ELUCIDATING THE FUNCTION OF THE HISTONE H4 BASIC PATCH IN SAGA-MEDIATED HISTONE H2B DEUBIQUITINATION AND HISTONE ACETYLATION

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ABSTRACT

Hashem Adnan Meriesh: Elucidating the function of the histone H4 basic patch in SAGA-mediated histone H2B deubiquitination and histone acetylation
(Under the direction of Brian D. Strahl)

The eukaryotic genome is packaged into the nucleus by the wrapping of DNA around histones to form the nucleosome—the fundamental repeating unit of chromatin. In addition to its structural role, chromatin regulates DNA accessibility and thereby DNA-templated processes such as DNA replication and transcription. Chromatin structure and function is mediated by the covalent modification of the histone proteins with a vast array of post-translational modifications (PTMs) including acetylation, methylation, phosphorylation, and monoubiquitylation. Histone PTMs can affect chromatin structure directly or serve as binding sites for effector proteins and complexes that mediate a specific function. Proper regulation of histone PTMs is critical for many cellular and developmental processes and their aberrant placement or removal is a hallmark of many diseases including cancer and neurodegenerative disorders.

Histone H2B monoubiquitylation (H2Bub1) has central functions in multiple DNA-templated processes including gene transcription, DNA repair, and replication. In addition, H2Bub1 is required for the trans-histone regulation of H3K4 and H3K79 methylation. Although previous studies have elucidated the basic mechanisms that establish and remove H2Bub1, we have only an incomplete understanding of how H2Bub1 is regulated. We report a novel regulator of H2Bub1 – the histone H4 basic patch. The H4 basic patch is a hub for chromatin modifiers, yet the H4 basic patch regulates H2Bub1 levels independently of interactions with chromatin

remodelers and separately from its regulation of H3K79 methylation. To measure H2B ubiquitylation and deubiquitination kinetics in vivo, we used a rapid and reversible optogenetic tool, LINX (light-inducible nuclear exporter), to control the subcellular location of the H2Bub1 E3-ligase, Bre1. The ability of Bre1 to ubiquitylate H2B was unaffected by a H4 basic patch mutant. In contrast, H2Bub1 deubiquitination by SAGA-associated Ubp8, but not by Ubp10, increased in the mutant. Consistent with a function for the H4 basic patch in regulating SAGA deubiquitinase activity, we also detected increased histone acetylation by SAGA in H4 basic patch mutants. The work in this dissertation discusses the discovery a new regulatory mechanism of the H4 basic patch in SAGA-mediated functions.

This one’s for you, Mama.

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In God’s name, the All-Merciful, the Compassionate. “And my success [in my task] can only come from God” (Qur’an 11:88).

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I owe much to my beloved parents, Sahar Afani and Adnan Meriesh, who constantly prayed for my success and wellbeing, and whose contentment and praise I sought upon taking this journey. My success is equally theirs. To my brother, Mohammed Meriesh, who inspires me to keep pushing, and for always being there for me and my family.

I would not be here without the support of my beloved wife, Rana Badwan, who sacrificed much to join me when I started, sacrificed much throughout, and continues to do so, lovingly, while we raise our children. This effort would have been impossible without the support of my wife’s family who have supported us in endless ways.

PREFACE

This dissertation was written during March and April of the COVID-19 global pandemic of 2020. Much of my PhD was a struggle, but finding the time and energy to write a dissertation while raising and educating my 3 children at home was a challenge of unparalleled magnitude. Alas, this dissertation is finished and this pandemic continues.

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LIST OF ABBREVIATIONS

β-ME	2-Mercaptoethanol
C	Celcius
CC	Coiled coil domain
cm	Centimeter
CO_2	Carbon dioxide
CTD	C-terminal domain
C-terminus	Carboxy-terminus
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSB	Double-strand break
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
FLAG	Peptide tag with sequence DYKDDDDK
5-FOA	5-Fluoroorotic acid
GAL	Galactose
G6PDH	Glucose-6-phosphate dehydrogenase
h	Hours
H (his)	Histidine
HAT	Histone acetyltransferase
HCl	Hydrochloric acid
HDAC	Histone deacetylase
−His	Lacking histidine
HMT	Histone methyltransferase
H2A	Histone H2A

H2B	Histone H2B
H2Bub1	Histone H2B monoubiquitylation
H3	Histone H3
H3K4me	Histone H3 lysine 4 methylation
H3K4me3	Histone H3 lysine 4 trimethylation
H3K36me	Histone H3 lysine 36 methylation
H3K36me1	Histone H3 lysine 36 monomethylation
H3K36me2	Histone H3 lysine 36 dimethylation
H3K36me3	Histone H3 lysine 36 trimethylation
H3K56ac	Histone H3 lysine 56 acetylation
H3K79me	Histone H3 lysine 79 methylation
H3K79me2	Histone H3 lysine 79 dimethylation
H3K79me3	Histone H3 lysine 79 trimethylation
H4	Histone H4
k	Thousand
kDa	Kilodalton
KO	Knock-out (refers to a gene that is no longer expressed)
L (leu)	Leucine
LANS	Light activated nuclear shuttle
$LANS_{RLR}$	variant incorporating HVR to RLR mutations in residues 519-521
$LANS_{RVH}$	variant incorporating HVR to RVH mutations in residues 519-521
LED	Light-emitting diode
−Leu	Lacking leucine
LiCl	Lithium chloride
LINX	Light-inducible nuclear exporter

$LINX_{RLR}$	variant incorporating HVR to RLR mutations in residues 519-521
$LINX_{RVH}$	variant incorporating HVR to RVH mutations in residues 519-521
m	Minutes
M	Molar
mA	Milliamp
MCS	Multiple cloning site
me1	Monomethylation
me2	Dimethylation
me3	Trimethylation
µL	Microliter
µM	Micromolar
mg	Milligram
mL	Milliliter
mM	Millimolar
MOPS	3-(*N*-morpholino)propanesulfonic acid
NaCl	Sodium chloride
Na_2EDTA	Disodium ethylenediaminetetraacetate dihydrate
$NaHCO_3$	Sodium bicarbonate
ND	Not determined
NES	Nuclear export signal
NH_4Oac	Ammonium acetate
NLS	Nuclear localization signal
nm	Nanometer
nM	Nanomolar
NPC	Nuclear pore complex

NS	Not significant
N-terminus	Amino-terminus
OD_{600}	Optical density at 600 nm
ORF	Open reading frame
PAS	Per-Arnt-Sim
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pH	Potential of hydrogen
PMSF	Phenylmethylsulfonyl fluoride
PTM(s)	Post-translational modification(s)
PVDF	Polyvinylidene difluoride
R (arg)	Arginine
RAFF	Raffinose
RCF	Relative centrifugal force
RNA	Ribonucleic acid
RNAPII	RNA Polymerase II
RPM	Revolutions per minute
s	Seconds
SC	Synthetic complete
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SET	Su(var)3-9, Enhancer-of-zeste and Trithorax
SUMEB	Buffer containing SDS, urea, MOPS, EDTA, and bromophenol blue

TATA	DNA sequence found in core promoters
TCA	Trichloroacetic acid
TE	Buffer containing Tris and EDTA
Tris	Tris (hydroxymethyl) aminomethane
U	Enzyme unit
−Ura	Lacking uracil
v/v	Volume/volume
WT	Wild type
w/v	Weight/volume
YPD	Yeast Extract-Peptone-Dextrose medium

CHAPTER 1: INTRODUCTION

1.1 Introduction

The human body displays an impressive array of diversity amongst the cell types that comprise it. Cells in the eye, with help from the brain, convert light to useful images. Cells in the heart and lungs function continuously to circulate oxygenated blood throughout the body and recharge deoxygenated blood. Platelets circulate through the blood, poised for activation at the onset of injury to stop bleeding and begin healing immediately. In addition to these examples of the beautiful diversity of function within cells, our bodies are also able to sense and adapt to changes in our environment, such as the cellular cue to shiver from brown fat cells when cold and the response of our immune systems at the detection of a foreign pathogen. Yet, the most remarkable feature connecting all of these unique and specialized functions is that they all originated from the same cell and share the same blueprint—the genome.

The genome contains information in the form of genes that not only encode the basic functions of each cell such as energy production and replication, but also the information required for specialized functions. Regulated expression of genes determines cell identity and function, and the regulation of gene expression occurs to a large extent during the transcription of DNA into RNA. Given that every cell shares the same genomic blueprint, a few important questions arise. First, how do cells express the proper genes at the proper times to perform a specific cellular function? How do cells ensure that expression of the appropriate genes is maintained as long as necessary while, at the same time, maintaining the inactivation of genes

whose expression would be to detrimental to the cell? Lastly, how are these specific patterns of expression maintained between subsequent cell divisions and inherited between parent and offspring? These questions form the basis of the field of epigenetics. The classical definition of epigenetics involves understanding the mechanism behind heritable phenotypic differences that lack any difference in the underlying DNA sequence. Recently, epigenetics has come to refer to the collection of covalent modifications to DNA and histone post-translational modifications that alter gene expression and chromatin structure and function (R. Janke, Dodson, and Rine 2015). A brief overview of the fundamentals of epigenetics, particularly the recent definition, and the advancements in understanding the mechanisms regulating gene expression will be discussed in this chapter in further detail.

1.2 Chromatin Structure and Function

Every human cell faces a monumental task—how to package nearly 2 meters of linear DNA into a nuclear space approximately 10 microns wide. In eukaryotic cells, DNA does not exist as a naked molecule. Instead, it is found in a compacted structure called chromatin. The fundamental repeating unit of chromatin is the nucleosome—a molecule consisting of approximately 147 base pairs of DNA wrapped an octamer of histones proteins. The histone octamer is comprised of two copies each of the canonical histones H2A, H2B, H3, and H4. Functionally, an H3-H4 tetramer is capped on two sides by an H2A-H2B dimer to produce the nucleosome core particle (NCP) (Luger et al. 1997; Kornberg and Lorch 1999). Electrostatic interactions between the positively-charged histones and the negatively-charged DNA stabilizes the structure of chromatin and drives the assembly of higher-order chromatin structures (Horn and Peterson 2002). Adjacent nucleosomes are connected by linker DNA that is bound by the non-core histone H1 (Allan et al. 1980). The actions of proteins such as condensin and cohesin

further drive the organization of chromatin (Hagstrom and Meyer 2003). The chromatin polymer is thus an extended array of nucleosomes. The long-standing opinion in the field holds that these arrays undergo a progressive compaction from an 11-nm NCP to a 30-nm fiber and ultimately to a 300-700-nm fiber and finally the iconic mitotic chromosomes. This opinion has long generated controversy, not only of the specifics of these higher order structures but of their mere existence. Recent studies, taking advantage of new methods and technologies, have provided much needed insight on this matter (Ou et al. 2017).

Contrary to the belief of many scientists in the nascent years of chromatin research, chromatin is not simply a scaffold for the efficient packaging of DNA. Chromatin serves two critical functions. Firstly, chromatin-bound DNA renders the bound DNA less accessible than free DNA thus protecting it from DNA damage while also providing the first layer of gene regulation by mediating accessibility. Secondly, chromatin functions as an active signaling hub in nearly all DNA-templated processes, from transcription to DNA replication and repair. This latter function occurs though the combined effects of varying the chemical landscape of the histones that make up each nucleosome and regulating the positioning of nucleosomes across the genome. Furthermore, DNA is also subject to chemical modification. Together, these features regulate the recruitment and occlusion of specific downstream effectors and allow for the precise control of DNA accessibility thus providing the foundational mechanism for specifying and maintaining cell identity.

The mechanisms summarized above result in two general subtypes of chromatin, namely euchromatin and heterochromatin, classified by their degree of compaction and accessibility and are thus considered “open” or “closed”, respectively. Euchromatin is found at gene-rich and actively transcribed loci. Euchromatin displays various levels of “openness” depending on the

nature of the gene (e.g. inducible; housekeeping) and its transcriptional activity. Conversely, heterochromatin is found at regions such as the telomeres and centromeres where maintaining a repressed state is critical. Within heterochromatin are two classifications based on the level and duration of repression required. Constitutive heterochromatin is found at regions of the genome that must be repressed in all cell types of an organism at all stages of the cell cycle. Facultative heterochromatin is a form of transcriptionally silenced chromatin that may become active due to cell-type differentiation or in response to external stimuli.

The function of chromatin in maintaining the integrity of the genome throughout the life time of an organism and its ability to transition between states to regulate and respond to external stimuli is a critical component of cellular development, adaptation, and survival. Fittingly, this transition between states is a complex and highly regulated process involving many factors. What follows is a description of the mechanisms involved in establishing, altering, and maintaining the chromatin state.

1.2.1 Histone Modifications and the Histone code hypothesis

Chromatin structure and function is largely regulated through the post-translational modification (PTMs) of histones. Since the earliest reports of histone acetylation and methylation in the 1960s, an astonishing number of histone PTMs have been discovered through the advancement of mass spectrometry approaches (Allfrey, Faulkner, and Mirsky 1964; Zhao and Garcia 2015). These modifications include methylation of arginine, histidine, and lysine residues, acetylation and ubiquitylation of lysine residues, and phosphorylation of serine, threonine and tyrosine residues. While most of these modifications occur at the flexible N- and C-terminal tails of the NCP, a number of significant modifications occur in the globular domain. While our knowledge

of the existence of these modifications expanded, advancements in our understanding of how these modifications regulate chromatin structure and function have been a recent phenomenon.

Functionally, histone PTMs alter chromatin structure both directly and indirectly. The histone tails contain many positively-charged residues, such as lysine and arginine, and acetylation of these residues neutralizes the charge and may affect DNA-histone interactions (Bannister and Kouzarides 2011). Additionally, histone PTMs are recognized by effector proteins that contain specialized domains including chromodomains, bromodomains, PHD fingers, Ankyrin repeats, WD40 repeats and others (Taverna et al. 2007; Yun et al. 2011). Importantly, these effector proteins contain multiple recognition domains suggesting the possibility of unique combinational patterns of PTMs at different genomic loci and the ability of these effector proteins to perform unique functions based upon these patterns. Considering the astonishing number of PTMs on both canonical and variant histones, the number of possible histone patterns across the genome is astonishing (Zhao and Garcia 2015). These advancements in our understanding of chromatin structure and function led to the formulation of the 'histone code' hypothesis—wherein distinct PTMs, on one or more histone tails, function sequentially or in combination to bring about distinct downstream events (Strahl and Allis 2000). In summary, the chromatin landscape is regulated by "writers" that modify histones, "readers" that recognize specific modifications, and "erasers" that remove these modifications in a dynamic manner to bring about a specific downstream effect (Figure 1.1).

The role of histone modifications in regulating the chromatin landscape is well established. The following sections will discuss how the histones and regions on the NCP also function to regulate both histone-modifying activities and the chromatin landscape.

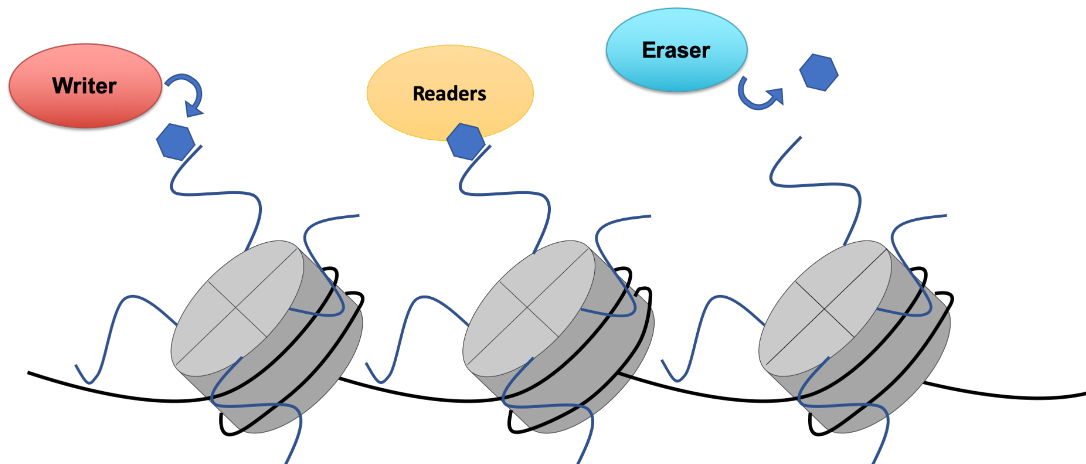

Figure 1.1. The writers, readers, and erasers of the Histone Code hypothesis

1.2.2 The histone H4 basic patch is a hub for chromatin-modifying factors

The flexible N- and C-terminal tails of the histone proteins are most well-known for harboring the sites of histone PTMs involved in regulating chromatin structure and function. The PTMs found on the N-terminus of histone H4, namely acetylation on lysine residues 5,8, 12 and 16, and their functions are well studied. In addition to these PTMs, a stretch of basic residues on histone H4 (i.e. arginine, histidine, and lysine), known as the H4 basic patch, functions as an interaction hub for multiple chromatin modifying enzymes and complexes (Figure 1.2). Dot1, the histone H3 lysine-79 methyltransferase, requires both arginine residues of the basic patch to efficiently catalyze di- and trimethylation (Fingerman, Li, and Briggs 2007). Additionally, the H4 basic patch activates the ISWI chromatin remodeling complex (CRC). ISWI contains a mimic of the H4 basic patch that inhibits ATPase activity through an autoinhibitory mechanism and this inhibition is relieved by the antagonistic binding of the authentic H4 basic patch (C. R. Clapier et al. 2001; Hamiche et al. 2001; C. R. Clapier 2002; Cedric R. Clapier and Cairns 2012). Moreover, the binding of the budding yeast silencing protein, Sir3, depends on the acetylation

state of histone H4 lysine-16 (H4K16), connecting the H4 basic patch to the maintenance of heterochromatin boundaries (Altaf et al. 2007; Onishi et al. 2007). The function of the H4 basic patch, and H4K16 in particular, in the regulation of higher-order chromatin organization will be discussed in greater detail in the following sections.

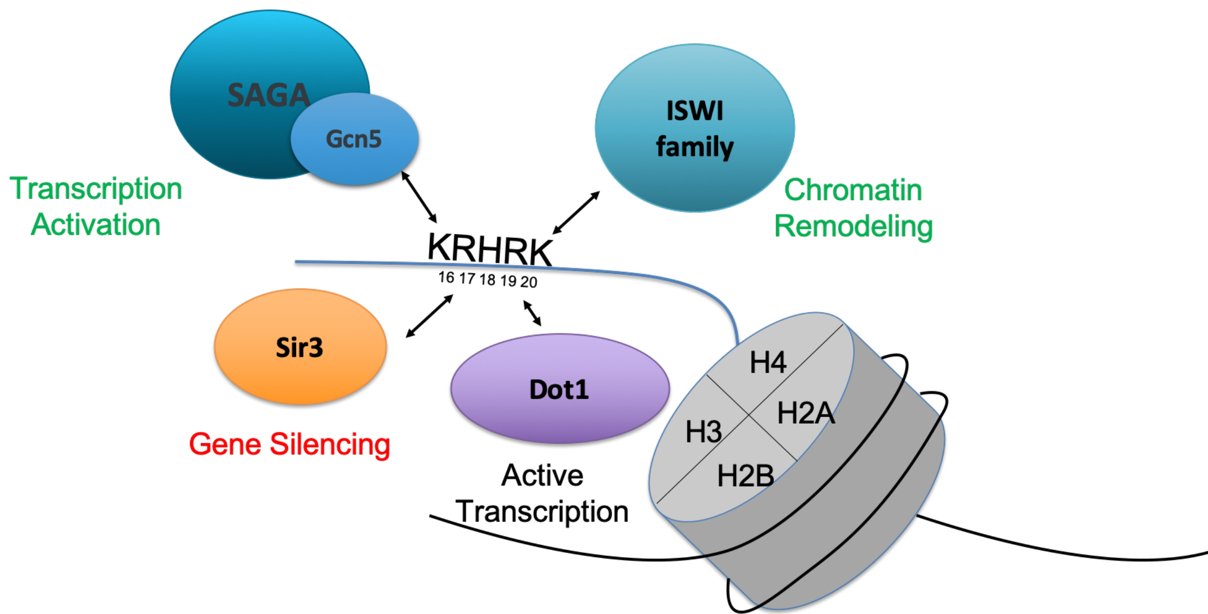

Figure 1.2. The histone H4 basic patch serves as a hub for chromatin-modifying activities and transcription associated modifications

1.2.3 The nucleosome acidic patch is a hub for chromatin-modifying factors

As is expected of a nucleoprotein complex, the nucleosome contains a large number of positively charged amino acids to facilitate DNA binding. The nucleosome is also a highly contoured and asymmetrical molecule. Interestingly, a negatively charged cavity is formed at the interface of histones H2A and H2B and is known as the "nucleosome acidic patch" (Kalashnikova et al. 2013). The acidic patch is formed from six histone H2A residues (E56, E61, E64, D90, E91, and E91) and two histone H2B residues (E102 and E110). Interestingly, nearly all published crystal structures of a nucleosome-bound macromolecule share the same interaction motif—an arginine residue bound to the nucleosome acidic patch (McGinty and Tan 2015).

The groundbreaking publication of the structure of the NCP by Luger *et al* was the first report of a protein binding the nucleosome acidic patch. In their crystal structure, residues 16 to 25 of the histone H4 tail from one nucleosome contact the nucleosome acidic patch on an adjacent nucleosome (Luger et al. 1997). This interaction is fundamental to chromatin compaction and the formation of higher order structure and will be discussed in more detail below. To date, the structures of at least 10 proteins bound to the nucleosome acidic patch have been solved and the list continues to grow. But what makes this small region of a complex as large as the nucleosome such a poignant motif for recognition? In the words of Robert McGinty and Song Tan, whose work on the nucleosome acidic patch has been seminal to our understanding of this motif, "The simple answer is that the acidic patch is the most unique region of the nucleosome surface [and] carries the greatest net charge of the solvent exposed region of the histone octamer disk surface." (McGinty and Tan 2015)

Of particular interest to the scope of work presented in this thesis is the association of factors that function to regulate histone H2B monoubiquitylation with the nucleosome acidic

patch. Both the ubiquitylation machinery (Rad6-Bre1) and deubiquitination machinery (SAGA DUB module member, Sgf11) have been shown to bind and compete for the nucleosome acidic patch (Gallego et al. 2016; Morgan et al. 2016). Additionally, residues in the nucleosome acidic patch have been shown to be important for the addition of ubiquitin to histone H2B (Cucinotta et al. 2015).

1.2.4 Inter-nucleosomal interactions and higher-order chromatin structure

Understanding the mechanisms that regulate the formation of metaphase chromosomes from a string of nucleosomes has eluded the field of chromatin biology for decades. Nucleosomes have been shown to condense into compact arrays under various ionic conditions (Bednar et al. 1995; Arya and Schlick 2009). The structure of the NCP by Luger *et al* revealed the interaction of residues 16-25 of the histone H4 N-terminus with the nucleosome acidic patch and a recent report by Wilkins *et al* used site-specific crosslinking to validate this interaction *in vivo* (Luger et al. 1997; Wilkins et al. 2014). It has also been shown experimentally that the histone H4 tail mediates the largest number of internucleosomal interactions (Arya and Schlick 2009) and that acetylation of histone H4 lyinse-16 (H4K16ac), but not histone H3 tail acetylation, disrupts higher-order folding and compaction (Tse et al. 1998; Shogren-Knaak et al. 2006).

The nucleosome and chromatin condensation are both barriers to the transcription machinery and relieving these barriers is critical for proper gene expression. In agreement with the above observations, it has been shown that H4K16ac stimulates transcription *in vitro* and *in vivo* (Akhtar and Becker 2000). Additionally, approximately 80% of the histone H4 in yeast is acetylated at H4K16 and most of the yeast genome exists in a decondensed state (Lohr, Kovacic, and Van Holde 1977; Smith et al. 2003).

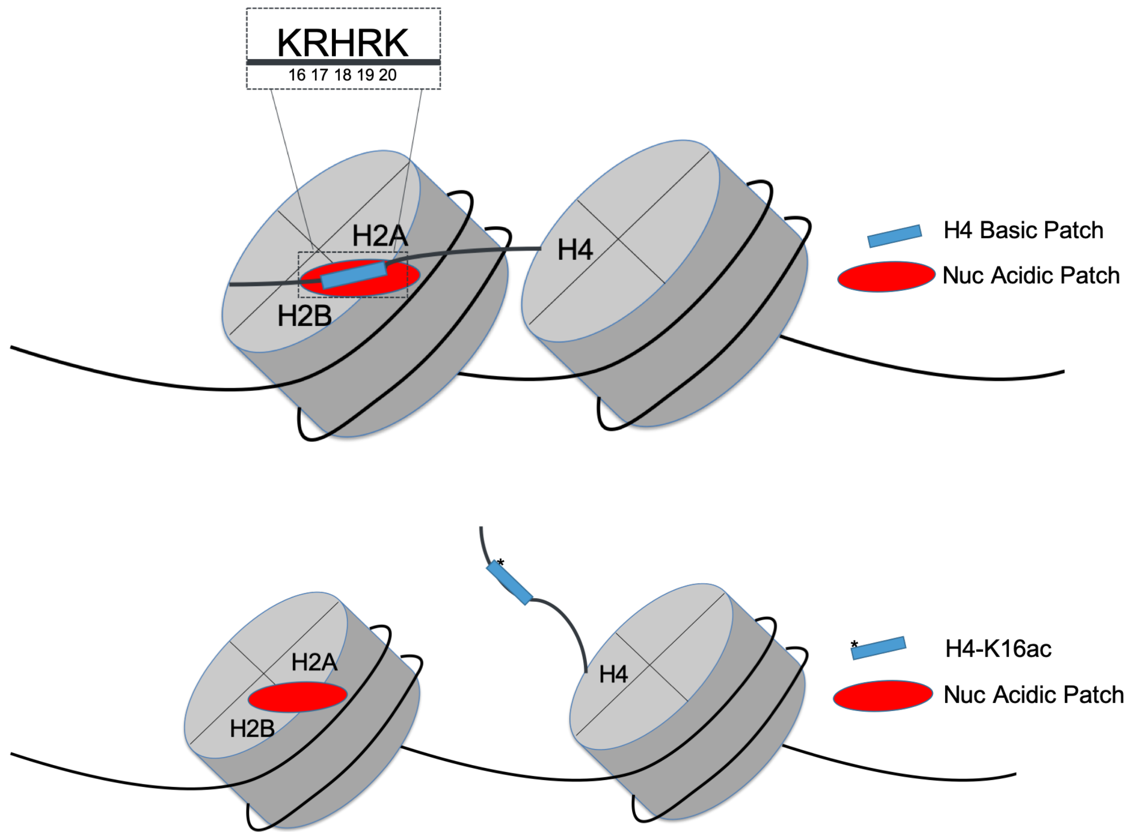

Figure 1.3. Internucleosomal interactions and higher-order chromatin structure are mediated by the H4K16 acetylation

In summary, the regulation of chromatin structure and function is a key determinant in nearly every DNA-templated process. Of particular interest to the Strahl lab is how chromatin structure and function regulate and are regulated by the process of transcription. The next section will provide an overview on the principles of eukaryotic transcription.

1.3 Overview of Eukaryotic Transcription

1.3.1 The Transcription cycle

As multicellular organisms develop, cell-specific genes must be carefully expressed to establish the different cell types. The regulation of genes occurs largely during the transcription of genic DNA to RNA. Therefore, understanding gene regulation requires an in-depth understanding of the process of transcription. In the five decades since the publication of the discovery of three unique eukaryotic RNA polymerases in 1969 (Roeder and Rutter 1969), biochemical, cellular and molecular studies have defined the structural organization of the transcription machinery and provided the mechanistic insights into the regulation of these complexes.

RNA polymerase (RNAP) enzymes catalyze the DNA-dependent synthesis of RNA. In general, RNAP transcription is initiated by the recognition of promoter DNA by RNAP. As described previously, access to promoter DNA is impaired by the presence of nucleosomes and must be removed or shifted (Knezetic and Luse 1986). While many active promoters are found in nucleosome-free regions (NFR), different classes of promoters regulate chromatin opening differently. “Pioneer” transcription factors (TF) bind nucleosomal DNA and enable chromatin opening, often through the recruitment of histone acetyltransferases (HATs) and chromatic remodeling complexes (CRCs) (Iwafuchi-Doi and Zaret 2016; Lorch and Kornberg 2017). Nuclear TFs bind free DNA in a sequence-specific manner and act to guide RNAPs to their target promoters as RNAPs are unable to recognize promoter elements by themselves. Promoter recognition by transcription initiation factors (TFIIs) functions as a bridge between RNAPs and their promoters (Table 1.1) to form pre-initiation complexes (PICs). In the case of RNAP II, the TATA box-binding protein TBP is loaded onto upstream DNA upstream by either TFIID or the

coactivator SAGA in conjunction with TFIIA, and assembles with TFIIB. TFIIB binds the "dock" and "wall" domains of RNAP II and is critical for the recruitment of the RNAPII-TFIIF complex. TFIIB and TFIIF binding to RNAP II functions to stabilize RNAP II in the PIC, facilitate start-site recognition and position DNA in the RNAP II active site, and stabilize the RNA-DNA hybrid in early elongation. Lastly, two activities of TFIIH, DNA unwinding by the ATP-dependent DNA translocase XPB and RNAP II-CTD phosphorylation by CDK7 (Kin28 in yeast), facilitate the transition from transcription initiation to elongation (Reviewed in Cramer 2019).

While many factors are involved during transcription initiation, transcription regulation also occurs during the elongation phase and until recently were poorly understood (Table 1.1). The transition from an initiation to elongation complex occurs when the synthesized RNA reaches a critical length. At this point, the complex can extend the RNA molecule in a processive manner. When RNAP II encounters certain DNA sequences, the addition of nucleotides is interrupted and RNAP II pauses, leading to backtracking and then arrest (Landick 2006). In this situation, TFIIS prevents the backtracking of RNAP II, realigns the DNA-RNA hybrid, and stimulates the nuclease activity of RNAP II to cleave the backtracked transcript so that transcription can be restarted (Cheung and Cramer 2011). Oftentimes in metazoan cells RNAP II pauses approximately 50 base pairs downstream of the TSS and a highly conserved mechanism regulates the release of this promoter-proximal pausing (Core and Adelman 2019). Paused RNAP II involves a shift in the RNA-DNA hybrid that inhibits extension of the RNA molecule and escape. Paused RNAP II is bound and stabilized by the hypophosphorylated forms of DRB-sensitivity inducing factor (DSIF) (Spt4-Spt5 in yeast) and negative elongation factor (NELF) (Yamaguchi, Shibata, and Handa 2013). DSIF binds RNAP II near the exit site of the DNA and

RNA (Ehara et al. 2017; Bernecky, Plitzko, and Cramer 2017) and NELF binds on the opposite side of RNAP II to prevent binding of TFIIS and restrict RNAP II mobility (Vos, Farnung, Urlaub, et al. 2018). Release of paused RNAP II requires positive elongation factor b (P-TEFb) and the kinase activity of its subunit, CDK9. Phosphorylation of DSIF and NELF by P-TEFb allows for the release of paused RNAP II and the phosphorylation of the RNAP II C-terminal domain (CTD) yields an activated elongation complex (Marshall and Price 1995; Zhou, Li, and Price 2012; Vos, Farnung, Boehning, et al. 2018).

Table 1.1. Selected examples of factors for human RNAP II transcription

Transcription Phase	Factor (subunits)	Function
Initiation	TFIIB (1)	Functions as a bridge between RNAP II and promoter DNA
	TFIID (14)	Functions to recognize promoter DNA and to load TBP
	TFIIE (2)	Activates TFIIH and stabilizes the open promoter complex
	TFIIF (2)	Stabilizes TFIIB and the PIC
	TFIIH (10)	Helicase activity promotes DNA opening and kinase activity functions is RNAP II CTD phosphorylation to stimulate promoter escape
	SAGA (18 to 20)	Coactivator complex that functions in TBP loading and has chromatin-modifying activities important for initiation and early elongation
Elongation	DSIF (2)	Promotes RNAP II pausing and active elongation; recruits elongation and 3′ processing factors
	Capping enzymes (3)	Catalyzes 5′ RNA cap formation, to prevent degradation of pre-mRNA by 5′ exonucleases
	NELF (4)	Stabilizes paused RNAP II
	P-TEFb (2)	phosphorylates RNAP II and negative elongation factors to released paused RNAP II
	PAF (5 or 6)	Recruits chromatin-modifying enzymes involved in stimulating elongation
	CHD1 (1)	Co-transcriptional chromatin remodeler
	FACT (2)	Histone chaperone that destabilizes nucleosome for RNAP II passage and reassembles them
	TFIIS (1)	Stimulates RNA cleavage restarts arrested RNAP II with backtracked RNA

1.3.2 RNA Polymerase II C-terminal domain (CTD) Phosphorylation

Critical to the proper transcription of genes is the coordinated phosphorylation and dephosphorylation of the RNAP II C-terminal domain (CTD) (Jeronimo, Bataille, and Robert 2013; Jeronimo, Collin, and Robert 2016). The C-terminal extension of the largest subunit of RNAP II distinguishes it from the other RNAP enzymes. The RNAP II CTD is composed of tandem repeats of the heptapeptide $Y_1S_2P_3T_4S_5P_6S_7$ and this repeat scales with organism complexity, from 26 repeats in yeast to 52 repeats in humans. The CTD is not required for the catalytic activity RNAP II and instead functions as a means to respond to regulatory cues as well as to couple transcription and co-transcriptional events such as RNA processing and chromatin organization. Five of the seven residues can be phosphorylated, including serine 2, 5 and 7, tyrosine 1, and threonine 4. Similar to histone residues and their PTMs, the CTD is believed to serve as a landing pad for various transcription-associated proteins and to date dozens of proteins have reported to bind the CTD (Hsin and Manley 2012).

In yeast, where the CTD cycle has been most extensively characterized, RNAP II is initially recruited to promoters in a non-phosphorylated state. Soon thereafter, the CTD is phosphorylated on Ser5 and Ser7 preceding initiation. As RNAP II clears the initiation site, Ser5-P levels decrease to nearly half their initial levels within the first few hundred base pairs of transcription (Komarnitsky, Cho, and Buratowski 2000). Concurrently, Ser2-P and Tyr1-P levels increase at a steady rate and peak after approximately 1 kb and remain constant throughout the gene until the 3’ end. Dephosphorylation of the CTD at the 3’ end gene commences with Tyr1-P dephosphorylation and is followed by Ser5, Ser7 and Ser2, resetting the CTD and allowing the transcription cycle to restart.

1.3.3 The SAGA coactivator complex

The activation of transcription is an important mechanism for regulating gene expression and is a common endpoint of signaling pathways involved in responses to environmental stimuli and metabolic stress. In addition to the general transcription factors described above, transactivator complexes enhance activated transcription by making direct contact with the basal transcription machinery thereby regulating the formation of the PIC complex and facilitating the transition of the transcription machinery from initiation to elongation through chromatin-modifying activities (Hahn and Young 2011). Two conserved and well-characterized members of this group is the Mediator complex and the Spt-Ada-Gcn5-acetyltransferase (SAGA) coactivator complex.

The Spt-Ada-Gcn5-acetyltransferase (SAGA) coactivator complex is a 1.8 mDa complex composed of 18-20 subunits that is involved in transcription from the early stages of PIC formation through elongation (Hahn and Young 2011). SAGA is highly conserved from yeast to humans and while SAGA subunits are non-essential in yeast, mutation of SAGA subunits lead to developmental defects in drosophila and mice (Weake et al. 2008; Xu et al. 2000). SAGA is comprised of 4 distinct modules: histone acetyltransferase (HAT) activity; deubiquitination (DUB) activity; TBP binding; transcription activator binding (Papai et al. 2020; Wang et al. 2020). The HAT module is comprised of the catalytic subunit, Gcn5, along with Ada2, Ada3 and Sgf29. This module is responsible for acetylating multiple lysine residues on the histone H3 N-terminal tail, including H3K9, K14, K18, K23 and K27 (Cieniewicz et al. 2014). The DUB module consists of the ubiquitin-specific protease, Ubp8, along with Sgf11, Sgf73 and Sus1 to catalyze the removal of H2Bub1. The Spt3 and Spt8 subunits bind TBP and along with the

transactivator binding of Tra1, recruitment the SAGA complex to promoters to function in RNAP II recruitment and PIC formation (Weake and Workman 2012).

Early genome-wide studies in the field established two classes of genes in yeast based upon the preference for SAGA or TFIID to assemble the transcription machinery. The first class known as “SAGA-dominated genes” were those genes that were down-regulated upon deletion of the yeast SAGA-specific subunit, Spt3, and account for approximately 10% of the yeast genome and correspond to mainly stress response genes with a TATA box in their promoters. The remaining 90% were known as “TFIID-dominated genes” and were down-regulated upon deletion of the yeast TFIID-specific subunit, Taf1 (Huisinga and Pugh 2004). However, these observations did not agree with studies reporting colocalization of SAGA subunits with RNAP II and a global effect on H2Bub1 and H3K9ac upon inactivation of SAGA HAT and DUB activities. To address this discrepancy, Bonnet *et al* monitored the distribution of H3K9ac and H2Bub1in yeast and human cells upon activation of their respective enzymes and discovered that SAGA acetylates the promoters and deubiquitinates the transcribed region of all expressed genes and is critical for RNAP II recruitment at all expressed genes (Bonnet et al. 2014). This study established SAGA as a bona fide cofactor for all RNAP II transcription.

While the mechanisms outlined above describe the events that take place in the vicinity of RNAP II, the cell must also contend with the challenges of RNAP II transcription throughout the rest of the gene and the genome. Precise mechanisms are involved with altering the chromatin landscape to regulate these processes and will be described in the following section.

1.4 Mechanisms for altering the chromatin landscape

1.4.1 Histone H2B monoubiquitylation

In budding yeast, monoubiquitylation of histone H2B at lysine-123 (H2Bub1) is catalyzed by the E2 ubiquitin-conjugating enzyme, Rad6, and the E3 ubiquitin ligase, Bre1 and requires the protein Lge1 (Hwang et al. 2003). This modification is conserved through higher eukaryotes. H2Bub1 is associated with active transcription and is enriched at promoters and throughout the gene body. H2Bub1 is a highly dynamic modification due to the competing activities of the ubiquitylation machinery and the presence of deubiquitinating enzymes. In budding yeast, H2Bub1 deubiquitination is catalyzed by two specific H2Bub1 ubiquitin proteases—Ubp8 and Ubp10 (Henry et al. 2003; Emre et al. 2005). The cycling between ubiquitylated and unmodified H2B, referred to as the ubiquitin cycle, plays an important regulatory function in the transition from transcription initiation to elongation (Wyce et al. 2007).

The mechanisms regulating the recruitment of the ubiquitylation machinery vary by location. Upon gene activation, Bre1 is recruited to its target promoter via an activator-dependent interaction. Rad6 is subsequently recruited to Bre1 and ubiquitylation is stimulated by Lge1. In the gene body, Rad6 is associated with the RNA polymerase II associated factor complex (PAFc) and this association was believed to couple ubiquitylation activity to RNAP II activity at actively transcribed genes (Weake and Workman 2008; Fuchs and Oren 2014). However, recent studies have shown that ubiquitylation does not strictly depend upon binding of Rad6-Bre1 to RNAP II (Van Oss et al. 2016). While it was known that Lge1 copurifies with Bre1 and is required for H2Bub1, its biochemical function remained unclear. Lge1 is composed of two domains—a long intrinsically disordered region (IDR) and a short coiled-coil (CC) domain. IDRs are known to

participate in multivalent interactions and this can lead to the phenomenon of liquid-liquid phase separation (LLPS) and the production of biomolecular condensates (Banani et al. 2017; Alberti 2017). *In vitro*, Lge1, via its IDR, functions as a scaffold protein that promotes Bre1 oligomerization and also phase separates under physiological salt conditions (Gallego et al. 2020). In the presence of Lge1 condensates, Bre1 penetrates the condensate and forms a catalytic E3 shell around a Lge1 liquid-like core. Through a direct interaction with Bre1, Rad6 is recruited to the catalytic outer shell and is able to accelerate H2B ubiquitylation. Importantly, this effect is lost upon deletion of the Lge1-IDR. Additionally, yeast cells expressing Lge1 lacking the IDR exhibit reduced H2Bub1 levels downstream of the +1 nucleosome by sequencing exonuclease-treated chromatin immunoprecipitates (ChIP-exo), suggesting LLPS plays a significant role in regulating H2Bub1 in the gene body (Gallego et al. 2020).

Studies on the effect of H2Bub1 on chromatin structure and function have yielded opposing viewpoints. *In vitro* studies suggest that H2Bub1 is a destabilizing mark that impacts chromatin compaction and renders chromatin, and the underlying DNA, more accessible (Fierz et al. 2011). In contrast, multiple *in vivo* studies show that nucleosomes are stabilized and the DNA less accessible due to H2Bub1(Fleming et al. 2008; Mahesh B. Chandrasekharan, Huang, and Sun 2009). Whether these effects are due to the presence of the ubiquitin moiety itself or due to the effectors recruited by H2Bub1 is an important area of open investigation.

H2Bub1 regulates chromatin structure and function in multiple ways. H2Bub1 is required by the histone H3 methyltransferases Set1 and Dot1 to catalyze di- and trimethylation of H3K4 and H3K79, respectively. This phenomenon of one histone PTM regulating a different histone PTM has been described as histone crosstalk (Briggs et al. 2002; Sun and Allis 2002; Dover et al. 2002). The methylation of H3K4 and H3K79 have well-defined functions in transcription and

around actively transcribed genes and will be discussed in further detail below. While the reports of H2Bub1-dependent H3 methylation are nearly two decades old, only recently have we come to understand the mechanism of this crosstalk from structural studies of these enzymes bound to ubiquitylated nucleosomes (P. L. Hsu et al. 2019; Anderson et al. 2019; Worden et al. 2019; Worden, Zhang, and Wolberger 2020).

Elegant structural studies have shown that the ability of Set1 (as a part of the COMPASS complex) to be fully stimulated depends on the COMPASS subunit Spp1, and the RxxxRR motif in the *n*-SET domain of Set1(Kim et al. 2013; P. L. Hsu et al. 2019). COMPASS interacts with the ubiquitinated nucleosomes through multiple subunit interactions with DNA and three of the core histones. Intriguingly, this interaction with the ubiquitinated nucleosome does not cause a conformational shift in the complex that positions the active site in the correct orientation. Rather, this interaction causes a restructuring of the RxxxRR motif to form a helix that enables the complex to interact with the nucleosome acidic patch (Worden, Zhang, and Wolberger 2020). Additionally, COMPASS methylates H3K4 on the opposite side of the nucleosome of the H2Bub1 it interacts with, providing the first known examples of trans-nucleosome histone crosstalk. The asymmetric activity of COMPASS is distinct from Dot1L (the human homolog of yeast Dot1), which methylates H3K79 on the same side of the nucleosome as the H2Bub1 it recognizes. In the case of Dot1L, binding to H2Bub1 contributes to limiting Dot1L mobility stabilizing productive conformations on the nucleosome (Valencia-Sánchez et al. 2019). Similar to COMPASS, H2Bub1binding does not cause a change in the affinity of Dot1L for the nucleosome, instead it has been suggested that the energy required to reorient H3K79 into an accessible through a conformational change of histone H3 is offset by the binding of Dot1L to H2Bub1(Worden et al. 2019). These are two examples of just how rich and complex of a binding

surface H2Bub1 contributes to the nucleosome. Ubiquitin is large and surprisingly flexible and, as our understanding of how H2Bub1 regulates other chromatin-modifying enzymes, reveals the remarkable plasticity of how H2Bub1 interacts and activates these different enzymes.

H2Bub1 is also required for the efficient reassembly of nucleosomes in the wake of elongating RNAP II. This function occurs in cooperation with the histone chaperone FACT and both factors are involved in the regulation of the other (Fleming et al. 2008).

1.4.2 Histone H3 methylation

Methylation of histone lysine residues represents a major mechanism of transcription regulation. Interestingly, lysine methylation can lead to both activation or repression depending on the modification and its genomic locus. Well established examples of activating lysine methylation include H3K4 and H3K79. Repressive lysine methylation in higher eukaryotes is exemplified by the nucleating marks at H3K9 and H3K27. While some histone PTMs such as phosphorylation and acetylation alter the charge of the residue, therefore impacting electrostatic interactions of the histone with DNA or intra- and internucleosomal contacts, lysine methylation is a net neutral modification and functions to recruit or occlude the binding of effector proteins. Below is a description of the importance of histone H3 methylation.

Histone H3 Lysine-4 Methylation

Histone H3 lysine-4 methylation (H3K4me) is involved in regulating transcription initiation and elongation through the precise recruitment of effector proteins to methylation-specific regions (Wozniak and Strahl 2014b). In yeast, all three state of H3K4me is catalyzed the SET-domain containing histone methyltransferase, Set1. H3K4me is a highly conserved histone PTM and in humans is catalyzed by multiple enzymes including SET1A/B and the mixed lineage

leukemia proteins MLL1-4 (Wu et al. 2008; Shilatifard 2012). Whether by a single yeast enzyme or multiple human enzymes, the regulated recruitment of the H3K4 methyltransferases is critical in establishing the pattern of H3K4 mono-, di- and trimethylation at genes and ultimately recruiting effector proteins involved in regulating transcription. In this regard, H3K4me is perhaps the best characterized example of a transcription-associated histone PTM.

Early genome-wide studies defined a H3K4me3 peak near the TSS of many actively transcribed genes, suggesting a role for this PTM in the regulation of transcription initiation. Further studies identified the association of the general transcription factor TFIID with H3K4me3 and that this association was mediated by the PHD finger of the TFIID subunit, TAF3 (Vermeulen et al. 2007; van Nuland et al. 2013; Lauberth et al. 2013) Additionally, H3K4me3 is important for the recruitment and stabilization of chromatin remodelers with their genomic targets. While human CHD1 is recruited to chromatin through an interaction with the mediator complex, it is believed that CHD1 binding H3K4me3 through its tandem chromodomains (Flanagan et al. 2005; Sims et al. 2005) may stabilize its interaction with chromatin (Lin et al. 2011). Another remodeler, the NURF complex, associates with H3K4me3-rich regions through the PHD finger-containing subunit, BPTF. Lastly, H3K4me3 is also responsible for its own histone crosstalk activity. In this scenario, subunits of two histone acetyltransferase complexes, NuA3 and SAGA, have been shown to bind H3K4me3 to effect HAT activity on the histone H3 N-terminal tail (John et al. 2000; Taverna et al. 2006; Martin et al. 2006; Vermeulen et al. 2010; Bian et al. 2011). Although the focus of this section has been on the role of H3K4me3, it is interesting to point out that, in contrast to H3K4me3, H3K4me1 is a PTM commonly associated with enhancer elements (Calo and Wysocka 2013) but that our overall understanding of this PTM deserves further investigation.

Histone H3 Lysine-79 Methylation

Unique amongst histone KMTs is the histone H3 lyisne-79 (H3K79) methyltransferase, Dot1. Originally discovered in a genetic screen to identify genes that disrupted telomeric silencing upon overexpression (Singer et al. 1998), yeast Dot1 and its homologs in other organisms, such as human DOT1L, contain a catalytic methylase fold conserved amongst class I SAM-dependent methylases (Min et al. 2003; Sawada et al. 2004). Dot1 is responsible for catalyzing mono-, di- and trimethylation of H3K79 (Ng, Feng, et al. 2002; Feng et al. 2002; Lacoste et al. 2002; Van Leeuwen, Gafken, and Gottschling 2002) in a nonprocessive manner (Frederiks et al. 2008). Unlike all other known methylated histone lysine residues, H3K79me has no known demethylase and the mechanism for removing this modification remain poorly understood. Yet, several reports suggest that H3K79me may be dynamically regulated as H3K79me2 levels have been shown to fluctuate with the cell cycle (Feng et al. 2002; Schulze et al. 2009). Dot1 catalytic activity is regulated by two regions on the NCP, the histone H4 basic patch and H2Bub1 (Fingerman, Li, and Briggs 2007; Ng, Xu, et al. 2002; Sun and Allis 2002; Briggs et al. 2002) and the details of this regulation have been recently characterized by structural studies (Valencia-Sánchez et al. 2019; Anderson et al. 2019; Worden et al. 2019).

H3K79me is enriched throughout the transcribed regions of genes and is associated with active transcription due to the dependence of Dot1 on H2Bub1 (Briggs et al. 2002). As such, it has been long assumed that Dot1 and H3K79me function in regulating transcription elongation. As previously mentioned, Dot1 was discovered in a genetic screen for genes that disrupt telomeric silencing. Yeast telomeric silencing is mediated through the binding and spreading of the SIR (Silent Information Regulator) complex composed of Sir2, Sir3, and Sir4. Sir3 binds the NCP through the histone H4 basic patch and unmodified H3K79 and recruits the HDAC Sir2 to

deacetylate H4K16ac leading to nucleation of the SIR complex and compaction of the chromatin fiber (Katan-Khaykovich and Struhl 2005; Altaf et al. 2007; Onishi et al. 2007; van Welsem et al. 2008). The competition between Dot1 and Sir3 to bind the H4 basic patch and alter the methylation of H3K79 is delicately balanced to prevent heterochromatin spreading into gene bodies and to regulate heterochromatin formation at telomeres, although to a lesser extent than originally thought (Rossmann et al. 2011; Takahashi et al. 2011).

Recently, Lee *et al* reported that Dot1, via its histone binding domain, can assemble nucleosomes from core histones and facilitate chromatin remodeling and that this activity is independent of its methyltransferase activity (S. Lee et al. 2018).

1.4.3 Histone acetylation

Histone acetylation is the oldest described histone modification and arguably one the best characterized. Histone acetylation is a dynamic process that is regulated by the opposing effects of histone acetyltransferases (HATs) and histone deacetylases (HDACs). The addition of an acetyl group to the ε-amino group of lysine side chains neutralizes the lysine residue's positive charge, thereby altering the electrostatic interactions between the nucleosome and DNA. In general two types of HATs exist: Type-B HATs that acetylate free histones and is important for the deposition of newly synthesized histones and Type-A HATs that acetylate histones in the NCP (Bannister and Kouzarides 2011). Type-A HATs are known to modify multiple residues on the N-terminal tails of H3 and H4 as well residues on the globular histone core, such as histone H3-lysine 56 (Tjeertes, Miller, and Jackson 2009).

Histone acetylation is often associated with active transcription due to its ability to neutralize the histone-DNA interaction, making the chromatin more accessible to the transcription machinery. Additionally, histone acetylation functions to recruit transcription-

associated complexes via their bromodomain-containing subunits, such as TFIID and the RSC chromatin remodeling complex (Jacobson et al. 2000; Kasten et al. 2004). In the coding regions, specific sites in the promoter and the 5' end of genes are enriched in acetylation. The discovery of the HAT activity of Gcn5, the catalytic subunit of the SAGA coactivator complex, was a seminal discovery that served to bridge the distinct fields of chromatin biology and transcription and catalyze the pursuit towards understanding the role of histone PTMs in transcription (Kuo et al. 1996). Gcn5 acetylates multiple lysine residues on the histone H3 N-terminal tail and is involved, through the multifaceted function of the SAGA complex, regulating transcription initiation and chromatin remodeling (Kuo et al. 1996; Grant et al. 1997).

The opposing activity of HDACs is also important for proper gene expression. Although histone acetylation is important for increasing chromatin accessibility, proper regulation is required to ensure that sites within the gene body that look like promoters, known as "cryptic" promoters, are not made accessible to the transcription machinery and initiate aberrant transcription. In yeast, Rpd3 is a RNAP II-associated HDAC that deacetylates histones in the gene body during transcription elongation. Therefore, the function of HDACs to reduce chromatin accessibility in a transcription-coupled manner is a mechanism to increase transcription efficiency and maintain chromatin integrity (Carrozza et al. 2005; Keogh et al. 2005; Li et al. 2007; 2009).

1.4.4 Nucleosome Dynamics

Histone Chaperones

The essential function of chromatin—to package the cell's genetic material—ultimately results in a challenge of accessibility for the transcription machinery. For proper transcription to occur, nucleosomes must be disassembled ahead of the traveling RNAP II and reassembled in its

wake. Making and breaking nucleosomes is carried out by histone chaperones in an ATP-independent manner. Broadly defined, histone chaperones can be defined as proteins involved in the storage, transport, disassembly and assembly of histones. While all histone chaperones bind histones generally, the specificity of their interactions and their structure and function in the cell vary greatly. Structural and biochemical studies have revealed the ability of histone chaperones to bind histone dimers, tetramers, and nucleosome assembly intermediates (Hammond et al. 2017). Binding nucleosome assembly intermediates is of critical importance to the proper disassembly and correct reassembly of nucleosomes during transcription elongation. Transcription-associated histone chaperones, such as Spt6 and FACT, are essential to resetting the chromatin landscape and preventing the initiation of aberrant transcription from cryptic promoters within the gene body.

Chromatin Remodelers

In contrast to the ATP-independent function of histones chaperones, chromatin remodelers harness the energy of ATP hydrolysis to drive a DNA translocase resulting in the sliding or eviction of nucleosomes from a specific region of the genome (Clapier et al. 2017). The regulation of genome-wide nucleosome occupancy and composition by chromatin remodelers serves three functions. Firstly, chromatin remodelers maintain the proper density and spacing of nucleosomes across promoters and gene bodies contribute to gene repression. Secondly, chromatin remodelers facilitate the binding of TFs to DNA by cooperating with site-specific TFs and histone writers to slide or displace histones. Lastly, chromatin remodelers establish functionally unique regions across the genome through the replacement of canonical histones with histone variants. Chromatin remodelers can be classified into four subfamilies,

imitation switch (ISWI), chromodomain helicase DNA-binding (CHD), switch/ sucrose non-fermentable (SWI/SNF) and INO80 (Clapier et al. 2017).

1.5 Importance of study and description of work

The introduction presented here is by no means an exhaustive study of chromatin biology but a snapshot into one of the most fundamental mechanisms regulating nearly every DNA-templated process. As our understanding of how histones and their modifications regulate chromatin structure and function advances, we are beginning to understand how histones mutants and aberrant regulation of chromatin are involved in the development and progression of human diseases such as cancer and neurological disorders (Zoghbi and Beaudet 2016). Yet, our understanding of the function of the vast majority of histone modifications, and even less so of their regulation, is still in its infancy. Therefore, pursuing the study of the fundamental mechanisms regulating histone modifications and chromatin structure are essential to understanding their dysregulation in disease and how to develop effective treatments where possible.

The work presented in this thesis focuses on understanding how a small stretch of a basic residues on the histone H4 tail, known as the H4 basic patch, regulates one particular histone PTM, histone H2B monoubiquitylation (H2Bub1). H2Bub1 is conserved from yeast to humans and is involved in multiple DNA-templated processes, including transcription, DNA repair, and replication. Importantly, proper regulation of the enzymes involved in placing and removing H2Bub1 are necessary for maintaining genomic stability whereas their dysregulation is involved in tumorigenesis (Johnsen 2012). This work began as an attempt to understand how the H4 basic patch regulates the H3K79 methyltransferase, Dot1, where I discovered that loss or mutation of the H4 basic patch led to reduced Dot1 protein levels and a significant reduction in global

H2Bub1 levels. This work is presented in Chapter 2. I then pursued the goal of determining the mechanism behind this newly discovered function of the H4 basic patch in regulating H2Bub1. This study identified the function of the H4 basic patch as a regulator of the histone-modifying activities of the SAGA coactivator complex. This work is presented in Chapter 3. Lastly, in Chapter 4 I attempt to posit a few hypotheses regarding the work presented in the preceding chapters and place them into the larger context of chromatin regulation. Overall, the study presented here defines a new function for the histone H4 basic patch and greater insights into the regulation of H2Bub1.

CHAPTER 2: IDENTIFICATION OF THE H4 BASIC PATCH AS A REGULATOR OF DOT1 PROTEIN LEVELS AND HISTONE H2B UBIQUITYLATION

2.1 Overview

Histone "cross-talk" can be defined as the phenomenon of a histone post-translation modification (PTM) regulating the placement or removal of a different histone PTM. This cross-talk is a fundamental mechanism by which histone PTMs regulate the structure and function of chromatin. One of the earliest characterized examples of histone cross-talk involves the H3K79 methyltransferase, Dot1. In order to achieve H3K79 di- and trimethylation, Dot1 requires monoubiquitylation of histone H2B (H2Bub1) and a stretch of basic residues of the histone H4 N-terminal tail called the H4 basic patch. In the absence of either of these factors, H3K79me2/3 is absent. However, it remains unclear if this regulatory cross-talk occurs in multiple directions. Here we report that the histone H4 basic patch is involved in the regulation of Dot1 protein levels and in the maintenance of global H2Bub1 levels. Loss of the entire H4 basic patch (residues 16-20) or arginine to alanine mutations of the two arginine residues leads to a significant decrease in Dot1 protein levels. Additionally, these same mutations also lead to a significant decrease of global H2Bub1 levels through a Dot1 and H3K79methylation independent mechanism.

2.2 Introduction

The nucleosome is the fundamental repeating unit of chromatin and the first level of chromatin organization. Composed of two copies each of histones H2A, H2B, H3 and H4, the

histone octamer is wrapped by approximately 147 base pairs of DNA to form the nucleosome core particle (NCP), and is composed of a globular core and the N-terminal "tail" domains. While forming the basis of all subsequent forms of higher-order chromatin structure, the nucleosome regulates accessibility to the DNA elements wrapped around it, serving as a gatekeeper to many DNA-templated processes by regulating access to these elements. One important mechanism in regulating the structure and function of chromatin is through the post-translational modification (PTM) of the histone tails that serve to recruit or occlude effector protein binding. These histone PTMs include acetylation, methylation, phosphorylation, and ubiquitylation amongst others (Zhao and Garcia 2015) While the first studies reporting histone acetylation were published over 50 years ago (Allfrey, Faulkner, and Mirsky 1964), we are still discovering new PTMs at a faster rate than we can determine the function of.

In addition to histone PTMs recruiting chromatin-modifying enzymes, specific regions of the nucleosome itself are involved in regulating the structure and function of chromatin. One region of interest is a short stretch of basic residues ($K_{16}R_{17}H_{18}R_{19}K_{20}$) on the N-terminal tail of histone H4 known as the H4 basic patch. This portion of the H4 tail was described in the original 2.8Å crystal structure by Luger *et al* due to its interaction with an acidic patch on the H2A/H2B interface of a neighboring nucleosome (Luger et al. 1997). Additionally, competition for binding of the H4 basic patch between the yeast silencing protein, Sir3, and the yeast histone H3 lysine-79 (H3K79) methyltransferase, Dot1, is believed to function in defining the boundary between euchromatin, containing actively transcribed genes, and heterochromatin, or regions of silenced or repressed genes (Fingerman, Li, and Briggs 2007; Altaf et al. 2007). Lastly, the H4 basic patch positively regulates the ATPase activity of the ISWI chromatin remodeling complex (C. R. Clapier et al. 2001; C. R. Clapier 2002; Hamiche et al. 2001; Cedric R. Clapier and Cairns 2012).

ISWI is involved in properly spacing nucleosomes and binding of the H4 basic patch relieves an autoinhibition and stimulates ATPase activity.

H3K79 methylation is a highly conserved histone PTM that is associated with active transcription. Dot1 is the methyltransferase responsible for H3K79me and it is able to catalyze mono-, di-, and trimethylation of H3K79. Dot1 is unique amongst lysine methyltransferases (KMT) because it is the only known KMT that does not contain a SET domain. Dot1 was originally discovered in a genetic screen for genes whose overexpression disrupted telomeric silencing and was subsequently identified as the H3K79 methyltransferase. H3K79 di- and trimethylation by Dot1 requires the histone H4 basic patch and another histone PTM, histone H2B monoubiquitylation (H2Bub1) (Ng, Xu, et al. 2002; Briggs et al. 2002; Fingerman, Li, and Briggs 2007). The regulation of the placement of one histone PTM by the presence of another histone PTM has been termed histone "cross-talk" and is one of the defining features of the histone-code hypothesis (Strahl and Allis 2000).

The function of Dot1 and H3K79me in transcription has puzzled the field. There are not many well defined effector proteins that bind H3K79me, although it has been shown that the yeast silencing protein, Sir3, preferentially interacts with the unmodified H3K79 and that methylation disrupts this interaction. However, H3K79me is definitely associated with active transcription, as its catalysis requires H2Bub1, also a mark of active transcription, and ChIP-seq experiments have verified its localization to the gene body (Weiner et al. 2015). To try and address this gap in the field, we sought to understand the function of H2Bub1 and the H4 basic patch in regulating H3K79me. We found that Dot1 protein levels were significantly reduced upon deletion of Paf1 (*paf1*Δ), one of the core subunits of the Paf1 complex that is responsible for recruiting the H2Bub1 machinery. Additionally, we found that complete loss of the H4 basic

patch ($H4_{\Delta 16\text{-}20}$) or an arginine-to-alanine mutation within the H4 basic patch ($H4_{2RA}$) also resulted in a significant reduction of Dot1 protein levels. There were no previous reports of either regulatory arm of Dot1 affecting the stability of the protein and this led us to investigating whether the crosstalk between these modifications was multidirectional. Strikingly, we found that both the $H4_{\Delta 16\text{-}20}$ and the $H4_{2RA}$ mutants result in a significant reduction in H2Bub1, identifying a novel regulator of this critical histone PTM.

2.3 Materials and methods

2.3.1 Yeast Strains

Strains and plasmids used in this study are listed in Tables 2.11 and 2.2. Gene disruptions and endogenous overexpression were performed as described (C. Janke et al. 2004) and verified by PCR and immunoblotting. Plasmids were generated using a standard site-directed mutagenesis protocol and primers were designed with the Agilent QuikChange Primer Design tool (www.agilent.com/store/primerDesignProgram.jsp).

2.3.2 Preparation of whole-cell extracts and immunoblots

Equivalent OD_{600} units of each asynchronous log phase culture were collected by centrifugation and frozen at -80 ˚C. Cells were subjected to glass bead lysis in SUMEB (1% SDS, 8 M urea, 10 mM MOPS, pH 6.8, 10 mM EDTA, 0.01% bromophenol blue) by vortexing at 4 °C for 3 minutes. Extracts were retrieved and clarified by centrifugation at max speed for 2 minutes and boiled at 95 °C for 5 minutes. 10 µL of whole cell extract were loaded on 8% and 15% SDS-PAGE gels. Proteins were transferred to 0.45 µm PVDF membranes using a Hoefer Semi-Dry Transfer Apparatus at 45 mA per membrane. Primary antibodies were incubated in 5% milk at 4 °C overnight and secondary antibodies were incubated in 5% milk for 1 h.

2.3.3 Antibodies

Immunoblots were developed using ECL Prime (Amersham RPN2232). Antibodies:G6PDH (Sigma-Aldrich A9521; 1:100,000), FLAG-M2 (Sigma-Aldrich F1804; 1:5,000), Ubiquityl-Histone H2B (Cell Signaling Technologies 5546; 1:5,000), H2B (Active Motif 39237; 1:5,000), Dot1 (Strahl Lab; 1:2,500). Rabbit (Amersham NA934; Donkey anti-Rabbit) and mouse (Amersham NA931; Sheep anti-mouse) secondary antibodies were used at 1:10,000.

2.4 Results

The H4 basic patch regulates Dot1 protein levels

In the absence of the H4 basic patch, Dot1 is unable to catalyze H3K79 di- and trimethylation, yet the mechanism behind this regulation was poorly understood. Studies published from the Strahl lab on Bre1, the H2Bub1 E3-ligase, highlighted the important role that regions of the histone required by Bre1 for catalysis has on the stability of the protein (Wozniak and Strahl 2014a). Therefore, we hypothesized that the interaction between the H4 basic patch and Dot1 was important for the stability of the protein and that in the absence of efficient catalysis of H3K79 di- and trimethylation, the protein may be degraded. To this effect we generated an endogenous C-terminal FLAG-tagged Dot1 construct in $H4_{WT}$, $H4_{\Delta 16\text{-}20}$ and $H4_{2RA}$ cells. After validating the constructs by immunoblot analysis (data not shown), we performed immunoblot analysis for Dot1 (FLAG) and G6PDH of whole-cell extracts prepared from cells grown to mid-log phase. Immediately, we noticed a significant reduction in Dot1 protein levels in both the $H4_{\Delta 16\text{-}20}$ and $H4_{2RA}$ cells (Figure 2.1). We observed this reduction in multiple repeats of the experiment, suggesting that this was not an artifact. Additionally, observing this reduction in both the $H4_{\Delta 16\text{-}20}$ and $H4_{2RA}$ cells suggests that the two arginine residues in the basic patch are

the most critical for regulating Dot1 protein levels, in agreement with these two residues being the most critical for regulating H3K79 di- and trimethylation (Fingerman, Li, and Briggs 2007).

The Histone H4 basic patch is involved in the regulation of H2Bub1

Little is known about the regulation of Dot1 protein levels and whether regulation of the stability of this protein is involved in regulating its catalytic activity or recruitment. Because Dot1 also requires H2Bub1 for its catalytic activity, we performed a similar immunoblot analysis for Dot1 levels in a strain background containing individual deletions of members of the Paf1 complex. This complex is directly involved in regulating H2Bub1 levels by recruiting the ubiquitylation machinery and mutations affecting the integrity of the complex are deficient in H2Bub1 and H3K79me. Interestingly, deletion of Paf1 (*paf1*Δ) an integral subunit of the complex also leads a significant decrease in Dot1 protein levels (Figure 2.2).

In *paf1*Δ cells, both H2Bub1 levels and Dot1 protein levels are affected, which prompted us to question whether loss or mutation of the H4 basic patch, which led to reduced Dot1 protein levels, could have an effect on H2Bub1. Strikingly, immunoblot analysis for H2Bub1 and H2B in $H4_{WT}$, $H4_{\Delta16\text{-}20}$ and $H4_{2RA}$ cells show a significant decrease in global H2Bub1 levels in the $H4_{\Delta16\text{-}20}$ and $H4_{2RA}$ cells (Figure 2.3). Additionally, we measured H2Bub1 levels in cells with a Dot1 deletion (*dot1*Δ) or expressing a H3K79R mutant and found no effect on H2Bub1 levels (data not shown). To our knowledge this is the first description for a role of the Histone H4 basic patch in regulating H2Bub1.

2.5 Conclusion

In this study we report two new regulatory functions of the histone H4 basic patch: maintenance of Dot1 protein levels and histone H2Bub1. The requirement of the histone H4

basic patch and H2Bub1 for di- and trimethylation of H3K79 by Dot1 has been known for over a decade, yet only recently has the mechanism been elucidated (Worden et al. 2019; Anderson et al. 2019; Valencia-Sánchez et al. 2019). The H4 basic patch and H2Bub1 function to position Dot1 onto the nucleosome in an optimal position to increase the efficiency of its catalytic activity. However, no reports have established a link between Dot1 stability and binding or catalysis. It is conceivable that the regulation of Dot1 stability involves the binding and stabilization of Dot1 on the nucleosome, independent of its catalytic activity, thereby protecting it from degradation by the proteasome. Interestingly, loss of H2Bub1 also led to a decrease in Dot1 protein levels suggesting again that the inability of Dot1 to be bound and positioned properly on the nucleosome may lead to its degradation. Future studies will be required to determine the extent of this regulation.

Of considerable interest is the novel finding that the loss or mutation of the H4 basic patch significantly reduces global H2Bub1 levels. This regulation has not been described previously and is of utmost importance considering the various functions H2Bub1 performs in regulating chromatin structure and function as well as in transcription. A detailed study of the regulation of the H4 basic patch on H2Bub1 is presented in chapter 3.

2.6 Figures

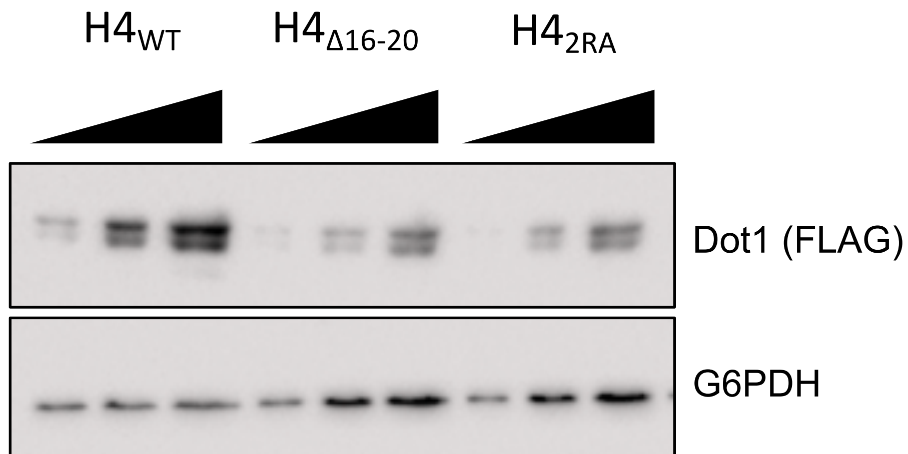

Figure 2.1. Dot1 protein levels are reduced upon loss or mutation of the H4 basic patch. Immunoblotting was performed for the indicated proteins using extracts from cells that expressed wild-type H4 ($H4_{WT}$), H4 basic patch delete ($H4_{\Delta 16\text{-}20}$) or the H4 basic patch mutant ($H4_{2RA}$). Primary antibody against the FLAG epitope was used to probe for endogenously tagged Dot1. G6PDH was used as a loading control.

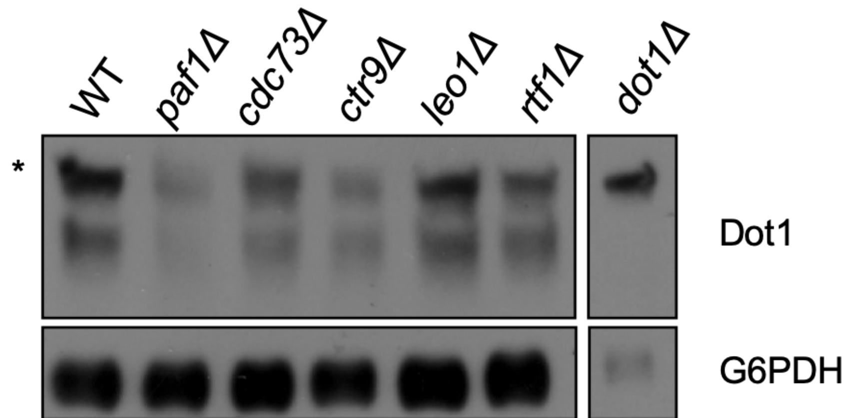

Figure 2.2. Dot1 protein levels are reduced in *paf1*Δ and *ctr9*Δ cells. Immunoblotting was performed for Dot1 and G6PDH using extracts from cells that with the indicated gene deletions of the individual PAF1 complex members. Primary antibody against Dot1 was used to probe for Dot1 levels and G6PDH was used as a loading control.

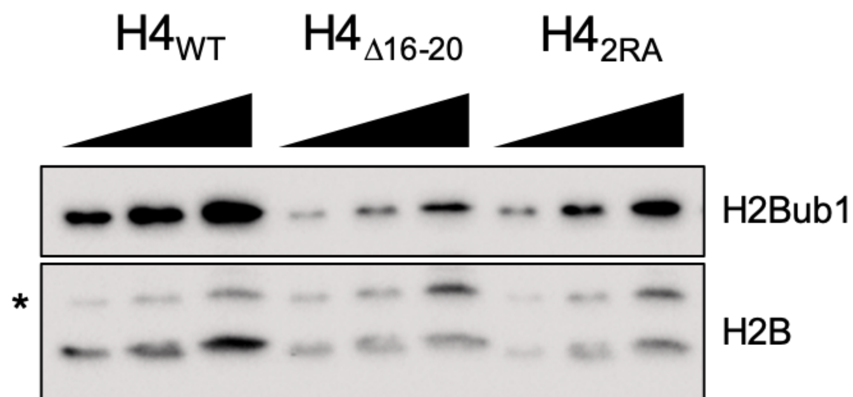

Figure 2.3. Global H2Bub1 levels are reduced upon loss or mutation of the H4 basic patch. Immunoblotting was performed for H2Bub1 and H2B using extracts from cells that expressed wild-type H4 ($H4_{WT}$), H4 basic patch delete ($H4_{\Delta 16\text{-}20}$) or the H4 basic patch mutant ($H4_{2RA}$). Primary antibody against H2Bub1 was used to measure global H2Bub1 levels and H2B was used as a loading control.

2.7 Tables

Table 2.1. Yeast strains used in this study

Name	Genotype
YJJ577	*MAT*α *his3*Δ*200 ura3-52 leu2*Δ*1 paf1*Δ::*HIS3*
YJJ662	*MATa his3*Δ*200 ura3-52 leu2*Δ*1*
YJJ665	*MATa his3*Δ*200 ura3-52 leu2*Δ*1 cdc73*Δ::*HIS3*
YJJ1197	*MATa his3*Δ*200 ura3-52 leu2*Δ*1 ctr9*Δ::KanR
YJJ1303	*MATa his3*Δ*200 ura3-52 leu2*Δ*1 rtf1*Δ::KanR
YJJ1336	*MATa his3*Δ*200 ura3-52 leu2*Δ*1 leo1*Δ::KanR
YGW187	*MATa his4-912 lys2-128 ura3-52 leu2*Δ*1 his3*Δ*200 trp1*Δ*63 hht1-hhf1::LEU2 hht2-hhf2::HIS3 dot1*Δ*::hygro* [YCp(TRP1)::HHT2-HHF2]
YHM007	MATa his4-912∂ lys2-128∂ ura3-52 leu2Δ1 his3Δ200 trp1Δ63 *hht1-hhf1*Δ::LEU2 hht2-hhf2Δ::HIS3 *DOT1-3xFlag::KanMX* [pJH18]
YHM009	MATa his4-912∂ lys2-128∂ ura3-52 leu2Δ1 his3Δ200 trp1Δ63 *hht1-hhf1*Δ::LEU2 hht2-hhf2Δ::HIS3 *DOT1-3xFlag::KanMX* [YCp(TRP1)::HHT2-HHF2(Δ16-20)]
YHM016	MATa his4-912∂ lys2-128∂ ura3-52 leu2Δ1 his3Δ200 trp1Δ63 *hht1-hhf1*Δ::LEU2 hht2-hhf2Δ::HIS3 *DOT1-3xFlag::KanMX* [pHM01]

CHAPTER 3: THE HISTONE H4 BASIC PATCH REGULATES SAGA-MEDIATED H2B DEUBIQUITINATION AND HISTONE ACETYLATION[1]

3.1 Overview

Histone H2B monoubiquitylation (H2Bub1) has central functions in multiple DNA-templated processes including gene transcription, DNA repair, and replication. In addition, H2Bub1 is required for the trans-histone regulation of H3K4 and H3K79 methylation. Although previous studies have elucidated the basic mechanisms that establish and remove H2Bub1, we have only an incomplete understanding of how H2Bub1 is regulated. We report a novel regulator of H2Bub1 – the histone H4 basic patch. Yeast cells with mutations in the H4 basic patch ($H4_{2RA}$) exhibited significant loss of global H2Bub1. $H4_{2RA}$ mutant strains also displayed chemotoxin sensitivities similar to, but less severe than, strains containing a complete loss of H2Bub1. The H4 basic patch regulates H2Bub1 levels independently of interactions with chromatin remodelers and separately from its regulation of H3K79 methylation. To measure H2B ubiquitylation and deubiquitination kinetics in vivo, we used a rapid and reversible optogenetic tool, LINX (light-inducible nuclear exporter), to control the subcellular location of the H2Bub1 E3-ligase, Bre1. The ability of Bre1 to ubiquitylate H2B was unaffected in the $H4_{2RA}$ mutant. In contrast, H2Bub1 deubiquitination by SAGA-associated Ubp8, but not by

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[1] This research was originally published in the Journal of Biological Chemistry. Meriesh HA, Lerner AM, Chandrasekharan MB, Strahl BD. The histone H4 basic patch regulates SAGA-mediated H2B deubiquitination and histone acetylation [published online ahead of print, 2020 Apr 3]. *J Biol Chem.* 2020;jbc.RA120.013196. doi:10.1074/jbc.RA120.013196. © the American Society for Biochemistry and Molecular Biology or © the Author(s).

Ubp10, increased in the $H4_{2RA}$ mutant. Consistent with a function for the H4 basic patch in regulating SAGA deubiquitinase activity, we also detected increased histone acetylation by SAGA in H4 basic patch mutants. Our findings uncover a new regulatory mechanism of the H4 basic patch in SAGA-mediated functions.

3.2 Introduction

The nucleosome – the fundamental repeating unit of chromatin – is the first level of chromatin organization, and it is essential for the regulation of nearly all DNA-templated processes. Composed of an octamer of histone proteins, two molecules each of histones H2A, H2B, H3 and H4, the nucleosome is a partial barrier to functions such as gene transcription and DNA repair; hence, nucleosomes must be disrupted transiently for these processes to occur. One major mechanism that contributes to the transient disruption of nucleosomes is histone post-translational modifications (PTMs). Histone PTMs are found both in the "tail" domains, which largely influence the recruitment of effector proteins (i.e., readers) and in their globular domains, which largely influence nucleosome-DNA interactions (Lawrence, Daujat, and Schneider 2016).

In addition to histone PTMs being major influencers of chromatin structure and function, histones also contain basic and acidic regions or "patches" that govern nucleosome-nucleosome interactions or the ability of readers to engage the nucleosome. One such region is the nucleosome acidic patch, a negatively charged cavity formed between histones H2A and H2B that contributes to chromatin function by regulating association of a multiple chromatin-modifying enzymes (Kalashnikova et al. 2013). Another region, the H4 basic patch located in the H4 tail domain (between residues 16 and 20), contains multiple basic residues, i.e., arginine, histidine, and lysine (Figure 1A). This segment of basic residues has important functions in regulating chromatin dynamics and multiple chromatin-modifying enzymes. For example, the H4

basic patch is required for efficient Dot1-mediated histone H3 lysine 79 di- and trimethylation (H3K79me2 and H3K79me3) (Fingerman, Li, and Briggs 2007; Altaf et al. 2007). Additionally, the basic patch has a key function in maintaining the balance of heterochromatin domains in yeast by interacting with the silencing protein Sir3 when H4K16 is unacetylated (Altaf et al. 2007). Acetylation of K16 within the basic patch is critical in chromatin organization (Shogren-Knaak et al. 2006). Recently, the H4 basic patch has been shown to function as a positive regulator of the ISWI family of chromatin remodeling complexes (Cedric R. Clapier and Cairns 2012) and as a regulator of Snf2 (Racki et al. 2014; Liu et al. 2017). Chd1, another remodeler that organizes nucleosomes across the coding regions of genes, also interacts with the H4 N-terminus (Sundaramoorthy et al. 2018).

H2Bub1 is a dynamic histone PTM enriched at promoters and across the transcribed regions of genes. This modification is associated with transcription elongation and maintaining chromatin integrity through its conserved trans-histone regulation of H3K79me and H3K4me (Dover et al. 2002; Sun and Allis 2002; Briggs et al. 2002; Ng, Xu, et al. 2002; Fleming et al. 2008; Mahesh B. Chandrasekharan, Huang, and Sun 2009; Weake and Workman 2008). In yeast, H2Bub1 is catalyzed by the E2/E3 ligases Rad6 and Bre1 (Robzyk, Recht, and Osley 2000; Wood et al. 2003; Kao et al. 2004) and is removed by the deubiquitinases Ubp8 and Ubp10. As a part of the SAGA coactivator complex, Ubp8 – the catalytic subunit of the SAGA deubiquitinase (DUB) module – deubiquitinates H2Bub1 near the promoters and transcription start sites (TSS) of virtually all expressed genes in eukaryotic cells to promote transcription by RNA polymerase II (Batta et al. 2011; Schulze et al. 2011; Bonnet et al. 2014; Baptista et al. 2017). This cycle of ubiquitylation and deubiquitination is important for the regulation of early elongation and the establishment of serine 2 phosphorylation on the C-terminal domain of RNA polymerase II

(Henry et al. 2003; Wyce et al. 2007). Monomeric Ubp10 regulates the pattern of H2Bub1 within gene bodies and intergenic regions. Initially identified for its role in maintaining silencing of subtelomeric genes, Ubp10 has been shown to be important in maintaining global H2Bub1 levels (Schulze et al. 2011; Emre et al. 2005; R. G. Gardner, Nelson, and Gottschling 2005; Orlandi et al. 2004). Nune *et al.* have described the coordinated activities of Ubp10 and the histone chaperone FACT in H2Bub1 deubiquitination and nucleosome disassembly and reassembly (Nune et al. 2019).

To further understand the function of the H4 basic patch, we investigated the possibility that there are yet-to-be elucidated aspects of histone PTM crosstalk involving this histone region. In this report, we show that the H4 basic patch is required to maintain proper H2Bub1 levels. Although the basic patch is required for Dot1-mediated H3K79me, we found that the ability of the H4 basic patch to regulate H2Bub1 is independent of Dot1 or H3K79 methylation. Furthermore, we found that the ability of the H4 basic patch to regulate H2Bub1 was also independent of its activity in regulating ATP-dependent chromatin remodelers known to be regulated by H4. We therefore examined the possibility that the H4 basic patch regulates some aspect of the H2Bub1 machinery that installs or removes this mark. Using an optogenetic tool to enable precise and rapid nuclear import or export of Bre1 (LINX-Bre1), we showed that, although Bre1 installed H2Bub1 in wild-type and H4 basic patch mutants at comparable rates, the rate of removal of H2Bub1 by Ubp8 (but not by Ubp10) increased when the H4 basic patch was mutated. Because Ubp8 is a DUB module of the SAGA complex, we further examined and found that the H4 basic patch also contributes to the levels of H3 acetylation by SAGA. Collectively, our findings reveal an unexpected function of the H4 basic patch in negatively regulating the histone-modifying activities of SAGA.

3.3 Materials and Methods

3.3.1 Yeast strains

Strains and plasmids used in this study are listed in Supporting Information Tables 3.1 and 3.2. Strains used in Figures 1 and 3A were derived from the parental histone shuffle strain, YAA524. All other histone shuffle mutants used were derived from the parental histone shuffle strain, yDT51. Gene disruptions and endogenous overexpression were performed as described (C. Janke et al. 2004) and verified by PCR and immunoblotting. Plasmids were generated using a standard site-directed mutagenesis protocol and primers were designed with the Agilent QuikChange Primer Design tool (www.agilent.com/store/primerDesignProgram.jsp).

3.3.2 Preparation of whole-cell extracts and immunoblots

Cells were collected by centrifugation and stored at -80 ˚C. Cell pellets were thawed on ice, resuspended in 200 µL ice-cold TCA buffer (10 mM Tris 8, 10% TCA, 25 mM NH4OAc, 1 mM EDTA), and moved to a microcentrifuge tube and incubated on ice for 10 minutes. Samples were centrifuged at 13K rpm for 5 minutes at room temperature to collect precipitated protein. After aspirating the supernatant, the pellet was resuspended 70-100 µL of 0.1 M Tris pH 11, 3% SDS and heated at 95 ˚C for 10 minutes followed by centrifugation at 13K rpm for 1 minute to collect cellular debris. Protein concentration was measured using the Bio-Rad DC Protein Assay kit (5000112) and diluted to normalize concentrations across samples. Samples were diluted in 2X loading dye and 10-15 µL of sample was subjected to SDS-PAGE followed by transfer to PVDF membrane for immunoblot analyses.

3.3.3 Yeast spotting assays

Saturated overnight yeast cultures were diluted to an OD600 of 0.5 and fivefold serial dilutions of the cells were plated onto YPD or SC medium containing the indicated chemotoxic agents. Plates were imaged after 2 to 4 days at 30 ˚C.

3.3.4 Optogenetic and in vivo DUB assays

LINX-Bre1 optogenetic time courses were performed as described (Yumerefendi et al. 2016). Briefly, colonies of LINX-Bre1 transformed in bre1Δ, bre1Δubp8Δ or bre1Δubp10Δ strains were grown overnight in SC-LEU-containing medium in the dark (Figure 5) or in the presence of blue light (Figure 4). Cell density was measured and diluted to an OD600 of 0.5 and grown for 4 hours in the same light condition as the overnight culture. Time courses began when cultures were moved from light to dark or vice versa. At each time point, the same volume of culture was collected and added to the appropriate volume of 100% TCA to yield a final TCA concentration of 20%, followed immediately by mixing and centrifugation at 5000 rpm for 5 minutes at 4 ˚C. The supernatant was aspirated and the pellets were stored at -80 ˚C until processed for Western blot analysis as detailed above.

3.3.4 Antibodies

Immunoblots were developed using ECL Prime (Amersham RPN2232). Antibodies: Ubiquityl-Histone H2B (Cell Signaling Technologies 5546; 1:5,000), H2B (Active Motif 39237; 1:5,000), H3K79me3 (Abcam 2621; 1:2,500), H3K79me2 (Active Motif 39143; 1:2,500), H3K4me3 (Epicypher 13-0004; 1:5,000), H3K9ac (Active motif 39917, 1:2,500), H3K14ac (Millipore 07-353; 1:2,500), H3K18ac (Millipore 07-354; 1:2,500), H3K27ac (abcam ab4729; 1:2,500), H3K56ac (Active motif 39281; 1:1,000), H3 (EpiCypher 13-0001; 1:5,000), G6PDH (Sigma-Aldrich A9521; 1:100,000), FLAG-M2 (Sigma-Aldrich F1804; 1:5,000), Myc (Millipore

;1:2,500). Rabbit (Amersham NA934; Donkey anti-Rabbit) and mouse (Amersham NA931; Sheep anti-mouse) secondary antibodies were used at 1:10,000.

3.3.5 Galactose induction

Yeast strains that expressed either the wild-type or mutant histone H4 were grown overnight in media containing 2% raffinose. Cell density was measured and diluted to an OD600 of 0.25 and grown for 4 hours in media containing 2% raffinose. The induction time course was begun by adding 20% galactose to a final concentration of 2%. At each time point the same volume of each culture was harvested and centrifuged at 4500 rpm for 5 minutes. The supernatant was aspirated and the cell pellets were stored at -80 ˚C.

3.3.6 Quantitative real-time PCR (qRT-PCR)

RNA was isolated by a hot acid phenol method as described (Collart and Oliviero 2001). Crude RNA was treated with DNase (Promega M6101) followed by RNA cleanup (QIAGEN RNeasy Mini Kit, 74106). cDNA was synthesized from 500 ng-1 ug of total RNA using random hexamer primers and Superscript Reverse Transcriptase III (Thermo-Fisher Scientific, 108-80044). The cDNA was diluted 1:25 before being subjected to real-time PCR (primers listed in Supporting Information). Quantitative RT-PCR was performed using the iTaq Universal SYBR Green Master mix according to manufacturer's instructions (Bio-Rad, 1725125), and the relative quantities of transcripts were calculated using the ΔΔCt method (Livak et al. 2013) and ACT1 as a control. The data shown are the replicates of three independent experiments with three technical replicates in each experiment.

3.3.7 In vitro DUB assays

Yeast strains that expressed either wild-type or mutant histone H4, FLAG-tagged histone H2B and lacking both UBP8 and UBP10 were grown overnight in 25 mL YPD and harvested by

centrifugation at 4500 rpm for 5 minutes. Cell pellets were frozen and stored at -80 ˚C. Pellets were resuspended in 500 uL 10 mM Tris.Cl, pH 7.4, 300 mM Sorbitol, 100 mM NaCl, 5 mM MgCl2, 5 mM EDTA, 10% glycerol, 0.1% Igepal-30 and split into 2 microcentrifuge tubes before being subjected to standard glass bead lysis by vortexing at 4 ˚C. The tubes were punctured with a push pin and placed into a clean tube and spun to 3000 rpm 3 times to separate the lysate from the beads. Final lysate was clarified by centrifugation at 13K rpm for 10 minutes at 4 ˚C. Protein concentration was determined using the BioRad Bradford assay. To perform the assay, 50-100 μg of total protein was diluted to a final volume of 20 μL. A master mix containing enough sample to complete the experiment was prepared and preincubated at 30 ˚C with mixing for 30 minutes before addition of enzyme. The time course was begun by addition of recombinant Ubp8 DUB module (rDUBm) or Ubp10 enzyme. At the indicated timepoints 20 μL of sample was removed and added to a microcentrifuge tube containing 5 μL of 5X loading dye and heated at 95 ˚C immediately. Twenty μL of the sample was subjected to 12% SDS-PAGE, which was blotted and probed with an M2-FLAG primary antibody to detect both the FLAG-H2Bub1 and FLAG-H2B signal from the same membrane.

3.4 Results

Mutation of H4 basic patch leads to reduced levels of H2B monoubiquitylation

Although studies have defined the relationship between H2Bub1 and H3K4 and H3K79 methylation, less is known about the nature of the histone crosstalk that regulates H2Bub1. In the course of our studies of the regulation of H3K79 methylation by the H4 basic patch (Figure 3.1A), unexpectedly, we discovered by immunoblotting that mutation of H4 basic patch residues R17 and R19 to alanine (hereafter $H4_{2RA}$) also resulted in a significant reduction in H2Bub1 (Figure 3.1B). As controls in these experiments, we included a deletion of BRE1 (bre1Δ), the

H2Bub1 E3 ligase, and a mutation of H3K79 (H3K79R); these mutants confirmed the specificity of the histone modification-specific antibodies and verified that the H4 basic patch regulates H3K79 di- and tri-methylation. We also observed the same reduction in H2Bub1 in a strain that expressed an H4 mutant lacking the entire basic patch (Δ16-20) and in a single H4-R17A mutant (data not shown). Because H4 basic patch regulation of H2Bub1 has not been reported, we sought to verify that this observation was not a property of a specific strain background. As shown in Figure 3.7A, mutation of the same H4 basic patch residues in another H2A/H2B shuffle strain likewise impaired H2Bub1 levels.

Given the well-established function of the H4 basic patch in regulating Dot1-mediated H3K79 methylation, we next assessed whether the regulation of H2Bub1 by the H4 basic patch might be indirectly due to the ability of this region to regulate Dot1 and H3K79 methylation that, in turn, regulates H2Bub1. This idea was supported by a study of van Welsem et al. who found that Dot1 promoted H2Bub1 formation when Dot1 levels were increased (van Welsem et al. 2018). However, we found that the deletion of DOT1 (dot1Δ) or mutation of H3K79 had no discernible effect on the levels of H2Bub1 (Figure 3.1B, 3.7A and 3.7B). Thus, the ability of the H4 basic patch to regulate H2Bub1 is independent of its regulation of Dot1 and H3K79 methylation. In sum, these data defined an important and previously unknown function for the H4 basic patch in regulating H2Bub1.

H4 basic patch mutant cells display phenotypes associated with the absence of H2Bub1

To determine the biological significance of the reduced levels of H2Bub1 in the H4 basic patch mutant, we spotted cells that expressed either $H4_{WT}$, $H4_{2RA}$, H3K79R, or H2BK123R histones onto solid media containing various compounds to identify drug sensitivity phenotypes.

As has been previously published, cells lacking all H2Bub1 (e.g., H2BK123R, rad6Δ, bre1Δ), display sensitivity to various toxic agents including caffeine and 6-azauracil (6-AU) (Song and Ahn 2010; Klucevsek, Braun, and Arndt 2012). Interestingly, the $H4_{2RA}$ mutant was also sensitive to these same agents as well as rapamycin (Figure 3.2). In contrast, the H3K79R mutant, lacking all forms of H3K79 methylation, was not sensitive to these agents, confirming that the mechanism by which the H4 basic patch regulates H2Bub1 is independent of H3K79 methylation.

H2Bub1 has a key function in the timely induction of the yeast GAL genes (Xiao et al. 2005). To determine whether the H4 basic patch was also required for the induction of GAL genes like H2Bub1, we grew $H4_{WT}$ and $H4_{2RA}$ cells in raffinose-containing media prior to the addition of galactose (2% final concentration). Total RNA was isolated across the induction time course and RT-qPCR of the GAL1 locus was performed. Relative to the $H4_{WT}$ cells, $H4_{2RA}$ cells displayed reduced accumulation of GAL1 transcripts during the time course (Figure 3.8), suggesting that the H4 basic patch, like H2Bub1, is important for the proper regulation of GAL gene transcription. Collectively, these studies showed that the H4 basic patch is physiologically important and its mutation phenocopies mutations in the H2Bub1 pathway.

The H4 basic patch and the ATP-dependent chromatin remodeler Chd1 regulate H2Bub1 by different mechanisms

Lee et al. demonstrated a function for the ATP-dependent remodeler Chd1 in regulation of H2Bub1 (J. S. Lee et al. 2012). Additional studies show that the H4 basic patch regulates the activity of multiple chromatin remodelers including Chd1, Snf2, and Isw1. Therefore, we reasoned that a possible mechanism for how the H4 basic patch regulates H2Bub1 could be by

H4 basic patch regulation of the activity of one of these remodelers, which, in turn, would regulate H2Bub1. To test this possibility, we measured global H2Bub1 levels in a panel of strains that contained individual deletions of the aforementioned chromatin remodelers (Figure 3.3A). Consistent with previous studies, deletion of CHD1 (*chd1*Δ) resulted in a reduction of H2Bub1. In contrast to chd1Δ, however, none of the other remodeler deletions exhibited any loss of H2Bub1. To determine whether the loss of H2Bub1 in the $H4_{2RA}$ strain was due to Chd1, we deleted CHD1 in our $H4_{WT}$ and $H4_{2RA}$ strains and measured H2Bub1 levels in all strain combinations. As expected, we observed a decrease in H2Bub1 in the individual chd1Δ and $H4_{2RA}$ mutants (Figure 3.3B). Intriguingly, however, the loss of H2Bub1 in the $H4_{2RA}$ *chd1*Δ double mutant was additive, suggesting that regulation of H2Bub1 by the H4 basic patch and Chd1 occurs by separate (non-epistatic) pathways.

H4 basic patch regulation of H2B ubiquitylation is independent of the ubiquitylation machinery

To define how the H4 basic patch regulates H2Bub1, we next considered the possibility that the region is important for Rad6/Bre1 to catalyze H2Bub1. Others have found that, in the absence of one or more of the H2Bub1 DUBs, the ability of Rad6/Bre1 to properly incorporate H2Bub1 can be monitored (M. B. Chandrasekharan et al. 2010; Cucinotta et al. 2015). More specifically, a defect in the ability of Bre1 to ubiquitylate H2B (e.g., if K123 is a poor substrate) resulted in reduced levels of H2Bub1 for which absence of a DUB could not compensate. Following this example, we created a deletion of UBP8 (ubp8Δ) in the context of either the $H4_{WT}$ or $H4_{2RA}$ mutant and examined the levels of H2Bub1 by immunoblotting. Upon deletion of UBP8, the H2Bub1 level in the $H4_{2RA}$ ubp8Δ cells was rescued to a level comparable to that in the $H4_{WT}$ ubp8Δ strain (Figure 4A). These findings suggested that the ubiquitylation machinery

in the $H4_{2RA}$ mutant was functional and that the H4 basic patch was not essential for H2Bub1 catalysis.

In addition to the above approach, another outcome that we observed for the absence of Bre1 catalysis was reduced Bre1 protein stability (Wozniak and Strahl 2014a). To determine if Bre1 protein level was reduced in the $H4_{2RA}$ mutant, we expressed a FLAG-BRE1 construct in $H4_{WT}$ bre1Δ and $H4_{2RA}$ bre1Δ cells and analyzed Bre1 levels (FLAG) and H2Bub1 levels by immunoblot (Figure 4B). As expected, we did not detect H2Bub1 in the bre1Δ cells. In contrast, and upon plasmid rescue with our FLAG-BRE1 construct, we observed a restoration of H2Bub1 level in $H4_{WT}$ that was reduced in the $H4_{2RA}$ strain. This result indicated that our rescue experiment recapitulated our original observations on H2Bub1 regulation by the H4 basic patch. Importantly, we did not find any significant difference in Bre1 protein levels between the $H4_{WT}$ and $H4_{2RA}$ strains, suggesting that Bre1 catalytic activity, specifically, and the ubiquitylation machinery, generally, were unaffected by the $H4_{2RA}$ mutation.

Although the foregoing studies were informative, they were limited by the fact that they captured only final, steady state levels of H2Bub1. Recently, for the study of dynamic epigenetic regulation, optogenetic tools have emerged that enable rapid and reversible nucleo-cytoplasmic shuttling to overcome the steady-state limitation. We developed an optogenetic tool LINX (light-inducible nuclear export) that uses blue light to control the cellular localization of Bre1 (Yumerefendi et al. 2016). Briefly, when cells lacking BRE1 and UBP8 are transformed with the LINX-Bre1 construct and grown in blue light, LINX-Bre1 is sequestered in the cytoplasm and phenocopies a bre1Δ strain. Removal of blue light results in import of LINX-Bre1 into the nucleus, rendering the cells BRE1+, and enabling precise measurement of the in vivo kinetics of ubiquitylation and deubiquitination with unprecedented control and resolution. Thus, we

measured the kinetics of LINX-Bre1-mediated ubiquitylation in $H4_{WT}$ and $H4_{2RA}$ cells that lacked endogenous BRE1 and UBP8. These cells were grown to log-phase in the presence of blue light before being switched to the dark to release Bre1 into the nucleus. As shown in Figure 4C, H2B ubiquitylation occurred rapidly, consistent with published results (Yumerefendi et al. 2016). Significantly, we did not observe any significant difference in the kinetics of ubiquitylation between the $H4_{WT}$ and $H4_{2RA}$ cells, providing strong, in vivo evidence that the ubiquitylation machinery in the $H4_{2RA}$ mutant was fully functional and that the H4 basic patch regulates H2Bub1 independent of Bre1.

The H4 basic patch regulates SAGA-associated Ubp8 deubiquitination

We next turned our attention to the effect of the H4 basic patch on H2Bub1 deubiquitination. We took advantage of the LINX-Bre1 system to measure the dynamics of deubiquitination. To distinguish between the activities of the two H2Bub1 DUBs, Ubp8 and Ubp10, we created $H4_{WT}$ *bre1*Δ and $H4_{2RA}$ *bre1*Δ strains that lacked either UBP8 (*bre1*Δ *ubp8*Δ) or UBP10 (bre1Δ ubp10Δ). Cells were grown to log phase in the dark to maintain normal H2Bub1 by LINX-Bre1. Then we exposed the cells to blue light to rapidly remove LINX-Bre1 from the nucleus, preventing any subsequent ubiquitylation and enabling us to measure the kinetics of DUB activity in vivo. In the H4WT cells, the half-lives of the H2Bub1 mark in the presence of Ubp8 or Ubp10 were approximately 2.58 and 2 minutes, respectively. The half-life of the H2Bub1 mark in $H4_{2RA}$ cells in the presence of Ubp10 was similar to that in H4WT, about 1.8 minutes. Strikingly, the half-life of the H2Bub1 mark in $H4_{2RA}$ cells in the presence of Ubp8 decreased 3-fold (to less than 1 minute; Figures 5A, 5C). This result suggested that the H4 basic patch regulates Ubp8, and it may limit the activity or accessibility of Ubp8. Given these

findings, we also employed an in vitro DUB assay; we used cell extracts from yeast strains doubly deleted for UBP8 and UBP10 to generate high levels of H2bub1 as a substrate for recombinant Ubp8 and Ubp10. We assayed an affinity-purified Upb8-DUB module previously shown to interact with the nucleosome and affinity-purified Ubp10. These in vitro assays did not reveal a difference in the rates of deubiquitination between H4WT and $H4_{2RA}$ cells (Figure 3.9). Thus, the regulation of Ubp8 by the H4 basic patch may involve either a regulatory mechanism that is not captured in in vitro assays or that depends on the full SAGA complex and not the SAGA-associated DUB module alone.

The H4 basic patch regulates SAGA-dependent H3 acetylation

The SAGA complex consists of four distinct modules and two enzymatic functions—deubiquitination and lysine acetyltransferase (KAT) activities (Weake and Workman 2012). The main target of the KAT module is the histone H3 N-terminus, specifically lysine residues 9, 14, 18, 23, 27, and 36 (Cieniewicz et al. 2014). Although the KAT and DUB modules are distinct structurally, they are in close proximity to one another and the DUB module modestly stimulates KAT activity (Han et al. 2014). To determine whether the H4 basic patch also influences SAGA-dependent histone acetylation, we analyzed the histone acetylation levels at H3K9, K14, K18, and K27 in H4WT and $H4_{2RA}$ cells alongside a gcn5Δ strain as a control (Figure 6). Intriguingly, in the $H4_{2RA}$ cells, we observed an increase in H3K18 and H3K27 acetyl levels but little change in H3K9 and H3K14 acetyl levels. However, H3K9 and H3K14 are also targets of other KATs (NuA3 and Rtt109, respectively). Of the acetylation sites tested, H3K18ac and H3K27ac are almost exclusively catalyzed by SAGA, as demonstrated by the complete loss of acetylation at these two sites. Thus, the selective increase in H3K18 and H3K27 acetylation in $H4_{2RA}$ cells was

likely due to SAGA-specific acetylation changes caused by the loss of H4 basic patch function. To eliminate the possibility that the $H4_{2RA}$ mutant alters KAT activity more generally, we analyzed H3K56ac levels in the H4WT and $H4_{2RA}$ cells; we did not find any difference between the strains (Figure 3.10). Taken together, these data suggest that, in addition to regulating the DUB model in SAGA, the basic patch also regulates the SAGA KAT module. Thus, these findings document an important inhibitory function of the H4 basic patch in SAGA-associated histone modification functions.

3.5 Discussion

We have shown that, by negatively regulating SAGA-associated activities, the H4 basic patch ensures proper levels of H2Bub1 and H3 acetylation. The H4 basic patch regulates many other chromatin-modifying activities, e.g., Dot1-mediated H3K79 methylation. However, we found that regulation of H2Bub1 and histone acetylation by the H4 basic patch were independent of its regulation of Dot1 or other chromatin remodelers that influence H2Bub1. Thus, these studies document a new function for the H4 tail and reveal another layer of cross-talk regulation of the SAGA complex.

Our study outlines a negative function of the basic patch on SAGA-associated histone-modifying activities. However, it remains unknown precisely how the H4 basic patch restrains the SAGA co-activator complex. Detailed Cryo-EM studies of SAGA and the DUB module in SAGA showed that the histone acetylation and DUB modules are in close proximity to the nucleosomal core (Samara et al. 2010; Samara, Ringel, and Wolberger 2012; Köhler et al. 2010; Han et al. 2014; Morgan et al. 2016); however, the H4 tail was not resolved and thus these studies do not provide insight into how the H4 basic patch interacts. Although our in vitro DUB assay with the Upb8-DUB module did not reveal a change in the H2Bub1 turnover rate when the

H4 basic patch was altered (Figure 3.9), our in vivo and time-resolved optogenetic studies did reveal a change in H2Bub1 turnover rate (Figure 5). Thus, the H4 basic patch may be involved in modulating an upstream regulatory pathway during transcription that regulates SAGA activity, perhaps by an indirect mechanism, instead of regulating enzymatic activity by a physical interaction that was perturbed in the mutant. Interestingly, both H4-R17 and SAGA DUB module member Sgf11-R78 interact with acidic patch residue H2A-E64, and DUB activity in the Sgf11-R78A mutant was severely reduced (Koehler et al. 2014; Morgan et al. 2016). We hypothesize that the H4 basic patch regulates H2Bub1 activity by competing with Sgf11 for association with the H2A acidic patch.

An additional, perhaps related possibility to explain this regulation is the fact that the H4 basic patch-H2A acidic patch interaction affects higher-order chromatin structure that may influence the accessibility of chromatin to chromatin-modifying enzymes. In this scenario, the mutation of the H4 basic patch would relieve its stabilizing effect on chromatin structure and "free" additional nucleosomes for SAGA binding, thereby increasing the overall H2Bub1 and H3 acetylation activities of the SAGA enzyme complex. The precise explanation for how this mechanism operates will be interesting to determine.

In sum, these studies reveal a new regulatory mechanism for the activity of SAGA that likely is important for proper gene regulation and other functions associated with SAGA. Given the highly conserved nature of yeast, we expect that this function of the H4 tail is conserved in more complex eukaryotes.

3.6 Figures

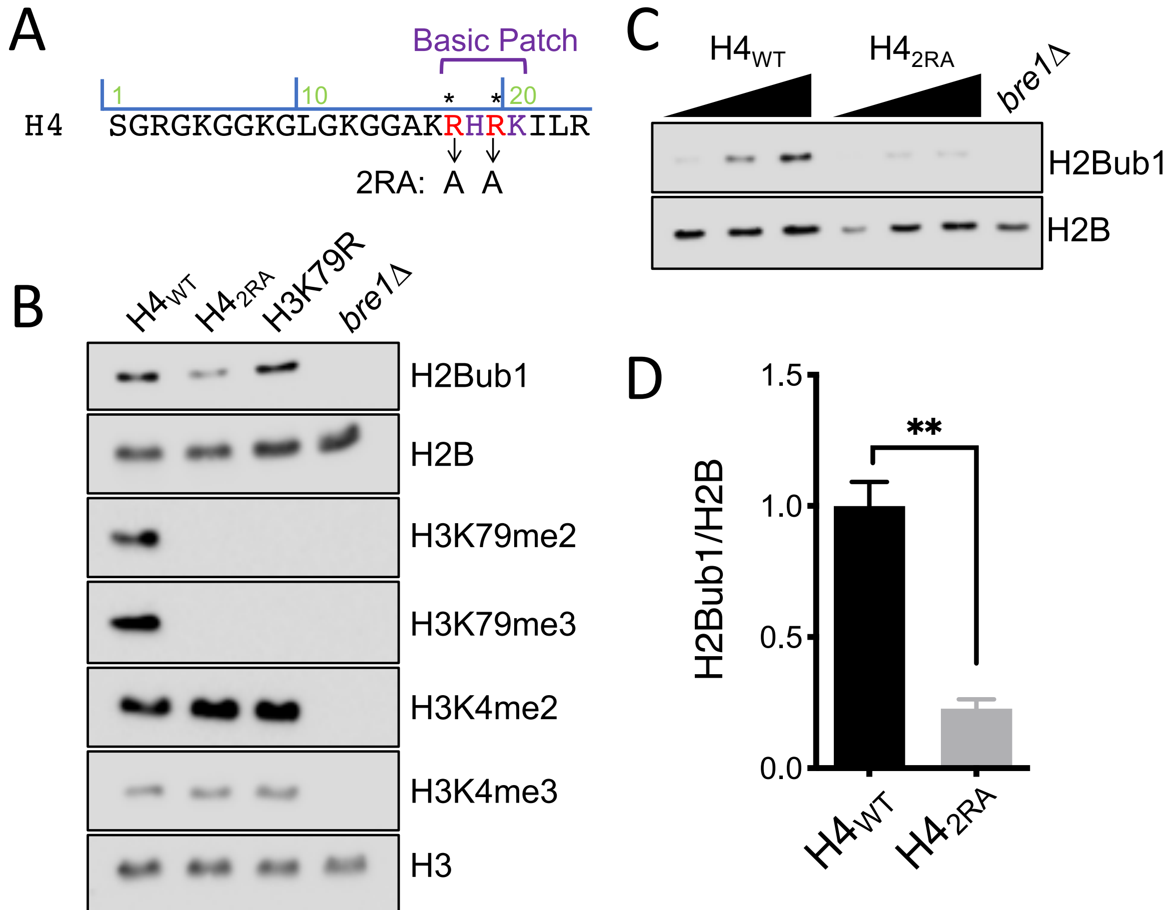

Figure 3.1. The H4 basic patch is required for proper H2BK123 monoubiquitylation (H2Bub1). A. Sequence of the H4 N-terminus with basic patch residues depicted. Arrows show the arginine residues mutated to alanine to create the basic patch mutant $H4_{2RA}$ used throughout the study. **B.** Immunoblotting was performed for the indicated proteins or modifications using extracts from cells that expressed wild-type H4 ($H4_{WT}$) or the basic patch mutant ($H4_{2RA}$). Strains that had a mutation at H3 lysine 79 (H3K79R) or were deleted for the E3 ubiquitin ligase Bre1 (*bre1*Δ) were used as antibody controls for H3K79 methylation and H2Bub1, respectively. H3 was used as a loading control. **C.** Representative immunoblot analysis of $H4_{WT}$ and $H4_{2RA}$ at increasing concentrations for H2B or H2Bub1. **D.** Relative quantification of H2Bub1/H2B from panel C. Means ± SEM were analyzed from three biological replicates.

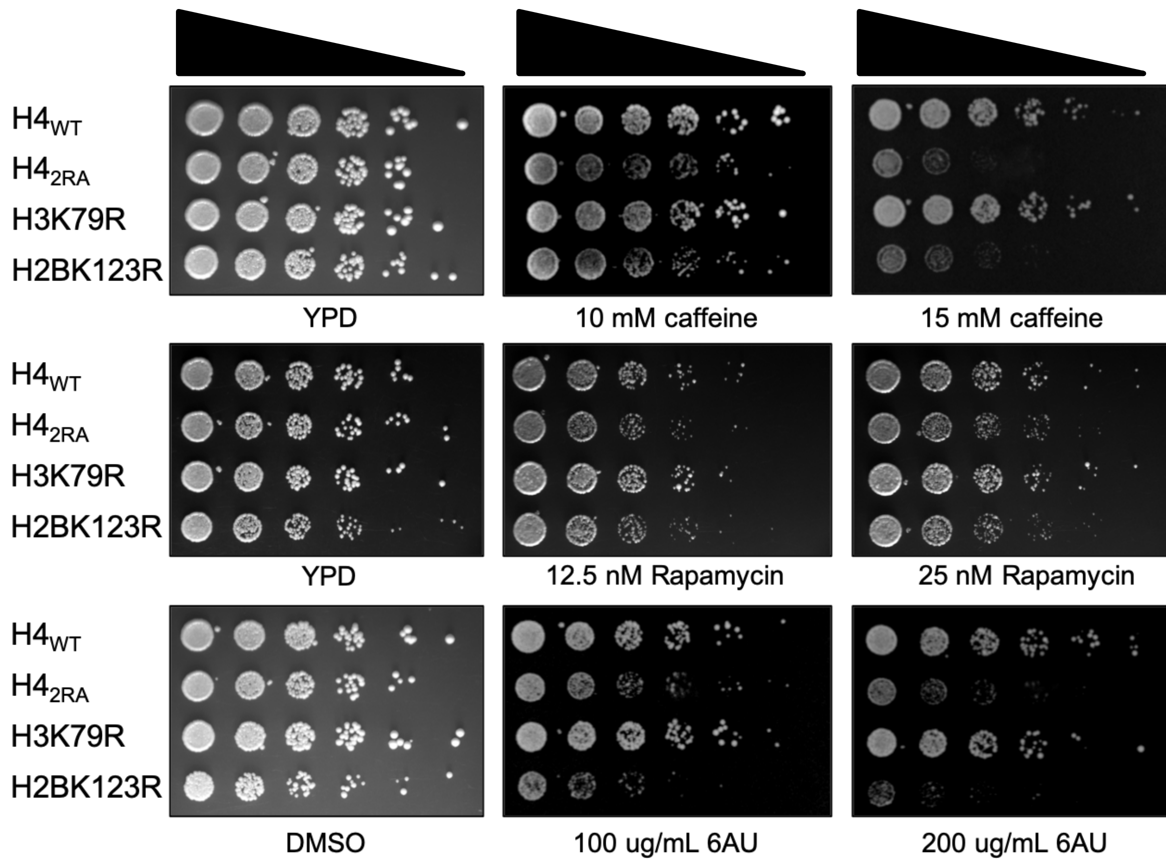

Figure 3.2. Mutation of the H4 basic patch and H2BK123 share phenotypes associated with H2Bub1 loss. Overnight cultures of the indicated strains were spotted at 5-fold dilutions onto solid rich (YPD) medium or media containing the indicated chemotoxic agents and imaged after 2-3 days.

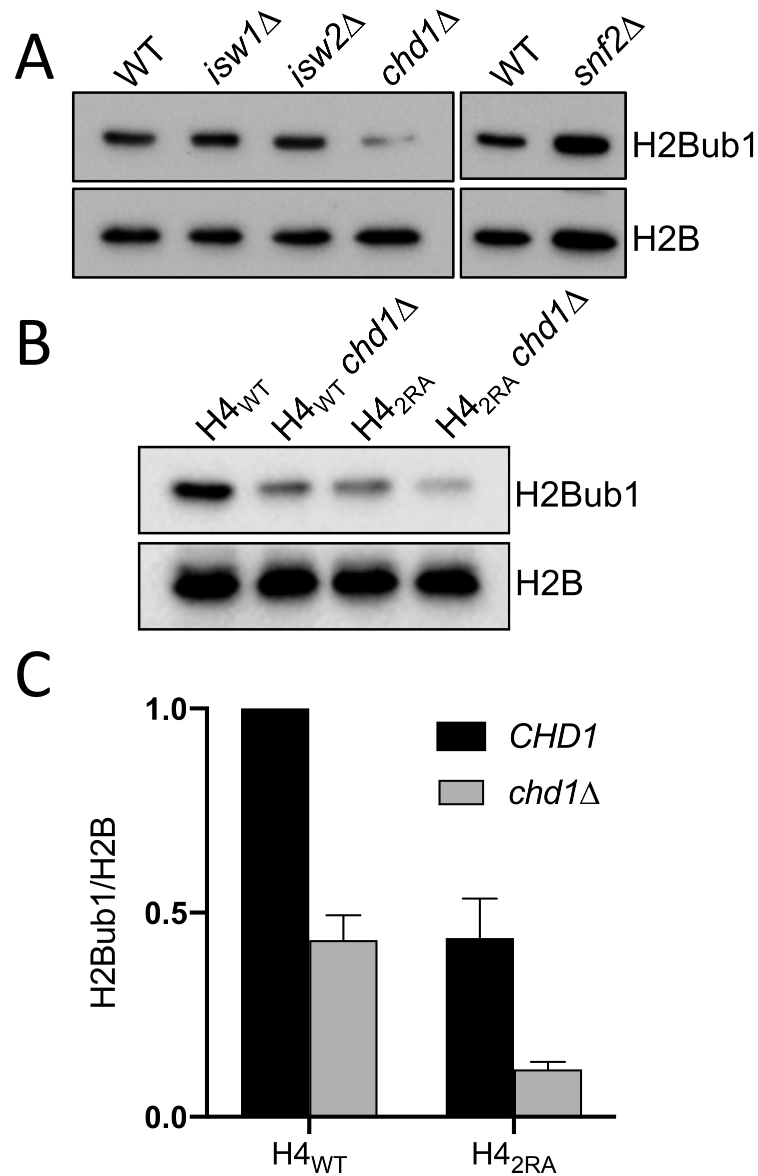

Figure 3.3. H4 basic patch regulation of H2Bub1 is independent of its interaction with chromatin remodelers. A. Immunoblotting for H2Bub1 and H2B was performed using whole cell extracts from wild-type or strains lacking the indicated chromatin remodelers. **B.** Blots for H2Bub1 and H2B were derived from cells that expressed $H4_{WT}$ or $H4_{2RA}$ in combination with a deletion of *CHD1* (*chd1*Δ). **C.** Relative quantification of H2Bub1/H2B from panel B. Means ± SEM were analyzed from three biological replicates.

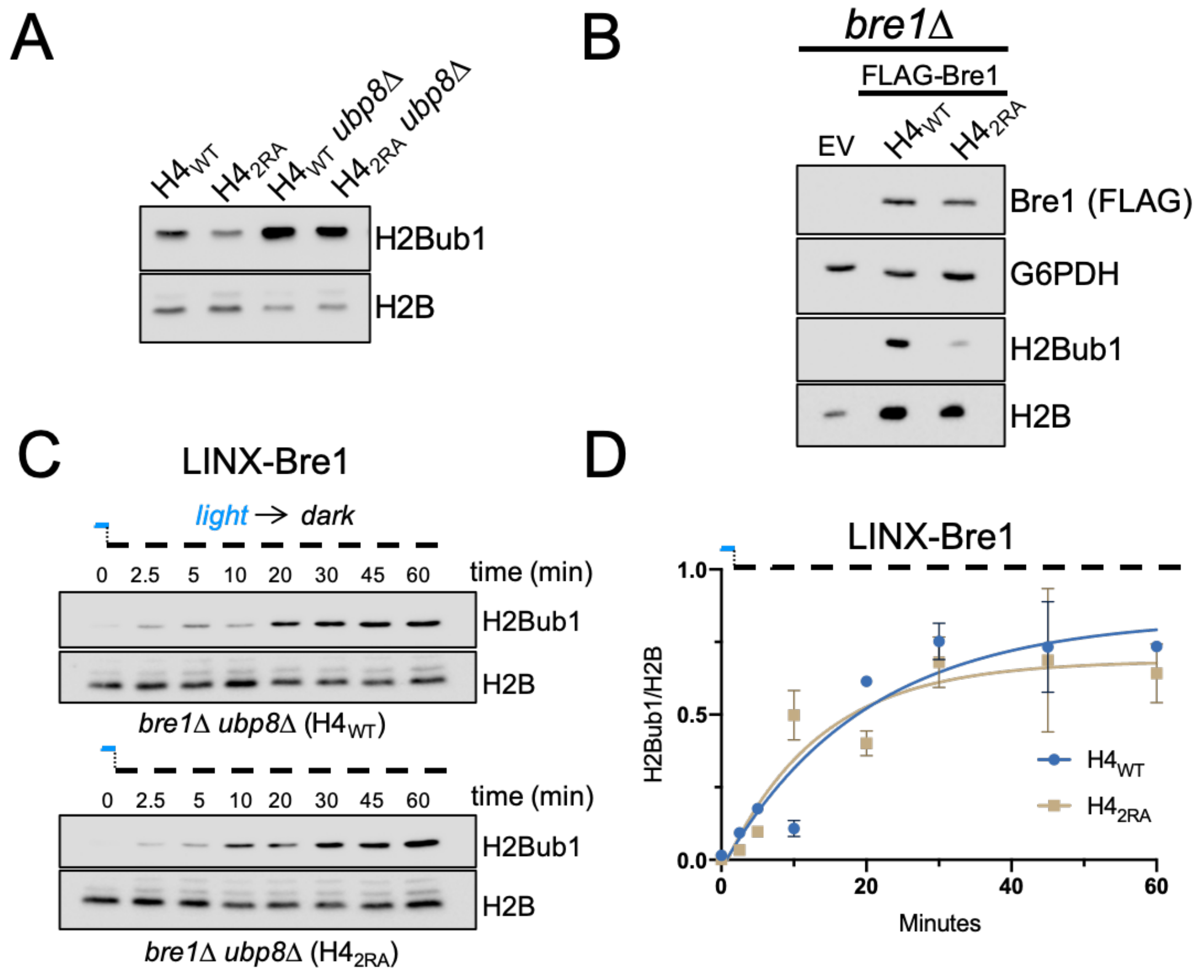

Figure 3.4. The H4 basic patch does not interfere with Bre1 ubiquitylation of H2B. A. Immunoblot analysis of H2Bub1 and H2B in $H4_{WT}$ and $H4_{2RA}$ in the presence or absence of *UBP8* (*ubp8*Δ). The ability of Bre1 to increase H2Bub1 in the $H4_{2RA}$ *ubp8*Δ strain suggested that the H4 basic patch mutant does not impair Bre1 catalysis. **B.** $H4_{WT}$ *bre1*Δ and $H4_{2RA}$ *bre1*Δ cells were transformed with empty vector or *ADH1*-driven Flag-*BRE1* and subjected to immunoblot analysis with the indicated antibodies. Visualizing normal levels of Bre1 protein in the $H4_{2RA}$ strain indicated that Bre1 function in catalysis was normal; Bre1 protein stability is normally diminished when Bre1 is unable to catalyze H2Bub1(Wozniak and Strahl 2014a) **C.** Optogenetic-mediated control of the E3 ligase Bre1 (LINX-Bre1) reveals similar kinetics of H2B ubiquitylation in $H4_{WT}$ and $H4_{2RA}$. **D.** Quantification of data from panel C. Means ± SEM were analyzed from two biological replicates.

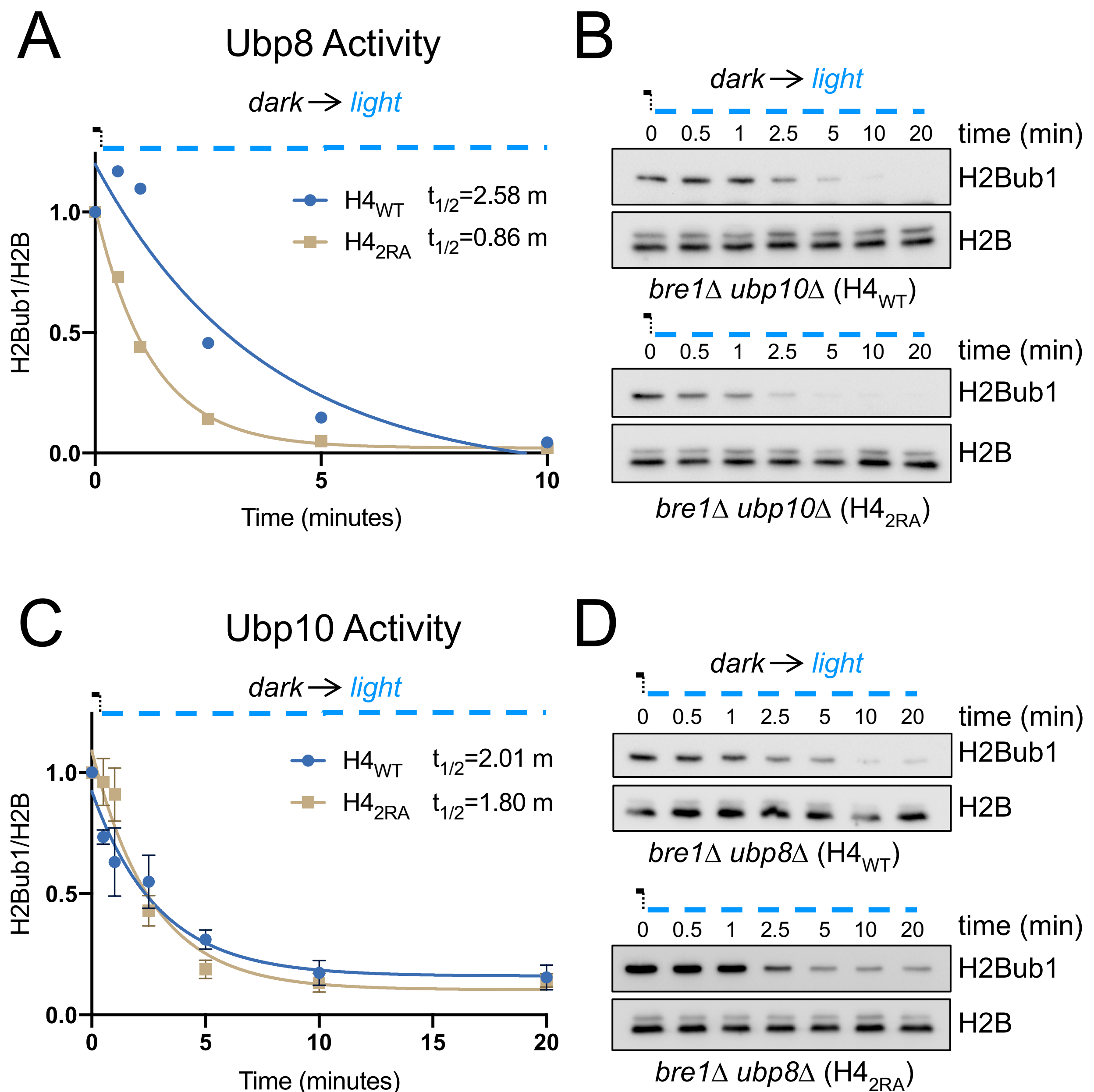

Figure 3.5. The H4 basic patch contributes to H2B deubiquitination by influencing Ubp8 but not Ubp10. A. $H4_{WT}$ and $H4_{2RA}$ cells lacking *UBP10* were grown to log phase in the dark to maintain LINX-Bre1 in the cytoplasm. Timepoint 0 was taken in the dark and subsequent timepoints were collected after exposure to blue light. Immunoblot analysis of H2Bub1 and H2B was performed and quantified. The half-life measured from each curve was determined from single exponential fits of the data. **B.** Representative immunoblots from panel A. **C.** $H4_{WT}$ and $H4_{2RA}$ cells lacking *UBP8* were grown to log phase in the dark to maintain LINX-Bre1 in the cytoplasm. Timepoint 0 was taken in the dark and subsequent timepoints were collected after exposure to blue light. Data were processed as above. **D.** Representative immunoblots from panel C.

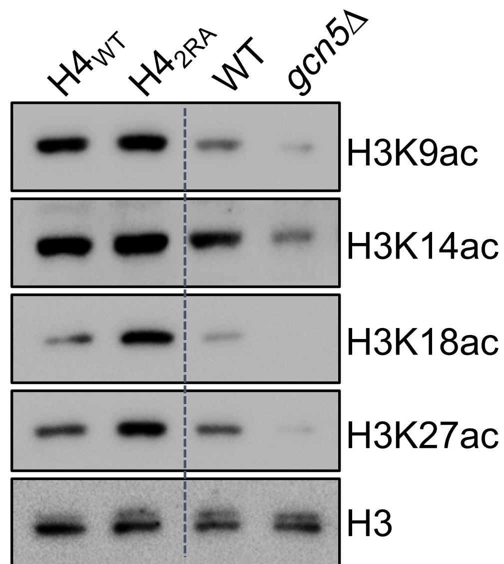

Figure 3.6. The H4 basic patch regulates SAGA-dependent H3 acetylation in addition to H2B deubiquitination. **A.** Immunoblot analysis of H3 N-terminal acetyl-lysine residues in $H4_{WT}$ and $H4_{2RA}$ cells. *gcn5*Δ and matched wild-type cells were included as controls.

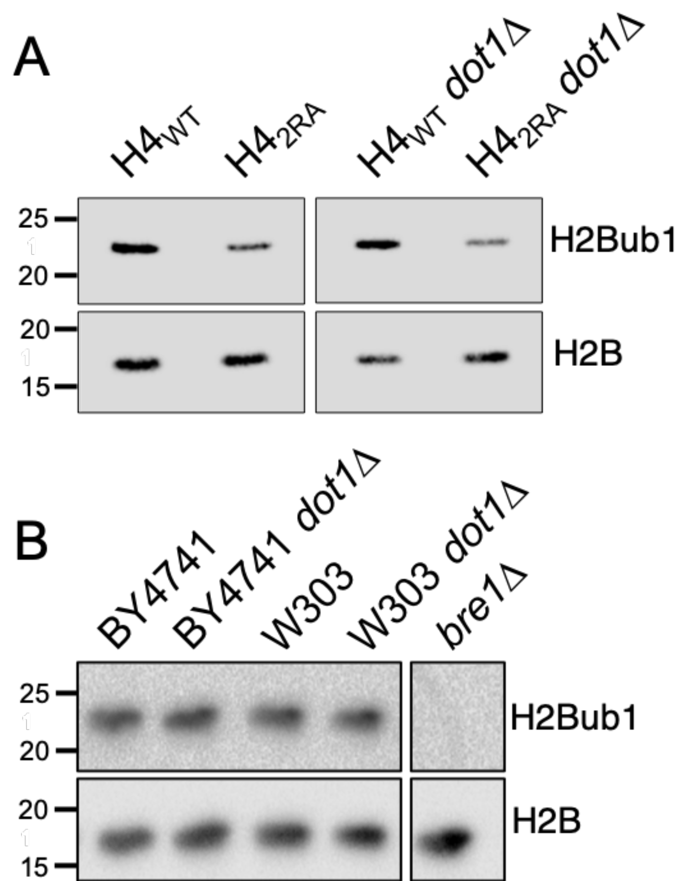

Figure 3.7. Effect of H4 basic patch mutant on H2Bub1 is not due to an effect on Dot1 mediated H3K79 methylation. A. Western blot of analysis of H2Bub1 and H2B in strains expressing wild-type or mutant histone H4 and in the presence or absence of the H3K79 methyltransferase Dot1 (*dot1*Δ). B. Western blot of analysis of H2Bub1 and H2B in two strain backgrounds, BY4741 and W303, and in the presence or absence of *DOT1* (*dot1*Δ).

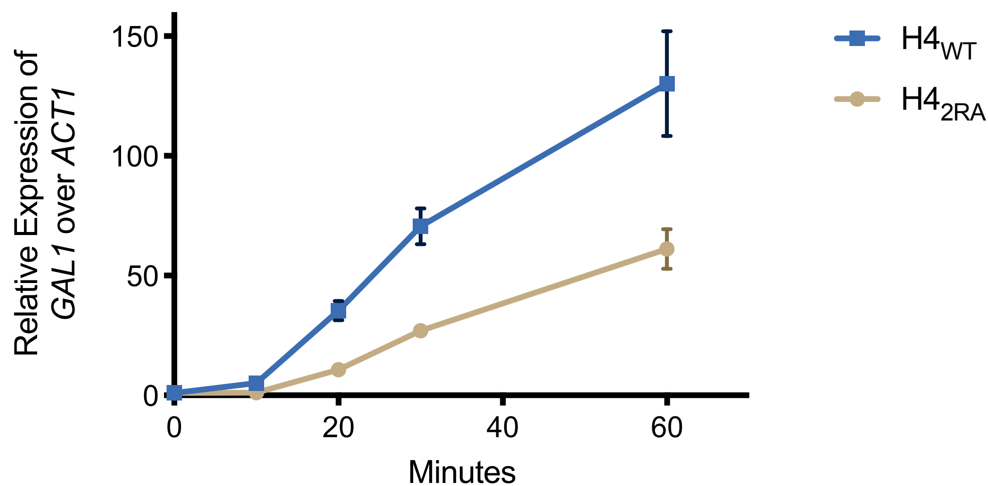

Figure 3.8. The basic patch of H4 is required for proper induction of *GAL1*. *GAL1* mRNA levels were measured for the H4-WT and H4-2RA strains. Strains were grown overnight in 2% raffinose-containing medium from which log cultures were started. Samples were removed before and at 10- to 60-min intervals after galactose induction, and RT-qPCR was performed on extracted RNA with primers against a region of the ORFs of the *GAL1* gene and the *ACT1* gene, which served as the internal control.

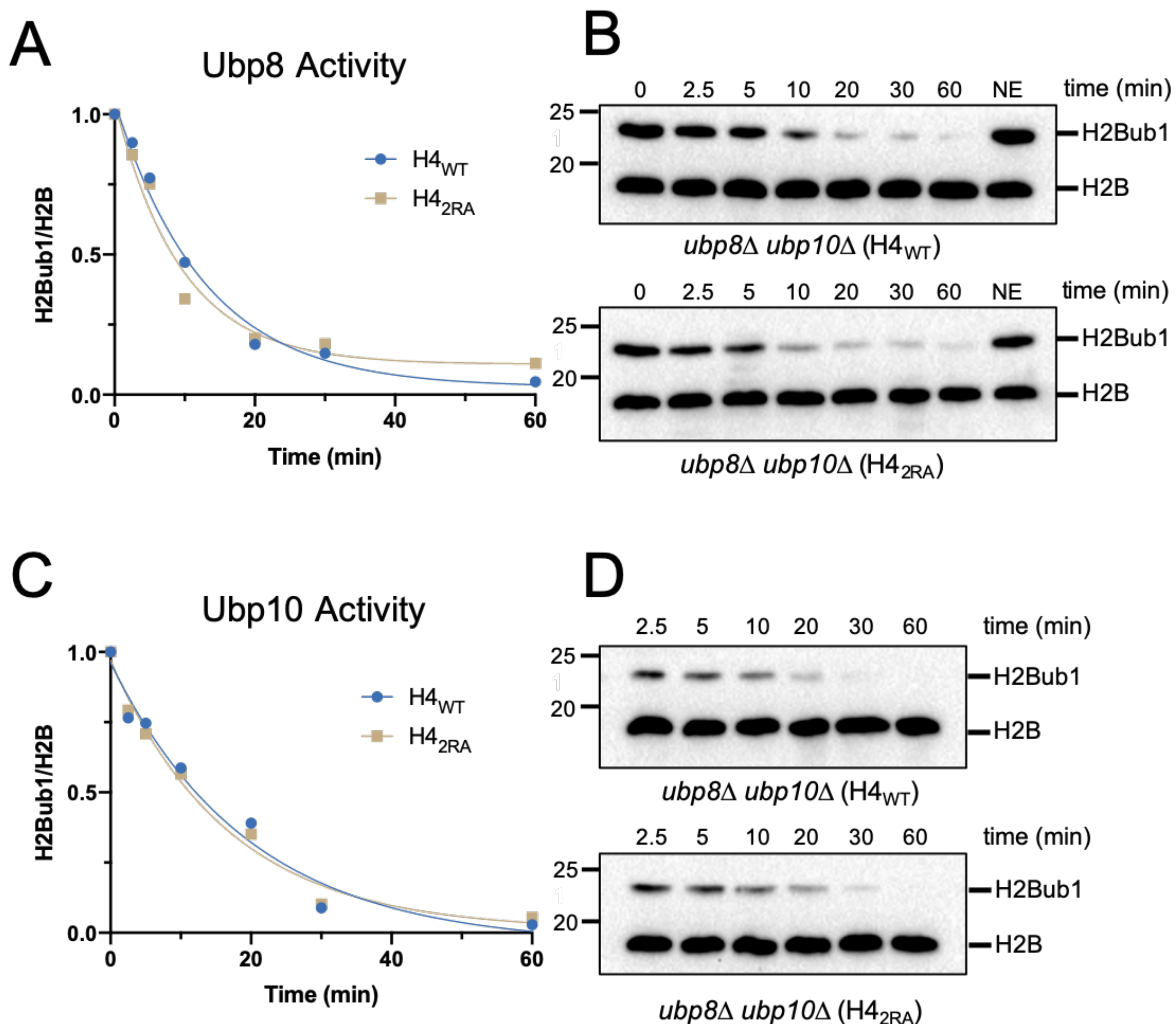

Figure 3.9. H4 basic patch mutant does not alter deubiquitylation activity *in vitro*. A. Whole cell extracts of $H4_{WT}$ and $H4_{2RA}$ cells lacking both *UBP8* and *UBP10* were incubated with recombinant Ubp8 DUB module (gift from C. Wolberger) and incubated at 30˚C. Samples were taken at the indicated time points and the reaction was quenched by addition of loading dye and boiling. NE indicates a sample of the same whole cell extract with no enzyme after 60 minutes of incubation. B. Western blots used to generate panel A. Blots were probed with anti-FLAG M2 antibody to identify both H2B and H2Bub1 from a single blot. C. Same as panel A but incubated with recombinant Ubp10. D. Western blots used to generate panel C.

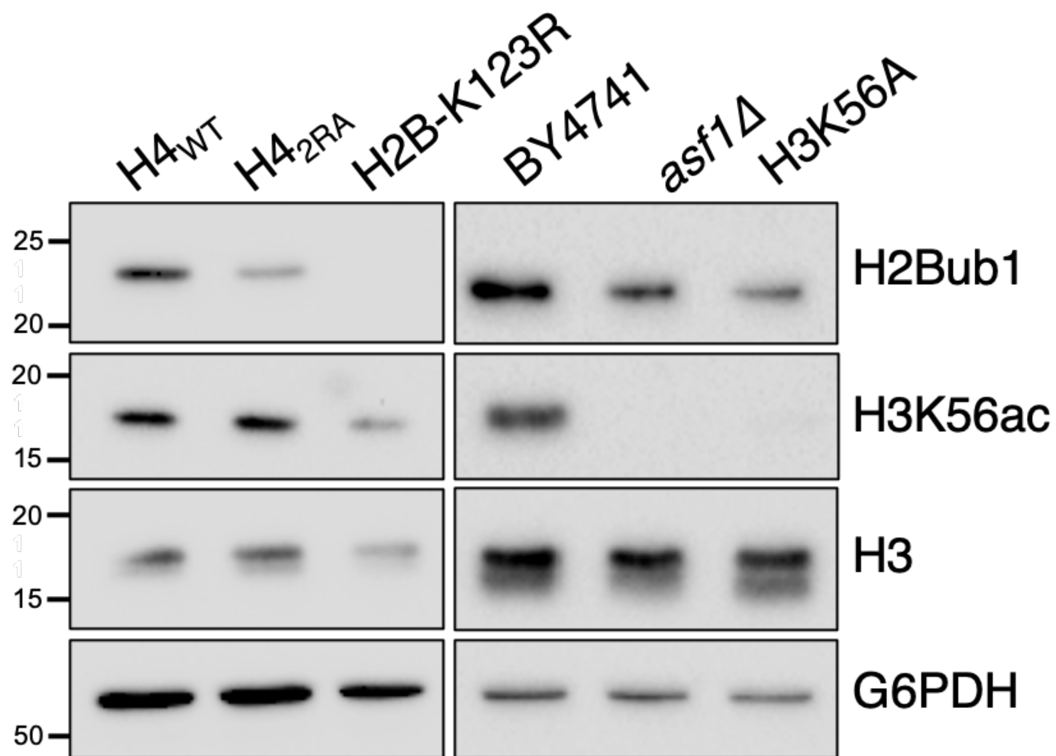

Figure 3.10. H4 basic patch regulation of H2Bub1 is not due to aberrant histone exchange. A. Western blot of analysis of H2Bub1, H3K56ac and H3 in strains expressing wild-type or the indicated mutant histones or gene deletions.

3.7 Supporting Information

Table 3.1. Yeast strains used in this study

Name	Description	Strain background	Genotype	Reference
W303-1A	WT	W303-1A	MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15	Thomas and Rothstein, 1989. (Thomas and Rothstein 1989)
W1588-4C	WT	W303-1A	W303-1A but RAD5	Tsukiyama et al.,1999. (Tsukiyama et al. 1999)
YTT186	isw1Δ	W303-1A	W1588-4C but isw1::ADE2	Tsukiyama et al.,1999.
YTT196	isw2Δ	W303-1A	W1588-4C but MATα isw2::LEU2	Tsukiyama et al.,1999.
JPY17	chd1Δ	W303-1A	W1588-4C but MATα chd1::TRP1	Tsukiyama et al.,1999.
YAA524	WT H3/H4 shuffle strain	YAA524	MATa his4-912∂ lys2-128∂ ura3-52 leu2Δ1 his3Δ200 trp1Δ63 hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3 [pHHT1-HHF1-URA3 CEN]	Gift from Fred Winston and Mary Bryk
YHM024	YAA524-H4WT	YAA524	MATa his4-912∂ lys2-128∂ ura3-52 leu2Δ1 his3Δ200 trp1Δ63 hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3 [pJH18]	This study.
YHM025	YAA524-H42RA	YAA524	MATa his4-912∂ lys2-128∂ ura3-52 leu2Δ1 his3Δ200 trp1Δ63 hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3 [pHM01]	This study.
YHM028	YAA524-K79R	YAA524	MATa his4-912∂ lys2-128∂ ura3-52 leu2Δ1 his3Δ200 trp1Δ63 hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3 [pK79R]	This study.
YHM022	YAA524-H4WT bre1Δ	YAA524	MATa his4-912∂ lys2-128∂ ura3-52 leu2Δ1 his3Δ200 trp1Δ63 hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3 bre1::hygro [pJH18]	This study.
YHM023	YAA524-H42RA bre1Δ	YAA524	MATa his4-912∂ lys2-128∂ ura3-52 leu2Δ1 his3Δ200 trp1Δ63 hht1-hhf1Δ::LEU2 hht2-hhf2Δ::HIS3 bre1::hygro [pHM01]	This study.

BY4741		BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	Open Biosystems
BY4741 dot1Δ	dot1Δ	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 dot1::kanMX4	Open Biosystems
BY4741 snf2Δ	snf2Δ	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf2::kanMX4	Open Biosystems
BY4741 asf1Δ	asf1Δ	BY4741	MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 asf1::kanMX4	Open Biosystems
H3K56A		YBL574	MATa leu2-1his3-200ura3-52trp1-63lys2-128(hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912Δ35-lacZ::HIS4 pWA414 [F12-hht2-K56A-HHF2]	Nakanishi et al., 2008. (Nakanishi et al. 2008)
YNL037	W303-dot1Δ	W303	MATα ade2 can1 his3 leu2 lys2 trp1 ura3 dot1::kanMX	Gardner et al., 2011. (K. E. Gardner et al. 2011)
yDT51		yDT51	MATα his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 hta2-htb2Δ0 hta1-htb1Δ0 hht1-hhf1Δ0 hht2-hhf2Δ0 [pDT83 (pRS416-HTA2-HTB2- HHT1-HHF1/URA3/CEN-ARS/Amp)]	Truong and Boeke, 2017. (Truong and Boeke 2017)
YHM045	H4WT	yDT51	MATα his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 hta2-htb2Δ0 hta1-htb1Δ0 hht1-hhf1Δ0 hht2-hhf2Δ0 [pJH18/pZS145]	This study.
YHM046	H42RA	yDT51	MATα his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 hta2-htb2Δ0 hta1-htb1Δ0 hht1-hhf1Δ0 hht2-hhf2Δ0 [pHM01/pZS145]	This study.
YHM047	H3K79R	yDT51	MATα his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 hta2-htb2Δ0 hta1-htb1Δ0 hht1-hhf1Δ0 hht2-hhf2Δ0 [pK79R/pZS145]	This study.
YHM048	H2BK123R	yDT51	MATα his3Δ200 leu2Δ0 lys2Δ0 trp1Δ63 ura3Δ0 met15Δ0 hta2-htb2Δ0 hta1-htb1Δ0 hht1-hhf1Δ0 hht2-hhf2Δ0 [pJH18/pZS146]	This study.
YHM049	H4WT chd1Δ	yDT51	H4WT chd1::nat	This study.
YHM050	H42RA chd1Δ	yDT51	H42RA chd1::nat	This study.
YHM051	H4WT ubp8Δ	yDT51	H4WT ubp8::nat	This study.
YHM052	H42RA ubp8Δ	yDT51	H42RA ubp8::nat	This study.

YHM053	H4WT ubp8Δubp10Δ	yDT51	H4WT ubp10::nat ubp8::hygro	This study.
YHM054	H42RA ubp8Δubp10Δ	yDT51	H42RA ubp10::nat ubp8::hygro	This study.
YHM055	H4WT ubp8Δ bre1Δ	yDT51	H4WT ubp8::nat bre1::hygro	This study.
YHM056	H42RA ubp8Δ bre1Δ	yDT51	H42RA ubp8::nat bre1::hygro	This study.
YHM057	H4WT ubp10Δ bre1Δ	yDT51	H4WT bre1::nat ubp10::kanMX	This study.
YHM058	H42RA ubp10Δ bre1Δ	yDT51	H42RA bre1::nat ubp10::kanMX	This study.
ZGYW303-1a	WT	W303-1A	MATa leu2-3, 112 ura3-1 his3-11,15, trp1-1, ade2-1, can1-100	Burgess et al., 2010. (Burgess et al. 2010)
ZGY690	gcn5Δ	W303-1A	MATa leu2-3, 112 ura3-1 his3-11,15, trp1-1, ade2-1, can1-100, gcn5Δ::kanMX6	Burgess et al., 2010.

Table 3.2. Plasmids used in this study.

Name	Parent	Features	Source
pJH18	pRS314	CEN TRP1 HHT2-HHF2	Hsu et al., 2000. (J. Y. Hsu et al. 2000)
pHM01	pJH18	CEN TRP1 HHT2-hhf2-R17A R19A	This study.
pK79R	pRS414	CEN TRP1 hht2-K79R-HHF2	Zhang et.al, 1998
pZS145	pRS313	CEN HIS3 HTA1-FLAG-HTB1	Sun and Allis, 2002. (Sun and Allis 2002)
pZS146	pRS313	CEN HIS3 HTA1-FLAG-htb1-K123R	Sun and Allis, 2002
pRS316-9mycBre1	pRS315-9mycBre1	CEN LEU2 pBRE1-9xMyc-BRE1	Wood et al., 2003. (Wood et al. 2003)
pRS316-9mycBre1 CD	pRS315-9mycH665A	CEN LEU2 pBRE1-9xMyc-bre1-C663A/H665A	Wood et al., 2003.
FLAG-Bre1 p416 ADH	pRS416-ADH	CEN URA3 pADH1-FLAG-BRE1	Wozniak and Strahl, 2014. (Wozniak and Strahl 2014a)
FLAG-Bre1 H665A p416 ADH	pRS416-ADH	CEN URA3 pADH1-FLAG-bre1-H665A	Wozniak and Strahl, 2014.

Table 3.3. qRT-PCR Primers used in this study.

Primer	Sequence
HM078-GAL1 F	GCGCAAAGGAATTACCAAGAC
HM079-GAL1 R	TTGACTCTACCAGGCGATCTA
ACT1 F	TTCTGGTATGTGTAAAGCCGG
ACT1 R	CCATACCGACCATGATACCTTG

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

When I began my journey as a graduate student in the Strahl lab, my goal was to understand the mechanisms behind the function of the histone H4 basic patch and histone H2B monoubiquitylation (H2Bub1) in regulating Dot1-mediated H3K79 methylation. The phenomenon of histone crosstalk, where the presence of a histone PTM regulates the addition of another histone PTM, had been well-described but poorly characterized and I was intent, perhaps overconfidently, on addressing this gap of knowledge and advancing the field. While I was hopeful my work would diverge from the regulation of H2Bub1, my pursuit led me to uncover a novel function of the histone H4 basic patch in regulating H2Bub1 through by regulating SAGA histone modifying activities. While the work in presented in this dissertation does not reveal the full mechanism behind the regulation of SAGA by the H4 basic patch, it does add to the already large body of literature about the elegant mystery that is the nucleosome and its regulation.

4.1 The histone H4 basic patch functions independently of the H2Bub1 machinery

As presented in Chapter 1, the histone tails, and their subsequent PTMs, play critical roles in regulating transcription and gene expression. Of particular interest is a short stretch of basic residues on the histone H4 N-terminus termed the H4 basic patch (Figure 3.1A). One of the earliest descriptions of the H4 basic patch is found in the original 2.8Å structure of the NCP that reported residues 16-25 of histone H4 interacting with a region of histone H2A on a neighboring nucleosome (Luger et al. 1997). Subsequent studies would show that the H4 basic patch is essential for chromatin condensation and compaction and that acetylation of histone H4 lysine-

16 disrupts chromatin compaction while stimulating transcription (Tse et al. 1998; Akhtar and Becker 2000; Shogren-Knaak et al. 2006). Additionally, the H4 basic patch was found to regulate the activity of the ISWI chromatin remodeling complex (C. R. Clapier et al. 2001; Hamiche et al. 2001; C. R. Clapier 2002), to bind the yeast Sir3 silencing protein (Altaf et al. 2007), and to bind and regulate the H3K79 methyltransferase, Dot1 (Fingerman, Li, and Briggs 2007). Importantly, nearly all of these functions are conserved throughout higher eukaryotes, highlighting the evolutionary importance of the H4 basic patch. While investigating the mechanism behind the H4 basic patch regulation of Dot1, we discovered that global levels of H2Bub1 were significantly reduced in an arginine to alanine mutation of the basic patch (Figure 3.1A, B). While the mechanisms regulating H2B ubiquitylation and deubiquitination have been well established, our understanding of how regions of the NCP itself regulate these mechanisms is unclear. Our discovery suggested two possibilities for the global decrease in H2Bub1: 1) the basic patch is a positive regulator of the ubiquitylation machinery, or 2) the basic patch is a negative regulator of the deubiquitination machinery.

There is precedence of the importance of specific regions of the nucleosome, and not specific histone PTMs, and the regulation of H2Bub1. Recent work by many, including the Wolberger and Arndt labs, have characterized the role of the nucleosome acidic patch in the regulation of both ubiquitylation and deubiquitination. Mutation of two adjacent residues in the acidic patch, H2A-E65A and H2A-L66A, result in a loss of H2Bub1 levels. Upon deletion of the H2Bub1 deubiquitinase (DUB), Ubp8, H2Bub1 levels are rescued in the H2A-E65A mutant but not in the H2A-L66A mutant suggesting that residue E65 is likely involved in the regulation of Ubp8 DUB activity (Cucinotta et al. 2015). Applying the same principle to our study of the H4 basic patch, deletion of Ubp8 also rescues H2Bub1 levels suggesting that the ubiquitylation

machinery is intact (Figure 3.4A). However, steady state levels of histone PTMs may provide an incomplete picture of the regulation of such dynamic processes, and the regulation of H2Bub1 is a prime example, being regulated by the subunits of two RNAP II-associated complexes. To address this question, we applied the use of the light-inducible nuclear exporter (LINX) fused to the H2Bub1 E3-ligase, Bre1, and measured the kinetics of H2Bub1 in the H4 basic patch mutant in real time (Figure 3.4C and D). Using LINX-Bre1 allowed us to conclude with certainty that the H4 basic patch is not involved in regulation the H2Bub1 ubiquitylation machinery. Interestingly, when the structure of the Ubp8 DUB module on a ubiquitylated nucleosome was solved, a member of the DUB module, Sgf11, was found to interact with H2A-E65 (Morgan et al. 2016). We hypothesized that a similar yet unidentified interaction may be affected in our H4 basic patch mutant.

4.2 The histone H4 basic patch and the SAGA coactivator complex

The H4 basic patch is known to regulate multiple chromatin-modifying enzymes through the direct interaction of these proteins with the H4 basic patch. For instance, recent structural studies have documented how the H4 basic patch interacts with Dot1L to properly orient Dot1L onto the nucleosome (Anderson et al. 2019; Valencia-Sánchez et al. 2019; Worden et al. 2019). Additionally, the binding of the H4 basic patch by the chromatin remodeler ISWI releases the AutoN inhibitory from the remodeler and activates its ATPase activity (Cedric R. Clapier and Cairns 2012). ISWI activity is critical for the proper assembly and spacing of nucleosomes (Cedric R. Clapier et al. 2017) that is believed to restrict access to DNA and enable gene repression (Parnell et al. 2015). With this in mind, we hypothesized that the H4 basic patch may function in a similar manner to regulate H2Bub1 DUB activity.

We pursued this inquiry using two methods: 1) an *in vitro* DUB assay using whole cell lysate as a source of nucleosomal substrate and recombinant Ubp8 or Ubp10 enzyme and, 2) an *in vivo* DUB assay using LINX-Bre1 to control the cellular localization of Bre1 and export it from the nucleus. Firstly, I was successful in establishing the *in vitro* experiments using whole cell lysates that lacked both H2Bub1 DUBs (*ubp8*Δ*ubp10*Δ) and that expressed either wild-type H4 ($H4_{WT}$) or the basic patch arginine-to-alanine mutant H4 ($H4_{2RA}$). For both enzymes, approximately 50% of H2Bub1 is removed within 10 minutes. However, both enzymes display very similar kinetics regardless of the status of the H4 basic patch (Figure 3.9). While I was initially disappointed by the lack of a difference in DUB activity for Ubp8 or Ubp10 in the $H4_{WT}$ and $H4_{2RA}$, I was reassured by the supportive comments of my advisor and committee members that *in vitro* assays often lack the contextual nuance that explains much of cellular biology. Accordingly, both Ubp8 and Ubp10 function in a co-transcriptional manner: Ubp8 as a part of the SAGA coactivator complex, and Ubp10 in cooperation with the histone chaperone, FACT (Henry et al. 2003; Orlandi et al. 2004; Daniel et al. 2004; Emre et al. 2005). As a result, we sought a means to measure H2Bub1 DUB activity *in vivo*, something that, to my knowledge, had not been successfully performed and reported.

We and others had already successfully used LINX-Bre1 to measure the kinetics of ubiquitylation so we hypothesized that performing the optogenetics assay in reverse (growing cells in the dark to maintain LINX-Bre1 in the nucleus and then switching to light leading to the rapid export of LINX-Bre1 to the cytoplasm) may provide us with the resolution necessary to measure the rapid kinetics of deubiquitination *in vivo*. By performing the experiment using cells expressing either $H4_{WT}$ or $H4_{2RA}$ and lacking either Ubp8 or Ubp10 (*ubp8*Δ or *ubp10*Δ), we could measure the specific activity of each DUB under nearly identical and physiological

conditions. To our astonishment, SAGA-associated Ubp8 deubiquitinates $H4_{2RA}$-containing nucleosomes at a significantly higher rate than wild-type nucleosomes, decreasing the half-life of H2Bub1 from ~2.6 minutes to less than 1 minute (Figure 3.5A and B). Additionally, $H4_{2RA}$ cells show no difference in the deubiquitination kinetics of Ubp10 (Figure 3.5C and D). This is the first report of histone H4 regulating the DUB activity of SAGA and the first example, to our knowledge, of histone deubiquitination activity measured *in vivo.* We hypothesize that the H4 basic patch functions as a negative regulator of SAGA DUB activity and mutation of the basic patch relieves SAGA of this regulation leading to aberrant H2Bub1 deubiquitination.

Structural and biochemical studies of the SAGA complex show that the DUB module and the HAT module are in close proximity to one another and that the DUB module stimulates HAT activity (Han et al. 2014). Therefore, we hypothesized that if the H4 basic patch is stimulating SAGA DUB activity, HAT activity will also be impacted. As expected, we measured a noticeable increase in SAGA HAT targets histone H3K18ac and H3K27ac (Figure 3.6). While Gcn5, the catalytic subunit of the SAGA HAT module, is reported to acetylate H3K9 and H3K14 as well, it appears that the level of these PTMs is regulated by the other histone H3 HATs, such as NuA3.

4.3 Possible mechanisms for the regulation of SAGA by the H4 basic patch

An important question still remains: what is the mechanism behind the regulation of the SAGA complex by the histone H4 basic patch? Because the loss of the H4 basic patch leads to enhanced activity of the SAGA DUB module, I suspect that the H4 basic patch in general, and the two arginine residues of the basic patch in particular, are important for an interaction that serves to "apply the brakes" on deubiquitination. The published structure of the SAGA DUB module bound to a H2Bub1-bound nucleosome makes no mention of the histone H4 N-terminal

tail (Morgan et al. 2016). In this structure, most of the contacts between the SAGA DUB module and the nucleosome are mediated through an arginine cluster on Sgf11 that interact with the nucleosome acidic patch, and two residues in Ubp8 that interact with H2B and the conjugated ubiquitin. This suggests that the H4 basic patch regulation of SAGA DUB activity occurs without physically interacting with the DUB module.

In considering how the H4 basic patch may regulate SAGA DUB activity without interacting with any members of the DUB module, it may be useful to consider how the histone H4 N-terminal tail contributes to chromatin structure. We know through structural and computational modeling studies that H4 basic patch interacts with the nucleosome acidic patch of a neighboring nucleosome. In the modeled structure, histone H4 residues R17 and R19 interact with H2A residues E56 and E64 (E57 and E65 in yeast), respectively (Yang and Arya 2011). Interestingly, the structure of the SAGA DUB module shows Sgf11 residues R78, R84, and R91 interacting with acidic patch residues H2A-E64, H2B-E107, and H2A-E61(Morgan et al. 2016). I hypothesize that the H4 basic patch and Sgf11 compete for binding of the same region of the nucleosome acidic patch and that doing so is critical for regulating the dynamic cycle of ubiquitylation and deubiquitination. In support of this hypothesis, mutation of Sgf11 residues R78, R84, and R91 or H2A-E64 reduces the rate of deubiquitination *in vitro* (Morgan et al. 2016) and substitutions of Sgf11 residues R84, and R91disrupt global deubiquitination *in vivo* (Köhler et al. 2010; Koehler et al. 2014). One possible method to test this hypothesis would be to use the well-defined amber codon suppression system to introduce a UV-light inducible crosslinking amino acid in the place of the H4 basic patch arginine residues to identify the interactome of the H4 basic patch. Additionally, it would be interesting to see whether a crosslinking residue could be used as a nucleosome acidic patch "trap", thereby preventing any

other proteins from interacting after UV-light induced crosslinking. If so, one may envision performing a time course pre- and post-UV-light induction to determine the effect of inhibiting binding to the parts of the nucleosome acidic patch bound by the H4 basic patch on H2Bub1 deubiquitination. Another approach that may allow us to distinguish between the functions of the nucleosome acidic patch and the H4 basic patch is to construct yeast strains expressing both a nucleosome acidic patch mutant in combination with the H4 basic patch mutant. Because mutation of many of the nucleosome acidic patch residues, including H2A-E65, significantly reduce global H2Bub1 levels, it will be important to know how these phenotypes will be affected by the H4 basic patch mutant.

Another nucleosome acidic patch residue implicated in the regulation of H2Bub1 by the SAGA DUB module is histone H2A-Y57/58 (human/yeast). This residue is phosphorylated by casein kinase 2 (CK2) and both the inhibition of CK2 and a mutation of the residue (Y57F) enhance SAGA DUB activity on H2Bub1(Basnet et al. 2014). Interestingly, this the H2A-Y58F mutation in yeast leads to a moderate increase in SAGA-mediated H3K27 acetylation (Suka et al. 2001), reminiscent of our observation on the effect of the H4 basic patch mutant on SAGA-mediated H3 acetylation (Figure 3.6). While the structural studies suggest this residue lies at the interface between the SAGA DUB module and the nucleosome, it will be important to assess the effect of the H4 basic patch mutant on H2A-Y58 phosphorylation levels.

Another important consideration for the mechanism behind the H4 basic patch regulation of SAGA DUB activity is the status of H4K16 acetylation (H4K16ac). This PTM is a key regulator of chromatin structure, especially in regards to higher-order chromatin structure, chromatin compaction and nucleosome accessibility for chromatin-modifying complexes. I attempted to address this question by performing immunoblot analysis for H4K16ac in the $H4_{WT}$

and $H4_{2RA}$. Interestingly, no H4K16 acetylation was detected the $H4_{2RA}$ cells, but it is unclear whether or not this was due to the loss of acetylation as a result of the mutation of the H4 basic patch or a failure of the antibody to recognize the mutated epitope (data not shown). It will be useful to apply more sensitive methods to determine the status of H4K16 acetylation in the H4 basic patch mutant in order to assess whether interactions mediated by the basic patch are important for regulating H4K16ac and therefore higher order chromatin structure.

Because the SAGA complex is so intimately involved in the regulation of transcription initiation and elongation, it will be critical to determine if the effects due to the H4 basic patch mutant impact the highly regulated steps in these processes. RNAP II CTD phosphorylation functions as one of the key checkpoints in transcription regulation and SAGA DUB activity is critical to this process (Wyce et al. 2007). Therefore, it would be useful to perform a chromatin immunoprecipitation (ChIP) experiment using antibodies against the various states of CTD phosphorylation at the promoters and 5' end of genes. Understanding if and how RNAP II CTD phosphorylation is affected due to the loss of the H4 basic patch may provide us with greater insight into understanding the mechanism behind the H4 basic patch regulation of SAGA activity as well as uncover new insights into SAGA regulation of transcription initiation and elongation.

4.4 Concluding Remarks

It is my hope that I have highlighted the uniqueness of the histone H4 basic patch in these preceding pages. Consisting of only five basic amino acids, the H4 basic patch is involved in regulating nearly all the different types of chromatin-modifying activities mentioned throughout this dissertation. From the earliest reports of the H4 N-terminal tail interacting with the nucleosome acidic patch to regulate higher-order chromatin structure, to its role as a positive regulator the ISWI chromatin remodeler and, as described in the work presented in this

dissertation, as a negative regulator of the chromatin modifying activities of the SAGA coactivator complex. This works adds to the already extensive body of knowledge on the regulation of H2Bub1 and adds yet another mechanism for regulating this important histone PTM. This is unsurprising given the importance of maintaining the proper balance of H2Bub1 ubiquitylation and deubiquitination and the dire consequences when this balanced in perturbed.

Identifying the H4 basic patch as a negative regulator of SAGA deubiquitylation and histone acetyltransferase activities also supports the growing body of evidence that the SAGA complex is a bona fide cofactor for all RNAP II transcription. The regulation of SAGA by a region of the nucleosome supports the idea of this being a general mechanism of regulation rather than an artifact of a specific scenario such as a response to stimuli or cellular stress. What remains to be determined is whether or not the regulation of SAGA is independent of the well-established role of the H4 basic patch in regulating ISWI chromatin remodeling activity or due to some effect on the structure of chromatin around the transcription start sites. Additionally, it will be of immense interest to determine the interaction profile of the H4 basic patch with the nucleosome acidic patch throughout the transcription cycle. The nucleosome acidic patch is, in my opinion, the only other region of the NCP that is more interesting than the H4 basic patch (to be fair it’s also much larger). To determine how the various chromatin-modifying enzymes, including those that regulate H2Bub1, possibly compete with the H4 basic patch to maintain their various functions will be interesting to see.

When I began my graduate school journey, I was amazed by the elegant mystery that is the nucleosome. Years later, I know more about the nucleosome and its functions than I ever thought I would, yet I remain humbled by the mystery of the nucleosome that continues to

unfold. I look forward to what the future brings in increasing our understanding of the most powerful macromolecule in the cell.

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