genes is the transcription of a non-coding RNA (ncRNA) through the *GAL10* coding sequence that originates from the gene's 3' region (Houseley et al., 2008). This transcript is produced in low levels when the *GAL* genes are either repressed or noninduced, and regulates the *GAL* gene in *cis*. Transcription of this ncRNA is stimulated by the binding of the transcription factor Reb1, which promotes the methylation of H3K4 at nucleosomes in the 3' end of *GAL10* (Houseley et al., 2008). One of the characteristics of octamers containing histone H3.3 is that they form less stable nucleosomes (Jin et al., 2011). Thus, the fragile H3.3 nucleosomes could promote increased transcription of the ncRNA, delaying the induction of the *GAL* genes.

Another interesting finding from our growth assay data was that yeast strains carrying the H3-hybrid histones, pH3.J or pH3.K, reached a lower optical density at saturation than yeast strains carrying wild type yeast histones. Two amino acids that have been implicated in transcriptional processes are different between human and yeast H3s: yH3S22 and yH3K42 which, in humans, are H3T22 and H3R42, respectively. Mutation of H3K42 to an alanine results in nucleosomes that are more mobile, due to the loss of the interaction between H3K42 and the DNA at the nucleosome dyad, resulting in hypertranscription (Somers and Owen-Hughes, 2009; Hyland et al., 2011). The change to an arginine would preserve this electrostatic interaction; however, H3K42 is a lysine that is di-methylated in yeast (Hyland et al., 2011). This methylation is mediated by the PAF1 elongation complex. It appears to be involved in regulating the mobility of nucleosomes during elongation, and yeast carrying the mutant H3K42Q (non-methyl) show significant transcriptome variation from the wild type (Hyland et al., 2011). Whether or not the arginine at H3R42 in humans plays a similar role remains to be determined, however, the inability of yeast to methylate this arginine may contribute to the phenotypes that we see in the growth assay.

In humans, hH3T22 is important for UTX/KDM6A targeted removal of H3K27 methylation through a novel Zn-binding domain in UTX/KDM6A (Kim and Song, 2011; Sengoku and Yokoyama, 2011). Yeast does not have H3K27 methylation, however, H3K23 is a residue that can be acetylated. Therefore, this serine to threonine substitution at H3S22 most likely changes the cell's ability to acetylate H3K23, which may also contribute to the growth assay survival and galactose induction phenotypes.

In this chapter, I, also, presented data that showing that neither full length human histone H3.1 nor H3.3 could substitute for yeast H3, and that this was because the yeast-codon optimized

human histones were not expressed in yeast. As mentioned above, using human-yeast hybrid histones, I showed that hybrid histones where the yeast N-terminal domain sequence, or globular domain sequences or both were replaced with the human sequences for the same regions could substitute for yeast histone H3. However, replacing the C-terminal domain sequence of yeast H3 with the human sequence resulted in a significant growth defect. Surprisingly, when we checked the total cellular protein levels of the C-terminal hybrid-protein, and its incorporation into chromatin we found that the hybrid was well expressed and in the chromatin fraction.

Further analysis of yeast and human C-terminal domain amino acids by our collaborator Dr. Nelson revealed that this growth defect could be recapitulated by a strain transformed with the H3-hybrid plasmid pyH3Q120M,K121P,K125Q. However, yeast strains transformed with single mutants or double mutant combinations of these three amino acids did not display similar growth defects. Therefore, it appears that the combined interactions of these three amino acids are important for proper yeast histone H3 function.

Although we found that yeast-codon optimized human histones were not expressed in yeast, we showed that the mechanism behind the C-terminal growth defect was unrelated to histone expression or deposition into chromatin. Data from Dr. Nelson's lab suggested that the yeast octamer is less stable, *in vitro,* than the human octamer, when reconstituted from purified nucleosomes. This implied that the growth defect in the strain containing the triple mutant was caused by changes to the nucleosome structure that resulted in nucleosomes that were difficult for the cell to disassemble. Crystallization and mutation studies examining the interactions of octamer amino acids have shown that mutation of yH3Q120 to an alanine disrupts histone:DNA interactions, *in vitro* (Sakamoto et al., 2009; Hall et al., 2009). Further, the mutation of yH3D123, which is conserved in all yeast and human histone H3 variants, to an alanine caused a

severe growth defect in yeast, and the interactions of this amino acid could have been perturbed by changes to yH3K121 and yH3K125 (Sakamoto et al., 2009). Although the other histones in the human octamer have evolved to accommodate these specific amino acid changes in human H3, it is possible that the interactions between these three mutated amino acids and the rest of the yeast octamer are responsible for the growth defect. Another possible explanation for the growth defect phenotype is that yeast chaperones cannot properly interact with the mutated H3 histones, and do not remove them efficiently from chromatin.

Chapter 6. Conclusions and Perspectives

Chromatin structure is mediated by many different factors, including the post-translational modification of histones, chromatin remodeling, histone variant incorporation into octamers, and the binding of linker histones to nucleosomes. In this thesis, I have presented data showing that changing the composition of the octamer, either by histone variant incorporation or through PTMs, is a key mechanism through which the cell regulates access to DNA. My research has focused on three different pathways involved in creating transcriptionally permissible, "open" chromatin: the acetylation of histone H3 and the RSC-complex by the HATs Gcn5 and Sas3, the regulation of linker histone binding through Htz1 deposition, and the function of the human H3 variants, H3.1 and H3.3, in transcription.

In Chapter 3, I showed that the synthetic lethal phenotype resulting from the combined deletion of *GCN5* and *SAS3* is due to the loss of both histone H3 acetylation and Rsc4 acetylation. Further, I showed that a yeast strain lacking endogenous H3 and Rsc4 and transformed with a plasmid carrying the mutant genes *hht2K9R,K14R,K18R,K23R* and *rsc4K25A* is inviable, whereas the same strain transformed with a plasmid carrying the mutant genes *hht2K9Q,K14Q,K18Q,K23Q* and *rscK25A* is viable. This suggested that the role of acetylated Rsc4K25 is independent of the ability of the RSC complex to bind to H3K14ac, as had been previously suggested based upon *in vitro* data (VanDemark et al., 2007, Kasten et al., 2004). In *S. cerevisiae,* the RSC-complex is an important member of the SWI/SNF family of chromatin remodelers and it has been shown to have roles in transcriptional activation, kinetochore function and cohesion association, and double-strand break repair (Cairns et al., 1996; Saha et al., 2006; Soutourina et al., 2006; Huang et al., 2004; Shim et al., 2005; Shim et al., 2007; Liang et al.,

2007). Work done by another lab, since the publication of Chapter 3, showed that the acetylation of Rsc4K25 has no impact on RSC's ability to bind acetylated nucleosomes; however, this acetylation was important for resistance to DNA damage (Charles et al., 2011). This group showed that the mutant *rsc4K25R* has synthetic interactions with genes coding for proteins in the DNA-damage repair pathway, and suggested that the function of the Rsc4K25ac:Rsc4BD1 interaction protects the acetyl-group from removal. They, further, hypothesized that the acetylation of Rsc4K25 acts as a switch that regulates RSC function in transcription and DNA-damage response (Charles et al., 2011).

The above data adds to our understanding of how post-translational modifications act as allosteric regulators that modulate the function of chromatin-associated complexes. Two other examples of this are the regulation of the deacetylatase Rpd3S through interaction with H3K36me3, and the regulation of the chromatin remodeler SWR1-C through interaction with H3K56ac (Li et al., 2007; Drouin et al., 2010; Watanabe et al., 2013). While allosteric regulation of protein complexes is a ubiquitious phenomena, post-translational modifications in chromatin have generally been thought to function in complex-recruitment pathways. Additionaly, our findings in Chapter 3 add to the data showing that individual acetylation marks on the histone tails may not each have a unique function, and suggest that the function of histone H3 acetylation is to neutralize histone:DNA charge interactions. Further, the viability of a *H3K9,14,18,23R.RSC4* strain shows that H3 tail acetylation is not required for transcription, and suggests that the high correlation of acetylation with transcription may be a consequence of transcriptional processes that play an important role in other cell functions, such as DNA repair. Currently, investigations into which histone PTMs are a consequence of transcription are being performed by Benjamin Martin, a fellow graduate student.

Our findings in Chapter 3, also, showed that the neutralization of the charged lysine residues on histone H3 is important when the acetylation of Rsc4 is compromised through mutation of Rsc4K25. Previous studies looking at the impact of histone H3 acetylation on nucleosome stability showed that acetylation of H3 resulted in decreased binding of the linker histone to nucleosomes (Mistelli et al., 2000). In order to investigate the role of acetylation in the regulation of the yeast linker histone we deleted *HHO1,* and showed that this deletion alleviates the synthetic sickness of a conditional *gcn5Δsas3Δ* mutant strain that expressed a temperature-sensitive version of Sas3. Strains with only the mutant Rsc4K25R protein were sensitive to MMS (Charles et al., 2011). Rescue of the *gcn5Δsas3Δ pSAS3.TS* strain, which should not have any Rsc4 acetylation, by deletion of *HHO1* suggests that RSC may help to remove the linker histone from nucleosomes during DNA repair.

In Chapter 4, I presented data that showed that Htz1 incorporation is a third mechanism, in addition to linker histone phosphorylation and histone acetylation (Vicent et al., 2011; Bhattacharjee et al., 2001; Koop et al., 2003; Lever et al., 2000; Misteli et al., 2000), for mobilizing the linker histone away from nucleosomes at the promoter of genes. Using ChIP-Seq, I showed that this mechanism had direct effects on Hho1 binding at the +1 and -1 nucleosomes, and had indirect effects on Hho1 binding at downstream nucleosomes.

Further, I hypothesized that the loss of Htz1 and subsequent increase in Hho1 binding at the promoter could cause decreased nucleosome "breathing" which could be responsible for the induction phenotypes seen in *htz1Δ* mutant strains (Halley et al., 2010; Wan et al., 2009; Adam et al., 2001). Since eviction of the linker histone is an important step in transcription, to test whether loss of Htz1 slowed galactose induction kinetics because of increased Hho1 binding we could investigate gene induction in yeast strains lacking both Hho1 and Htz1. If mobilizing Hho1

from the +1 nucleosome is an important function of Htz1, we would expect that an *htz1Δhho1Δ* strain would exhibit faster induction kinetics than an *htz1Δ* strain. The increased binding of Hho1 in an *htz1Δ* mutant could also be used to further investigate Hho1 interactions, such as whether Hho1 is phosphorylated in yeast. Our finding that Htz1 acts to keep Hho1 away from nucleosomes is especially interesting in the context of mammalian systems where there is a 1:1 ratio of nucleosomes to linker histones, and H2A.Z binding is correlated with transcription rate (Barski et al., 2007). However, whether this mechanism extends to mammalian systems is unknown.

Hho1 has been shown to increase 5-fold on chromatin and to play an important role in compacting the genome during sporulation (Bryant et al., 2012). Transcription profiles show that the many genes are downregulated during sporulation, including *HTZ1* (Klutstein et al., 2010). However, it is unknown whether Htz1 is lost from chromatin during sporulation. The importance of Htz1 for proper kinetics of galactose induction, suggests that Htz1 could facilitate rapid induction of germination genes by keeping the +1 nucleosome Hho1-free. Investigation into the patterns of Htz1 and Hho1 in spore-chromatin could help us to better understand how chromatin is regulated and packaged during sporulation, and unpackaged during germination.

Our ChIP-Seq data also provided other insights into Hho1 function *in vivo*. We showed that Hho1 does not protect two nucleosome repeat lengths of DNA, which suggests that the yeast linker histone binds similarly to linker histones in multicellular eukaryotes, despite its second globular domain. Additionally, although not discussed in Chapter 4, the genome wide profile of Hho1 binding in the wild type strain showed that Hho1 was enriched at the centromere. The *S. cerevisiae* centromere, unlike the centromeres of other eukaryotes, is a point centromere with a single Cse4 (yeast CENP-A)-containing nucleosome (Krassovsky et al., 2012; Dalal et al., 2007;

Furuyama and Biggins, 2007). In mammals, the linker histone has been found to interact with nucleosomes containing the centromeric H3 variant CENP-A, however it is present at levels consistent with H1 binding to the rest of chromatin (Orthaus et al., 2009). Therefore, enrichment of Hho1 at the yeast centromere could indicate a particular role for Hho1 at the centromere. A large number of genes identified as SDI phenotype exacerbating hits were genes encoding for proteins involved in chromosome and chromatid separation, which supports the hypothesis that Hho1 plays a role in centromeric chromatin function. A unique feature of the centromeric nucleosomes is that it is a hemisome: a tetramer containing one copy of each of Cse4, H4, H2A, and H2B (Dalal et al., 2007). Therefore, an interesting question is whether Hho1 binding helps to maintaining yeast centromere structure, and how it functions in chromosome segregation.

In Chapter 5 of this thesis, I showed that human-yeast histone hybrids containing the N-terminal and globular domain sequence of human H3.1 or H3.3, and the C-terminal domain sequence of yeast H3 could rescue the loss of yeast H3 *in vivo*. This confirmed findings, in *Drosophila,* that suggested that the most important differences between the canonical and variant H3s, regarding their function in transcription, were their expression timing during the cell cycle (Hödl and Basler, 2009; Hödl and Basler, 2012). I, further, showed that both of the hybrid-H3 histones allowed yeast to grow similarly under a number of different drug supplemented media conditions. However, there were subtle growth defects in the strains that were transformed with the human-hybrid histones, particularly with regards to their ability to metabolize galactose, indicating that the two variants were not truly functionally identical.

Our growth assay data showed that yeast containing the H3.3-hybrid histone took longer to induce galactose metabolism than either the wild type or the H3.1-hybrid histone containing strains. We hypothesize that the growth defect in the yeast strain containing the H3.3-hybrid

histone is due to an increase in transcription of the *GAL10* ncRNA. H3.3-containing nucleosomes have been shown to be more fragile than H3.1-containing nucleosomes, *in vitro* (Jin and Felsenfeld, 2007; Jin et al., 2009); therefore, this increase in transcription could be due to an increased ease in displacing H3.3-containing nucleosomes by the transcriptional machinery. If this is true, it would suggest that H3.3-containing octamers do have a different effect on nucleosome structure, *in vivo,* and that this may contribute to the ability of a gene to undergo subsequent rounds of transcription.

Rich media and high glucose conditions, while common in the laboratory, are not conditions that yeast would encounter in the wild. Therefore, it is probable that many growth differences between the two human-hybrids strains, and between the hybrid strains and the yeast wild type strain will only be elucidated under different media conditions. Our sucrose data, particularly, warrants further exploration since sucrose is a very common natural carbon source.

The absence of H3.3 in *Drosophila* renders the animals sterile, and this histone variant, also, has been implicated in male germline chromatin regulation in mice, plants, and *Caenorhabditis elegans* (Hödl et al., 2009; Ooi, et al., 2006; Ingouf et al., 2007; van der Heijden et al., 2005). Further, the defect appears to be unrelated to expression timing and cellular level (Hödl et al., 2009). Since our hybrid-proteins do not grow identically under all conditions, we are interested in testing whether or not there is a difference in their function during sporulation.

Additionally, in Chapter 5, we identified three amino acid residues in the yeast C-terminal tail domain that are important for histone H3 interactions in chromatin. We showed that these residues were not important for histone H3 incorporation into chromatin, using a chromatin association assay, and that yeast strains expressing these triple mutant histones as the sole source of H3 had a slow growth phenotype. Data from our collaborator Dr. Nelson suggested that yeast

octamers form less stable nucleosomes than human octamers; therefore, these three amino acids may be important to pathways that mediate octamer dissociation, and may be involved in yet to be identified histone:histone or histone:DNA interactions. Dr. Nelson's lab is, currently, investigating the *in vitro* stability of nucleosomes containing this triple mutant version of histone H3. Another potential cause of the slow growth phenotype could be the disruption of a histone chaperone interaction with the $(H3:H4)_2$ tetramer due to mutation at these three amino acids. To date, the only histone chaperone that has been shown to bind at the H3 C-terminal tail is Asf1 (Antczak et al., 2006; English et al., 2006; Agez et al., 2007, Lin et al., 2010). Yeast strains that are *asf1Δ* mutants, however, do not exhibit a slow growth phenotype (Tyler et al., 1999). We are, currently, in the process of creating plasmids that overexpress yeast histone chaperones to assay whether the over-production of any of the histone chaperones can rescue the H3 triple mutant slow growth phenotype.

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