Dynamic Regulation of Histone Lysine Methylation in *Saccharomyces cerevisiae*

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Abstract

Kathryn Elizabeth Gardner: Dynamic Regulation of Histone Lysine Methylation in *Saccharomyces cerevisiae*
(Under the direction of Dr. Brian D. Strahl)

DNA within eukaryotic nuclei is wrapped around histone proteins to form chromatin. Recent advances have greatly extended our understanding of both histone lysine methylation as an important post-translational modification that affects chromatin functionality and the enzymes responsible for placement of these marks. This particular modification plays important roles in maintenance of genome integrity, transcriptional regulation, and epigenetic memory. In the budding yeast *Saccharomyces cerevisiae*, histone lysine methylation has been shown thus far to occur on lysine residues 4, 36, and 79 of histone H3 (H3K4, H3K36, and H3K79, respectively) and is coupled tightly to the process of transcription. Prior to completion of the studies contained herein, both the reversibility of histone lysine methylation and the presence of methyl marks on other lysine residues remained poorly understood. Histone methylation was long considered to be a static modification. However, identification of a novel enzyme capable of removing methyl marks from modified lysine residues challenged this thought. Intriguingly, the identified demethylase activity was solely conferred by the enzyme's JumonjiC (JmjC) domain, a signature motif present in a large family of proteins, suggesting that other JmjC-domain-containing proteins could also act as histone demethylases. In budding yeast, the JmjC-domain-containing protein Jhd1 was the first identified histone demethylase with specificity for the mono- and dimethyl states of H3K36. To extend the concept of reversibility of histone methylation in yeast to other modified residues in distinct methylation states, here the budding yeast

JmjC-domain-containing proteins Rph1 and Jhd2 are characterized as active histone demethylases with specificity for di- and trimethylated H3K36 and H3K4, respectively. Importantly, evidence is provided that Rph1-mediated demethylation of H3K36 putatively functions in transcription elongation and that Jhd2 is necessary for proper silencing of telomeric regions. Beyond demonstrating that histone methylation can be actively reversed, evidence is also provided that additional sites of lysine methylation exist. Namely, lysine 37 of histone H2B is identified as a novel site of histone methylation in budding yeast with evolutionary conservation in humans. Altogether, the work described in this dissertation supports the dynamic nature of histone lysine methylation and existence of additional sites of lysine methylation in budding yeast.

"My father gave me life,
My mother nourished me.
Were it not for them,
I could not have come into being.
Their love for me cannot be repaid;
It is as boundless as the sky."

Songgang Jeong Cheol, 1536-1593

Dedicated with love to my parents

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List of Abbreviations

^{3}H-SAM	*S*-adenosyl-L-[methyl-^{3}H]methionine
5-FOA	5-fluoroorotic acid
6-AU	6-azauracil
AdoHcy	*S*-adenosyl-homocysteine
AdoMet	*S*-adenosyl-L-methionine
ChIP	chromatin immunoprecipitation
ChIP-chip	chromatin immunoprecipitation coupled with DNA microarrays analysis
ChIP-seq	chromatin immunoprecipitation coupled with deep-sequencing
cpm	counts per minute
CTD	carboxy terminal domain
Da	Dalton
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
Fe(II)	iron^{2+}
FTICR	Fourier transform ion cyclotron resonance
H2A	histone 2A
H2B	histone 2B
H2BK123	histone H2B lysine 123
H2BK37	histone H2B lysine 37
H3	histone 3
H3K27	histone H3 lysine 27
H3K36	histone H3 lysine 36
H3K4	histone H3 lysine 4

H3K79	histone H3 lysine 79
H3K9	histone H3 lysine 9
H4	histone 4
H4K20	histone H4 lysine 20
HAT	histone acetyltransferase
HDAC	histone deacetylase
HDMT	histone demethylase
HMT	histone methyltransferase
hr	hour
HU	hydroxyurea
JmjC	JumonjiC
kDa	kilodalton
mCi	millicurie
me1	monomethyl
me2	dimethyl
me3	trimethyl
min	minute
mM	millimolar
MMS	methyl methanesulfonate
MPA	mycophenolic acid
MS	mass spectrometry
MS/MS	tandem mass spectrometry
ORF	open reading frame
PBS	phosphate-buffered saline

PHD	plant homeodomain
PMSF	phenylmethylsulfonyl fluoride
PTM	post-translational modification
qPCR	quantitative PCR
RNAPII	RNA polymerase II
RT-PCR	reverse transcription-PCR
SC	synthetic complete
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
sec	second
SET	*Su(var)3-9*, *Enhancer of zeste*, and *Trithorax*
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
ub	ubiquitin
WCE	whole cell extract
WT	wild-type
YPD	yeast extract-peptone-dextrose
ZF	zinc finger
α-KG	α-ketoglutarate
β-ME	β-mercaptoethanol
µL	microliter
µM	micromolar

Chapter One

Introduction

Chromatin Structure and Function | The rich history of chromatin to date has been written by the laudable efforts of numerous scientists, and is defined by landmark discoveries spurred by great vision and even some serendipitous moments. In the late 1800s, Walther Flemming coined the term "*chromatin*" (*stainable material*, which was in reference to the observed fibrous scaffold in the nucleus that could be easily stained) to avoid any confusion that may arise from the more general term "nuclear substance" (PAWELETZ 2001). Despite the discovery of the phosphate-rich "*nuclein"* (now *nucleic acids*) by Friedrich Miescher in 1871 and the isolation of "histon" (now *histones*) by Albrecht Kossel in 1884 (OLINS and OLINS 2003), as well as the characterization of the double-helical structure of DNA by James Watson and Francis Crick in 1953 (WATSON and CRICK 1953), it was not until 1974 that the model for the structure of chromatin was proposed by Roger Kornberg, who posited that approximately 200 base pairs of DNA complexed with four histone pairs (KORNBERG 1974). In 1975, "*nucleosomes*" received their formal name from Pierre Oudet (OUDET *et al.* 1975), and in 1997, the elegant crystal structure of the nucleosome core particle was determined to 2.8 Å by Karolin Luger (LUGER *et al.* 1997).

From such elegant studies, we know today that in eukaryotes, DNA is assembled on histones to form chromatin. The basic unit of chromatin is the nucleosome, which consists of approximately 147 base pairs of DNA wrapped in 1.75 superhelical turns around an octamer containing two copies of each of the four core histones H2A, H2B, H3 and H4 (LUGER *et al.* 1997). Nucleosomes are packaged into progressively higher order structures, such as "beads-on-a-string" and the 30 nm chromatin fiber, to ultimately form metaphase chromosomes (ALBERTS and MANIS 2002). For mitotic chromosomes, such packaging results in a compaction ratio of nearly 10,000-fold (ALBERTS and MANIS 2002).

In broad terms, chromatin structure influences transcriptional regulation, maintenance of genomic integrity, and epigenetic inheritance. Because chromatin structure affects DNA-

templated processes, including transcription, DNA replication, recombination and repair, and chromosome segregation, access to DNA must be tightly controlled to allow factors that function in such processes to make appropriate contacts with the DNA template itself. Interphase chromosomes are still tightly packed (with a compaction ratio of approximately 1000-fold), but condense and decondense as is necessary to provide access for the cellular machinery to specific DNA sequences appropriate for a particular biological process (ALBERTS and MANIS 2002). Such fluidity within the levels of chromatin compaction thereby necessitates a means by which rapid and localized access to DNA can be accomplished.

Histone Post-translational Modifications and the Histone Code | One means by which alterations to chromatin structure is accomplished is through post-translational modifications (PTMs) of the histone proteins. Short, unstructured N-terminal tails (typically less than 40 amino acids long and rich in basic residues) protruding from the globular domains of the nucleosome core particle are subject to numerous PTMs (CAMPOS and REINBERG 2009). It is becoming increasingly evident that residues within the globular domains themselves are also subject to being post-translationally modified (CAMPOS and REINBERG 2009; FREITAS *et al.* 2004; MERSFELDER and PARTHUN 2006). Modifications thus far identified to occur on histone proteins include acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP-ribosylation, proline isomerization, citrullination, glycosylation, butyrylation and propionylation (CHEN *et al.* 2007; KOUZARIDES 2007; SAKABE *et al.* 2010).

Distinct domains exist within the genome, where condensed chromatin (heterochromatin) is generally inaccessible and open chromatin (euchromatin) is more accessible to cellular machinery. For a process such as transcription, such delineation between heterochromatin and euchromatin affects gene expression, and ultimately results in defined

transcriptionally silenced and active regions, respectively (CHOSED and DENT 2007; TAMARU 2010). As modifications help to demarcate such regions, modulation of chromatin architecture by PTMs therefore functions in establishing the appropriate local environment for normal cellular processes to occur. While the functional significance of some of the above-mentioned modifications remains to be determined, other modifications can be functionally categorized as *intrinsic*, *extrinsic*, and/or *effector-mediated* (CAMPOS and REINBERG 2009). Where *intrinsic* denotes effects caused by PTMs that directly alter the physical properties of the nucleosome (such as DNA contacts or stability), *extrinsic* refer to those PTMs that directly impact internucleosomal contacts. In both cases, modifications disturb contacts between histones in contiguous nucleosomes or histones with DNA, resulting in alteration of higher-order chromatin structure. For example, acetylation of lysine residues on histone tails neutralizes the basic charge of the residue on which it occurs, thereby disrupting histone contacts with other histones and/or DNA and in turn chromatin compaction (HONG *et al.* 1993; SHOGREN-KNAAK *et al.* 2006; WOLFFE and HAYES 1999). Alterations of nucleosomes by PTMs of histones can also promote the association of non-histone chromatin-binding proteins, and are thus termed *effector-mediated* (CAMPOS and REINBERG 2009). Here, recruitment of non-histone effector proteins is facilitated by the ability of specialized domains to recognize and bind to modifications in defined states. For example, bromodomains can recognize acetylated lysine residues, and the following have been identified as methyl-binding domains: chromodomain, tudor domain, PHD finger, MBT, Ankyrin repeat, PWWP domain and WD40 repeats (COLLINS *et al.* 2008; TAVERNA *et al.* 2007a; VEZZOLI *et al.* 2010; WANG *et al.* 2009).

The existence of numerous and diverse types of PTMs capable of recruiting non-histone effector proteins prompted the proposal of a histone code, which posits that combinatorial patterns of histone modifications lead to defined biological outcomes mediated by the

recruitment of effector proteins (STRAHL and ALLIS 2000). For instance, TAF1 (the largest subunit of the TFIID complex which is involved in initiating the assembly of the transcriptional machinery) preferentially binds to multiply acetylated histone H4 through its double bromodomain (JACOBSON *et al.* 2000) and functions itself as a histone acetylase (MIZZEN *et al.* 1996). It was believed that the repercussions of such a code could broadly impact diverse processes such as gene expression, epigenetic inheritance, and control of cellular growth, differentiation, and disease. Numerous elegant biochemical and genetic studies, as well as technological advancements, have dramatically expanded this concept since the time of its inception to demonstrate the many intricacies of the histone code (AGALIOTI *et al.* 2002; FISCHLE *et al.* 2005; HAKE and ALLIS 2006; NG *et al.* 2002b; SHI *et al.* 2006; SUN and ALLIS 2002; TAVERNA *et al.* 2006; WYSOCKA *et al.* 2006; ZIPPO *et al.* 2009).

Histone Lysine Methylation in *Saccharomyces cerevisiae* | Lysine methyltransferases catalyze the transfer of one to three methyl groups from *S*-adenosyl-L-methionine to the ε-amino group of the target lysine residue (FIGURE 1.1, panel A) (SHILATIFARD 2006). The budding yeast *Saccharomyces cerevisiae* encodes three known histone lysine methyltransferase enzymes, Set1, Set2, and Dot1, which modify histone H3 on lysine residues 4, 36, and 79, respectively (FIGURE 1.1, panel B) (MILLAR and GRUNSTEIN 2006). The histone methyltransferases are typically highly specific for their targeted substrate as well as the degree to which they methylate a specified lysine residue (SHILATIFARD 2006; XIAO *et al.* 2003a). As methylation of lysine residues does not change the overall charge of the histone molecule, this modification is largely believed to serve as a binding platform for effector proteins that recognize and interpret these marks to mediate downstream effects. Accordingly, beyond its position on the histone tail, the methylation state (mono-, di-, or trimethyl; me1, me2, or me3, respectively) of a modified lysine residue is also

important, as placement of a specific number of methyl marks on different lysine residues establishes diverse environments for effector proteins to recognize, in turn producing distinct functional outputs (KOUZARIDES 2007). Methylation of histone H3 on lysines 4, 36, and 79 largely correlates with euchromatic regions of chromatin (MARTIN and ZHANG 2005), and accordingly, histone lysine methylation in budding yeast is tightly coupled to the process of transcription, where the deposition of these modifications occurs during the initiation and elongation phases of RNA polymerase II-based transcription (MILLAR and GRUNSTEIN 2006).

Histone H3 Lysine 4 (H3K4) Methylation | The SET domain is an evolutionarily conserved domain (named after the *Drosophila* genes *Su(var)3-9*, *Enhancer of zeste*, and *Trithorax*) that has been shown to be involved in modulation of gene activity and histone lysine methylation (JENUWEIN *et al.* 1998; SHILATIFARD 2006; ZHANG and REINBERG 2001). There are hundreds of proteins harboring this motif in species ranging from bacteria to humans (ZHANG and REINBERG 2001), and 12 proteins in budding yeast contain a SET domain (PETROSSIAN and CLARKE 2009a). In *Saccharomyces cerevisiae*, methylation of H3K4 is mediated by the SET-domain-containing protein Set1 (BRIGGS *et al.* 2001; ROGUEV *et al.* 2001). Set1 is capable of methylating H3K4 in all three states, and deletion of *SET1* abolishes all H3K4 methylation (BRIGGS *et al.* 2001).

As Set1 mediates all three H3K4 methylation states, it is believed that targeting and/or regulation of the methyltransferase activity of Set1 is necessary for proper spatiotemporal patterns of H3K4 methylation (MARTIN and ZHANG 2005). In budding yeast, Set1 is part of a larger macromolecular complex named COMPASS (complex proteins associated with Set1). The subunits of COMPASS are termed Cps60 through Cps15 (according to their molecular weight), and include: Cps60, Cps50, Cps40, Cps35, Cps30, Cps25, and Cps15 (KROGAN *et al.* 2002; MILLER *et al.* 2001). COMPASS complex members are necessary for regulation of Set1 enzymatic activity.

For example, where Set1, Cps50 and Cps30 are necessary for all three methylation states, only Set1, Cps60, Cps40, and Cps25 are necessary for formation of trimethylated H3K4 (SCHNEIDER *et al.* 2005).

Set1 has been found to be predominantly localized to the coding regions of highly transcribed RNA polymerase II (RNAPII) genes, and associates with the carboxy terminal domain (CTD) of RNAPII in a manner that is dependent upon Kin28-mediated phosphorylation of serine 5 of the CTD (NG *et al.* 2003). Set1 association with the RNAPII CTD is also thought to be mediated in part by the Paf1 complex (KROGAN *et al.* 2003a). The development of modification-specific antibodies that are capable of recognizing modified lysine residues in a particular methylation state (me1, me2, or me3) has allowed for genome-wide mapping studies of histone methylation by chromatin immunoprecipitation combined with whole-genome microarrays (ChIP-chip) and more recently, coupled with deep sequencing (ChIP-seq) (MILLAR and GRUNSTEIN 2006). Genome-wide ChIP-chip analyses has revealed that H3K4 trimethylation is localized specifically to the 5' end of actively transcribed genes, where dimethylation is more enriched in the middle of genes, and monomethylation is found predominantly at the 3' end of genes (POKHOLOK *et al.* 2005). Such genome-wide patterning of the various methylated forms of H3K4 in general correlates well with Set1 occupancy and RNAPII association.

Trans- and *cis*-tail mechanisms also exist as a level of regulation for Set1-mediated methylation of H3K4. Monoubiquitylation of histone H2B on lysine 123 (H2BK123ub) catalyzed by the budding yeast E2 ubiquitin-conjugating enzyme Rad6 and E3 ubiquitin ligase Bre1 (ROBZYK *et al.* 2000) has been demonstrated to be a prerequisite modification for H3K4 methylation, as mutation of H2BK123 to an unmodifiable form (H2B K123R) or deletion of *RAD6* or *BRE1* results in a loss of H3K4me (BRIGGS *et al.* 2002; DOVER *et al.* 2002; SUN and ALLIS 2002). It has been suggested that the Cps35 subunit of the COMPASS complex is involved in regulation of the

crosstalk between H2BK123ub and methylation of H3K4, as Cps35 interacts with chromatin in a monoubiquitin-dependent fashion (TAKAHASHI and SHILATIFARD 2010). Use of the Scanning Histone Mutagenesis with Alanine (SHIMA) library has revealed that additional *cis*- and *trans*-tail regulatory mechanisms may exist in which H3K4 trimethylation is influenced by H3K14 and residues on histones H2A and H2B (namely E65, L66, N69, and D73 of histone H2B, and H112 and R119 of histone H2B) (NAKANISHI *et al.* 2008). The exact mechanism by which these residues function in regulating H3K4 methylation is unknown, but it is possible that acetylation of H3K14 and regulation of H2BK123ub function in these *cis*- and *trans*-tail histone crosstalk events, respectively (NAKANISHI *et al.* 2008).

Set1, a member of the yeast Trithorax group of proteins (a large family of proteins whose members have been implicated in transcriptional regulation (RINGROSE and PARO 2004)), was originally identified as a factor necessary for proper regulation of transcriptional silencing of telomeres and the silent mating-type loci (NISLOW *et al.* 1997). *SET1* is not an essential gene, but its deletion results in pleiotropic phenotypes, namely affecting growth, transcriptional activation, repression and elongation, regulation of telomere length, rDNA silencing, meiotic differentiation, DNA repair, and chromosome segregation (DEHE and GELI 2006), supporting a role for H3K4 methylation in these processes. It is also possible that the functional consequence of H3K4 methylation is largely dictated by the effector proteins that recognize and bind to this mark. For example, Yng1, a member of the NuA3 histone acetyltransferase (HAT) complex, is able to bind to H3K4me3 through its PHD finger, thus promoting acetylation of H3K14 (MARTIN *et al.* 2006a; TAVERNA *et al.* 2006). Additionally, Set3, the defining member of the Set3 complex (Set3C) containing the histone deacetylases (HDAC) Hos2 and Hst1, harbors a PHD finger that is capable of binding to H3K4me2, thereby functioning in localization of the Set3C HDAC activity (KIM and BURATOWSKI 2009).

Histone H3 Lysine 36 (H3K36) Methylation | Methylation of H3K36 is mediated by the budding yeast SET protein Set2 (STRAHL *et al.* 2002). Set2 is capable of catalyzing H3K36 methylation in all three states. *SET2* is a nonessential gene, and its deletion results in the loss of H3K36 methylation, indicating that it is the sole H3K36 methyltransferase (STRAHL *et al.* 2002). Further *SET2* deletion analysis has revealed phenotypes that are consistent with transcription elongation defects, including resistance to 6-azauracil (a compound that reduces intracellular levels of GTP, which itself is not lethal to yeast, but can be lethal when combined with mutations that affect transcription (EXINGER and LACROUTE 1992; HAMPSEY 1997; RILES *et al.* 2004)) (KIZER *et al.* 2005) and synthetic growth defects when combined with deletions of elongation factors such as the subunits of the Paf complex (KROGAN *et al.* 2003b). Consistent with a role in transcription elongation, many groups have shown that Set2 physically interacts with RNAPII (KROGAN *et al.* 2003b; LI *et al.* 2003; LI *et al.* 2002; SCHAFT *et al.* 2003; XIAO *et al.* 2003b). Collectively, these studies demonstrated that Set2 interacts with RNAPII in a manner that is dependent upon Ctk1-mediated phosphorylation of serine 2 of the CTD. Within the C-terminal domain of Set2, a recently identified domain termed the SRI (Set2-Rpb1 Interacting) mediates interaction with RNAPII and binds to RNAPII CTD repeats that are doubly modified by phosphorylation of both serines 2 and 5 (KIZER *et al.* 2005). Association with the CTD of RNAPII is necessary for Set2 catalyzed methylation of H3K36 (XIAO *et al.* 2003b), as deletion of the SRI results in a loss of all H3K36 methylation (KIZER *et al.* 2005). Consistent with an association of Set2 with RNAPII in the body of actively transcribed genes (KROGAN *et al.* 2003b), genome-wide ChIP-chip analyses have revealed that both di- and trimethylation mediated by Set2 are enriched at the 3' end of open reading frames (ORFs), and that H3K36me3 positively correlates with transcription rates (LI *et al.* 2007a; POKHOLOK *et al.* 2005; RAO *et al.* 2005). These data raise the intriguing possibility that different states of H3K36 methylation have different biological roles in gene regulation.

Given that deletion of *SET2* causes no overt cellular phenotype, it is difficult to extrapolate the functional significance of H3K36 methylation in distinct cellular events from deletion analysis alone. Thus, to date, the role of H3K36 methylation is best characterized in the context of transcription elongation. Eaf3, a subunit of the Rpd3S HDAC complex, has been shown to bind to di- and trimethylated H3K36 through its chromodomain (CARROZZA *et al.* 2005; JOSHI and STRUHL 2005; KEOGH *et al.* 2005). Recruitment of the Rpd3S HDAC complex to H3K36 that has been methylated cotranscriptionally results in a hypoacetylated environment within ORFs. Such deacetylation ultimately functions in preventing transcription initiation from cryptic promoter-like sequences within the gene bodies, as evidenced by the fact that disruption of the Set2-Rpd3S pathway leads to hyperacetylation within an ORF thereby enabling spurious initiation events that result in the formation of cryptic transcripts (CARROZZA *et al.* 2005; JOSHI and STRUHL 2005). The Eaf3 subunit of the Rpd3S HDAC complex is also a member of the NuA4 HAT complex. While a role for H3K36me-binding by Eaf3 in recruitment of the NuA4 complex to date remains to be shown, its paradoxical role as a subunit of both an HAT and HDAC complex suggests that H3K36 methylation could putatively function in localization of acetyltransferase activity in addition to deacetylation. Alternatively, it has been shown that Eaf3 can also bind to trimethylated H3K4 (XU *et al.* 2008), thereby providing an alternative mechanism by which one protein can function as a toggle for two complexes with opposing roles. In line with a general role of methylated H3K36 in recruitment of acetyltransferase activity, the NuA3 HAT complex subunit Nto1 has been shown to preferentially bind to H3K36me3 through its PHD finger (SHI *et al.* 2007). While the functional consequence of this binding remains to formally be demonstrated, it has been suggested that the interaction of NuA3 with chromatin and subsequent HAT activity of this complex is dependent upon the methyltransferase Set2 and its substrate H3K36 (MARTIN *et al.* 2006b).

A role for Set2-mediated H3K36 methylation has also been shown by mutational analysis in combination with mutations in other transcriptional elongation factors. For example, the Bur1/2 cyclin-dependent kinase complex promotes transcription elongation by RNAPII (CHU *et al.* 2006; KEOGH *et al.* 2003). While *BUR2* is a non-essential gene, deletion of *BUR2* renders a slow growth phenotype in yeast (YAO *et al.* 2000). Deletion of *SET2* can bypass this slow growth phenotype (KEOGH *et al.* 2005). Similarly, mutation of H3K36 to a non-modifiable form (H3 K36A) can support cellular viability in the absence of the essential gene *BUR1* (KEOGH *et al.* 2005). Another example of crosstalk between H3K36 methylation and transcription elongation is provided by the FACT histone chaperone complex, composed of Spt16, Pob3 and Nhp6 (BELOTSERKOVSKAYA *et al.* 2003; FORMOSA *et al.* 2001; MASON and STRUHL 2003; SAUNDERS *et al.* 2003; SCHWABISH and STRUHL 2004). Loss of the FACT subunit *SPT16* results in a slow growth phenotype, which can be bypassed by both deletion of *SET2* and mutation of H3K36 (BISWAS *et al.* 2006). Together, these findings support an antagonistic function for Set2-mediated H3K36 methylation and the BUR and FACT complexes.

Histone H3 Lysine 79 (H3K79) Methylation | Unlike lysine residues 4 and 36 that reside on the unstructured N-terminal tail of histone H3, lysine 79 is located within the globular domain of histone H3 on the accessible surface of the outside of the nucleosome core (LUGER *et al.* 1997). Methylation of H3K79 is mediated by the non-SET-domain-containing budding yeast protein Dot1 (disruptor of telomeric silencing 1) (NG *et al.* 2002a; VAN LEEUWEN *et al.* 2002). The majority of H3 in chromatin (estimated to be 90%) is methylated at H3K79 (SHILATIFARD 2006; VAN LEEUWEN *et al.* 2002). Dot1 catalyzes all three methylation states of H3K79, but in contrast to other protein lysine methyltransferases, does so in a nonprocessive fashion (FREDERIKS *et al.* 2008). Unlike Set1 and Set2, there is no evidence that Dot1 physically interacts with actively transcribing RNAPII, but genome-wide ChIP-chip studies have revealed that trimethylated H3K79

is enriched within the transcribed regions of genes, although such enrichment is not clearly correlated with levels of transcriptional activity (POKHOLOK *et al.* 2005; SCHULZE *et al.* 2009). Arguing that the overall level of H3K79 methylation is more important for downstream function than a methylation state in particular, previous work has suggested that Dot1-dependent methylation states of H3K79 are functionally redundant (FREDERIKS *et al.* 2008). However, a subsequent study has counterargued that di- and trimethylated H3K79 are differentially associated with promoters and ORFs, and that these marks are associated with distinct genomic regions thereby supporting a separation of function for the di- and trimethyl state (SCHULZE *et al.* 2009). In agreement with previous studies, H3K79me3 was uniformly enriched in the transcribed regions. However, unlike H3K79me3, H3K79me2 was found in both the ORF and promoter region of genes (particularly those expressed specifically in the M/G1 phase of the cell cycle) (SCHULZE *et al.* 2009).

Methylation of H3K79 by Dot1 has been shown to be regulated by at least two *trans*-tail pathways. Like methylation of H3K4 catalyzed by Set1, monoubiquitylation of lysine 123 on histone H2B is a prerequisite for efficient trimethylation of H3K79 by Dot1 (NG *et al.* 2002b; SHAHBAZIAN *et al.* 2005). In accordance with this *trans*-tail regulatory mechanism, ChIP-chip studies have also demonstrated that genome-wide localization patterns of H3K79me3 (but not H3K79me2) generally correlate with H2BK123ub, supporting the view that monoubiquitylated H2B functions as a major determinant for H3K79me3 (SCHULZE *et al.* 2009). Additionally, the N-terminal tail of histone H4 is necessary for methylation of H3K79 (ALTAF *et al.* 2007; FINGERMAN *et al.* 2007). A patch of basic residues (R17/H18/R19) within the H4 N-terminal tail is necessary for H3K79 methylation, and it has been shown that Dot1 can interact with the H4 N-terminal tail through this basic patch (FINGERMAN *et al.* 2007), thus providing a putative means by which Dot1 can be targeted to the chromatin template to modify nucleosomal H3K79.

Unlike methylated H3K4 and H3K36, there are no proteins identified to date that definitely bind to a specifically methylated state of H3K79 (FREDERIKS *et al.* 2008). Functional insight into the role of H3K79 has largely come from *DOT1* deletion and overexpression analyses. As its name suggests, Dot1 was originally identified in a screen for factors that affect telomeric silencing (SINGER *et al.* 1998). It was subsequently shown that both functional Dot1 methyltransferase activity and intact lysine 79 on histone H3 are required for proper telomeric silencing (NG *et al.* 2002a; VAN LEEUWEN *et al.* 2002). Dot1 has also been shown to function in meiotic checkpoint control (SAN-SEGUNDO and ROEDER 2000). Additionally, Dot1-mediated methylation of H3K79 has been shown to function in DNA repair pathways and G1 and S-phase DNA damage checkpoint signaling (CONDE *et al.* 2009; WYSOCKI *et al.* 2005).

Dynamic Nature of Histone Methylation | Unlike other histone modifications, the dynamic nature of histone methylation was long unclear, as previous studies indicated that methyl group turnover occurred at a rate similar to histone turnover, and the stability of the C-N bond between the methyl and ε-amino groups argued against active demethylation (AGGER *et al.* 2008; BORUN *et al.* 1972; BYVOET *et al.* 1972; KLOSE and ZHANG 2007). Alternative mechanisms proposed in lieu of active removal of methyl groups from histones included histone exchange with an unmodified histone or proteolytic cleavage of the modified histone tail (BANNISTER and KOUZARIDES 2005; BANNISTER *et al.* 2002; SANTOS-ROSA *et al.* 2009). Uncertainty about the dynamic nature of histone methylation was dissipated in 2004, when Yang Shi and colleagues identified Lysine Specific Demethylase 1 (LSD1) as the first active histone demethylase and showed specificity for H3K4me2/1 (SHI *et al.* 2004). LSD1 is an amine oxidase that uses a FAD-dependent oxidation reaction to remove methyl groups, producing hydrogen peroxide and formaldehyde as byproducts (FIGURE 1.2, top) (SHI *et al.* 2004). When associated with the androgen receptor,

LSD1 can change its substrate specificity from H3K4me2/1 to H3K9me2/1 (METZGER *et al.* 2005), supporting the notion that protein-protein interactions play a fundamental role in dictating substrate specificity for the histone demethylases. LSD1 is limited to mono- and dimethylated substrates only, as amine oxidation requires a protonated nitrogen to initiate the demethylation reaction. However, other lysine resides on histone tails are modified by methylation, and the trimethylated state is a common modification, supporting the existence of other enzymes capable of histone lysine demethylation.

Histone Demethylation by JumonjiC (JmjC)-domain-containing Proteins | Using an unbiased biochemical purification and an activity-based assay, Yi Zhang and colleagues demonstrated that JHDM1A (formerly FBXL11, an uncharacterized protein originally identified in a bioinformatics search for F-box-containing proteins (CENCIARELLI *et al.* 1999; WINSTON *et al.* 1999)) possesses histone demethylase activity with substrate specificity for H3K36me2/1 (TSUKADA *et al.* 2006). JHDM1A was shown to use an iron (Fe(II))- and α-ketoglutarate (α-KG)-dependent hydroxylation reaction in which the methylamine group of the targeted lysine residue is hydroxylated, thereby creating a highly unstable intermediate hydroxymethyl group that is spontaneously released as formaldehyde, thus resulting in the loss of a methyl group (FIGURE 1.2, bottom) (KLOSE and ZHANG 2007; TSUKADA *et al.* 2006). JHDM1A contains five curated domains (namely JmjC, FBOX, CXXC-ZF, PHD, and LRR), of which only the JmjC domain is absolutely necessary for demethylase activity (TSUKADA *et al.* 2006). Within the JmjC domain are five cofactor binding site – three amino acids that coordinate Fe(II) and two that bind to α-KG (KLOSE *et al.* 2006a).

Phylogenetic analysis of a non-redundant set of 98 JmjC-domain-containing proteins in six organisms spanning from yeast to humans revealed that these enzymes can be divided into seven subfamilies on the basis of conservation in the JmjC domain and overall protein domain

architecture (KLOSE *et al.* 2006a). The five predicted cofactor-binding sites are well conserved among many of the JmjC-domain-containing proteins, indicating that other members of this family of enzymes could also serve as histone demethylases. Additionally, JmjC-domain-containing proteins often contain other DNA- and/or chromatin-binding domains, suggesting that proteins that contain this domain could function in regulation of chromatin structure (BALCIUNAS and RONNE 2000; CLISSOLD and PONTING 2001; TAKEUCHI *et al.* 1995). Moreover, unlike the amine oxidation mechanism employed by LSD1, the Fe(II)- and α-KG-dependent hydroxylation reaction used for demethylation does not require a protonated nitrogen to initiate the reaction, and therefore has the capacity to target not only mono- and dimethyl marks, but also the trimethyl state. Given that JmjC-domain-containing proteins have a predicted role in modulating chromatin structure, it remained largely possible that the other JmjC-domain-containing proteins were responsible for demethylation of other modified lysine residues in varying methylation states. Indeed, following the initial identification of JHDM1A as an active histone demethylase, a flurry of publications was released demonstrating that JmjC-domain-containing proteins from the other subfamilies also function as evolutionarily conserved histone demethylases with particular substrate specificities (see TABLE 1.1, and (PEDERSEN and HELIN 2010)).

Histone Lysine Demethylation in *Saccharomyces cerevisiae* | Budding yeast has no LSD1 homologue. However, the identification of the JmjC-domain-containing protein Jhd1, the budding yeast orthologue of mammalian JHDM1A, as an H3K36me2/1 demethylase demonstrated the dynamic nature of histone methylation in this organism (FANG *et al.* 2007; TSUKADA *et al.* 2006). There are four other JmjC-domain-containing proteins in budding yeast: Rph1, Gis1, Yjr119c, and Ecm5 (FIGURE 1.3, panel A) (KLOSE *et al.* 2006a; KLOSE and ZHANG 2007).

Due to a high level of conservation within the JmjC domain, it is possible that other enzymes capable of histone demethylation exist within this group. Namely, the putative histone demethylase activity of the remaining four JmjC-domain-containing proteins can be predicted by looking at conservation of cofactor binding sites (FIGURE 1.3, panel B). Rph1 and Gis1 were originally identified as repressors of *PHR1*, a DNA repair gene encoding a photolyase that catalyzes the repair of pyrimidine dimers (JANG *et al.* 1999). Rph1 and Gis1 are the yeast homologues of the mammalian JHDM3/JMJD2 proteins (TABLE 1.1), which possess histone demethylase activity with dual-substrate specificity for H3K9me3/2 and H3K36me3/2 (CLOOS *et al.* 2006; KLOSE *et al.* 2006b; WHETSTINE *et al.* 2006). Whereas Rph1 maintains conservation of all five cofactor binding sites and is a likely candidate for histone demethylation, Gis1 has a mutation in one of the Fe(II)-binding sites that likely abrogates any activity (KLOSE *et al.* 2006a; KLOSE and ZHANG 2007). The mammalian H3K4me3/2 demethylases from the JARID subfamily (JARID1A-D) have two yeast orthologues: Yjr119c and Ecm5 (TABLE 1.1) (KLOSE *et al.* 2006a; KLOSE and ZHANG 2007). *YJR119C* is an uncharacterized ORF. Ecm5 was originally identified in a screen for genes involved in cell surface assembly, but its function remains unknown (LUSSIER *et al.* 1997). Like Gis1, mutations in Ecm5 cofactor binding sites likely abrogate any activity. Yjr119c, however, maintains conservation at all cofactor binding sites, thus making it probable that it functions as a H3K4me3/2 demethylase like its orthologues in higher eukaryotes (EISSENBERG *et al.* 2007; IWASE *et al.* 2007; KLOSE *et al.* 2006a; KLOSE *et al.* 2007b; KLOSE and ZHANG 2007; LEE *et al.* 2007; SECOMBE *et al.* 2007; SEWARD *et al.* 2007; YAMANE *et al.* 2007). On the basis of conservation of residues necessary for cofactor binding, it remains highly likely that the JmjC-domain-containing proteins Rph1 and Yjr119c function as histone demethylases in budding yeast, thus expanding upon the dynamic nature of histone methylation in budding yeast.

Additional Sites of Histone Lysine Methylation | The histone code hypothesis posits that combinatorial patterns of post-translational modifications of histone proteins act in concert to dictate downstream biological outcomes (STRAHL and ALLIS 2000). If the true breadth of such a code is ever to be completely understood, it is essential that the totality of all modifications that putatively contribute to it are identified. To date, only lysine residues 4, 36, and 79 of histone H3 have been characterized as sites of histone methylation in budding yeast (MILLAR and GRUNSTEIN 2006). In higher eukaryotes, methylation also occurs on lysines residues 9 and 27 of histone H3 and lysine 20 of histone H4 (MARTIN and ZHANG 2005). Sophisticated technological advancements, particularly in mass spectrometry (MS), have significantly moved forward efforts to identify novel histone modifications. For example, a recent comprehensive study designed to identify patterns of histone PTMs associated with each phase of the yeast cell cycle using tandem MS (MS/MS) revealed that lysine 111 of histone H2B (H2BK111), lysine 37 of histone H3 (H3K37), and lysine 31 of histone H4 (H4K31) are each monomethylated (UNNIKRISHNAN *et al.* 2010), in agreement with a previously published study also reporting methylation of H3K37 and H2BK111 in yeast (ZHANG *et al.* 2009). The latter study found monomethylation of lysine 22 of histone H2B as well. Another study looking at organismal differences in histone modifications reported that monomethylation of lysine residues 18 and 23 of histone H3 was conserved from yeast to humans (GARCIA *et al.* 2007a). Additional sites of histone lysine methylation have been reported in higher eukaryotes (namely, trimethylation of lysine 64 and monomethylation of lysine 122 of histone H3 in mice and monomethylation of lysine 5 on histone H2B in humans (BARSKI *et al.* 2007; COCKLIN and WANG 2003; DAUJAT *et al.* 2009; WANG *et al.* 2008)). It is likely that additional sites of histone lysine methylation remain to be identified, thereby necessitating additional investigations aimed at elucidating a complete atlas of histone PTMs, as much

remains to be discovered with regard to the intricacies of lysine methylation and how exactly it contributes to the histone code and cellular function.

Significance of Studies on the Dynamic Nature Histone Lysine Methylation in *Saccharomyces cerevisiae* | Across evolution, histone methylation has pleiotropic cellular roles regulating processes including transcriptional regulation, X-chromosome inactivation, heterochromatin formation, and homeotic-gene regulation (MARTIN and ZHANG 2005). Both enzymes that place and remove histone methyl marks are of fundamental importance to such processes, as they maintain appropriate levels of methylation necessary for normal cellular function. Aberrant regulation of gene expression is a central cause of many human diseases. Mutation or overexpression of both histone methyltransferases and histone demethylases resulting in misregulation of gene expression has been linked to human diseases including neurological disorders and cancer (ALBERT and HELIN 2010; SHI 2007). There is much speculation in the field as to whether HMTs and HDMTs could be potentially targeted for therapeutic purposes. However, such advanced applications necessitate a basic understanding of the underlying enzymology, molecular mechanism, and biological function of the appropriate histone modifying enzyme. To that end, cross-disciplinary investigations involving model organism studies will provide a more complete understanding of the workings of such enzymes, as models such as the budding yeast *Saccharomyces cerevisiae* provide an elegant system in which complementary biochemical and genetic analyses can be completed. Moreover, with particular regard to histone modifications, point mutants in specifically modified residues can be made straightforwardly in this organism, a feat not readily accomplished in higher eukaryotes (KOUZARIDES 2007). Thus, the studies completed in budding yeast will ultimately provide a more comprehensive picture of the dynamic regulation of histone lysine methylation, and contribute significantly to future studies

on how such regulation functions in more complex biological processes such as cellular growth, differentiation, and disease.

Synopsis of Work Contained within this Doctoral Dissertation | All the studies contained within the subsequent chapters are centered around the theme of "Dynamic regulation of histone lysine methylation in *Saccharomyces cerevisiae*". The majority of this work is aimed at demonstrating that lysine methylation is a dynamic modification in *S. cerevisiae* and is thus focused on the identification and characterization of histone lysine demethylases in this model organism. To that end, in Chapter Two, the budding yeast JmjC-domain-containing protein Rph1 is identified as an active histone demethylase with specificity for di- and trimethylated lysine 36 of histone H3 (KLOSE *et al.* 2007a). In continuing with the theme of histone demethylation, in Appendix One the protein product of the previously uncharacterized budding yeast ORF *YJR119C* is identified as a histone lysine demethylase with specificity for H3K4me3/2 (and was subsequently renamed Jhd2 for JmjC-domain-containing histone demethylase 2) (LIANG *et al.* 2007). Beyond the active removal of histone lysine methyl marks, regulation of their placement was investigated. Though it had been previously established that a *trans*-tail regulatory mechanism existed for placement of H3K4 methylation, a recent study questioned the validity of this histone crosstalk (FOSTER and DOWNS 2009). Conclusive evidence that monoubiquitylation of histone H2B on lysine 123 is indeed a prerequisite for methylation of both H3K4 and H3K79 is provided in Appendix Two, thus supporting the existence of such a *trans*-tail regulatory mechanism (NAKANISHI *et al.* 2009). To expand the field of chromatin biology's understanding of how histone methylation contributes to the histone code by identifying additional marks that must be factored into it, in Chapter Three, lysine 37 of histone H2B is identified as a novel site of histone methylation in budding yeast (GARDNER *et al.* 2011b). Lastly, reflections on how the

histone code hypothesis itself has evolved since it was first proposed in 2000 are included Chapter Four to provide readers with a current synopsis of how landmark studies in chromatin biology focused on placement, removal and/or interpretation of histone modifications (including lysine methylation) have influenced the many shapes this influential hypothesis has taken over the past ten years (GARDNER *et al.* 2011a). In total, this work endeavors to expand our understanding of the enzymology and regulation of histone lysine methylation to provide readers with a greater appreciation for the complexity and amazing potential of this unique histone modification.

Table 1.1 | JmjC-domain-containing histone lysine demethylases and their substrate specificity

Subfamily	Protein Name	Substrate Specificity	Reference(s)
JHDM1	JHDM1A (Hs)	H3K36me2/1	(Tsukada *et al.* 2006)
	JHDM1B (Mm, Hs)	H3K36me2/1	(He *et al.* 2008)
	dKDM2 (Dm)	H3K36me2	(Lagarou *et al.* 2008)
	Jhd1 (Sc)	H3K36me2/1	(Tsukada *et al.* 2006)
JHDM2	JHDM2A/JMJD1A (Mm, Hs)	H3K9me2/1	(Yamane *et al.* 2006)
	JHDM2B/JMJD1B (Mm)	H3K9me2	(Yamane *et al.* 2006)
JHDM3/ JMJD2	JHDM3A/JMJD2A (Hs)	H3K9me3; H3K36me3	(Cloos *et al.* 2006; Klose *et al.* 2006b; Whetstine *et al.* 2006)
	JHDM3B/JMJD2B (Mm, Hs)	H3K9me3/2	(Cloos *et al.* 2006; Fodor *et al.* 2006)
	GASC1/JMJD2C (Hs)	H3K9me3/2	(Cloos *et al.* 2006)
	ceJMJD2 (Ce)	H3K9me3; H3K36me3	(Whetstine *et al.* 2006)
	dKDM4A (Dm)	H3K36me3/2	(Lin *et al.* 2008)
	dKDM4B (Dm)	H3K9me3; H3K36me3/2	(Lin *et al.* 2008)
	Rph1 (Sc)	H3K9me3/2; H3K36me3/2	(Klose *et al.* 2007a)
JARID	JARID1A/RBP2 (Mm, Hs)	H3K4me3/2	(Christensen *et al.* 2007; Klose *et al.* 2007b)
	JARID1B/PLU-1 (Mm, Hs)	H3K4me3/2	(Yamane *et al.* 2007)
	JARID1C/SMCX (Hs)	H3K4me3/2	(Iwase *et al.* 2007)
	JARID1D/SMCY (Hs)	H3K4me3/2	(Christensen *et al.* 2007; Iwase *et al.* 2007)
	rbr-2 (Ce)	H3K4me3	(Christensen *et al.* 2007)
	Lid (Dm)	H3K4me3/2	(Christensen *et al.* 2007; Eissenberg *et al.* 2007; Lee *et al.* 2007b; Secombe *et al.* 2007)
	Jmj2 (Sp)	H3K4me3/2	(Huarte *et al.* 2007)
	Lid2 (Sp)	H3K4me3	(Li *et al.* 2008)
	Jhd2 (Sc)	H3K4me3/2	(Liang *et al.* 2007)
UTX/UTY	UTX (Mm, Hs)	H3K27me3/2	(Agger *et al.* 2007; Lan *et al.* 2007; Lee *et al.* 2007a)
	JMJD3 (Mm, Hs)	H3K27me3/2	(Agger *et al.* 2007; De Santa *et al.* 2007; Lan *et al.* 2007)
	XJ193 (Ce)	H3K27me3	(Agger *et al.* 2007)
	dUTX (Dm)	H3K27me3/2	(Smith *et al.* 2008)
PHF2/PHF8	PHF2 (Mm, Hs)	H3K9me1	(Wen *et al.* 2010)
	PHF8 (Hs)	H3K9me2/1, H4K20me1	(Kleine-Kohlbrecher *et al.* 2010; Qi *et al.* 2010)
	KIAA1718 (Mm)	H3K9me2/1; H3K27me2/1	(Tsukada *et al.* 2010)
	drKDM7a (Dr)	H3K9me2; H3K27me2	(Tsukada *et al.* 2010)
	drKDM7b (Dr)	H3K9me2; H3K27me2	(Tsukada *et al.* 2010)
	KDM7A (Ce)	H3K9me2; H3K27me2	(Kleine-Kohlbrecher *et al.* 2010)
JmjC Domain Only	NO66 (Mm)	H3K4me3/1; H3K36me3/2	(Sinha *et al.* 2010)
	JMJD5 (Hs)	H3K36me2	(Hsia *et al.* 2010)

Abbreviations: *Saccharomyces cerevisiae* (Sc), *Schizosaccharomyces pombe* (Sp), *Drosophila melanogaster* (Dm), *Caenorhabditis elegans* (Ce), *Danio rerio* (Dr), *Mus musculus* (Mm), *Homo sapiens* (Hs)

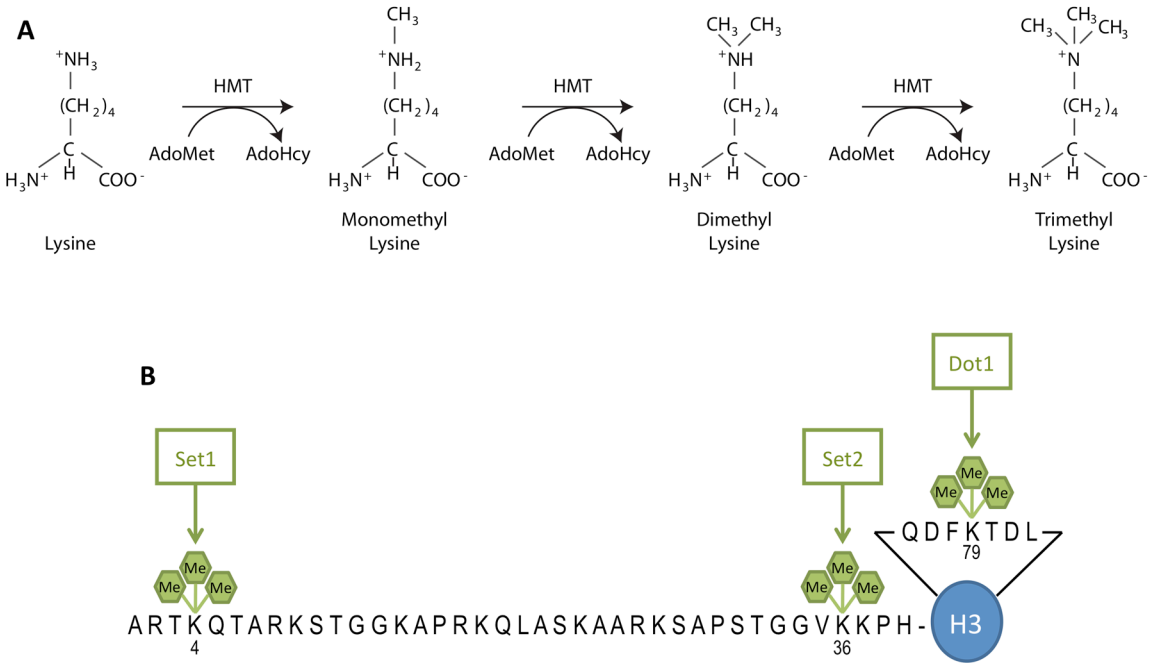


FIGURE 1.1 | Histone lysine methylation in *Saccharomyces cerevisiae*. (**A**) Reaction mechanism for histone lysine methylation. Histone methyltransferases (HMTs) catalyze the transfer of one, two, or three methyl groups onto the ε-amino group of target lysine residues using the cofactor *S*-adenosyl-L-methionine (AdoMet) as the methyl group donor, thereby producing mono-, di-, or trimethylated lysine residue, respectively, and the reaction byproduct *S*-adenosyl-homocysteine (AdoHcy). (**B**) Known sites of histone lysine methylation in the budding yeast *Saccharomyces cerevisiae* and the HMTs that catalyze placement of each methyl group (Me; depicted by green hexagons). In budding yeast, histone lysine methylation on histone H3 on lysine residues 4, 36, and 79 is catalyzed by the HMTs Set1, Set2, and Dot1, respectively.

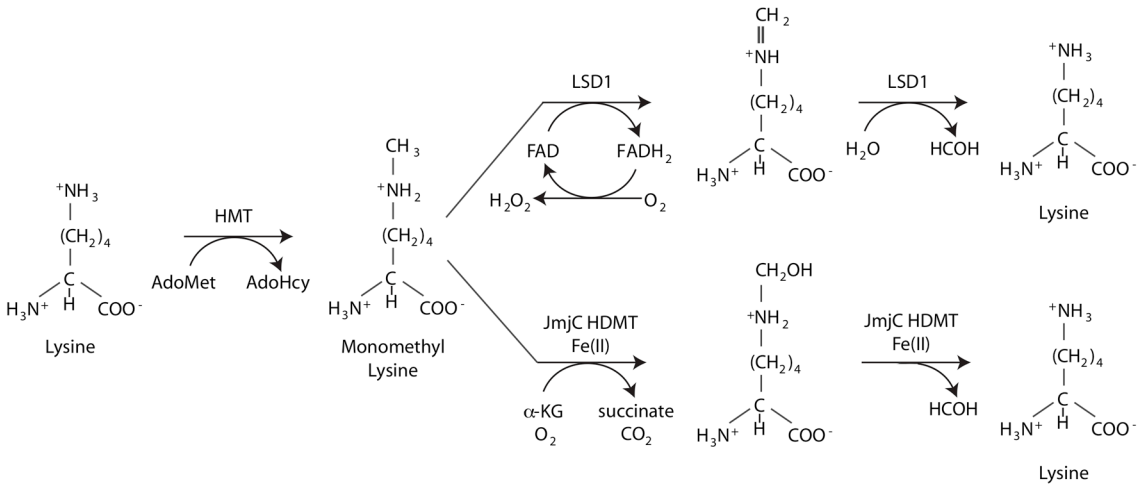
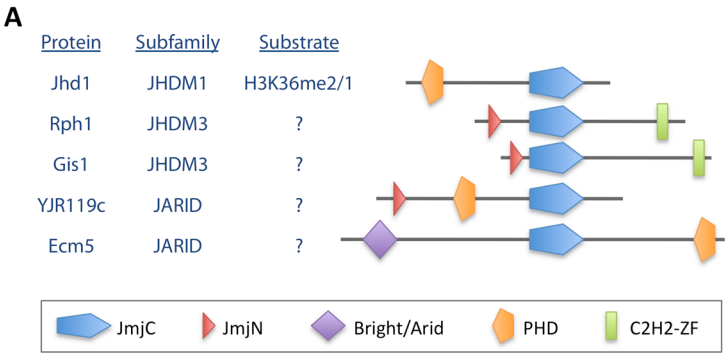


FIGURE 1.2 | Reaction mechanisms used by histone demethylases (HDMTs). The reaction scheme for formation of monomethyl lysine by histone methyltransferases (HMT) in a *S*-adenosyl-L-methionine (AdoMet)-dependent reaction is shown (**left**). Note that lysine residues can also be di- and trimethylated. Histone methylation can be reversed by an amine oxidase reaction catalyzed by LSD1 (**top**) or by a hydroxylation reaction catalyzed by JmjC-domain-containing HDMTs (**bottom**). LSD1 uses flavin adenine dinucleotide (FAD) as a cofactor in an amine oxidation reaction to demethylate methylated lysine residues, where removal of a methyl group occurs through an imine intermediate, which is hydrolyzed to form the reaction byproduct formaldehyde (HCOH) thereby resulting in the removal of a methyl group. JmjC-domain-containing HDMTs use the cofactors iron (Fe(II)) and α-ketoglutarate (α-KG) in an oxidation reaction that produces a hydroxylated intermediate. The hydroxymethyl group is spontaneously lost as formaldehyde (HCOH), resulting in the loss of a methyl group.



B

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                *       20        *       40        *       60        *       80        *      100        *
Jhd1 :   IEGLELERPTAVRQNDLVDKIWSFNGHLEKVNGEKAEENDPKPKVTKYILMSVKDAYTDFHLDFAGTSVYYNVISG-QKKFLLFPPTQSNIDKYIEWSLKEDQNSVFLGDILEDGIA : 116
Rph1 :   ------------------PEGLNVWNVAKLPNILDHMETKVPGVNDSYLYAGLWKASFSWHLEDQDLYSINYIHFGAPKQWYSIPQEDRFKFYKFMQEQFP--EEAKNCPEFLRHKM :  97
Gis1 :   ------------------PYDLTLWNLNNLPDSINSSNRR--------LLTGQSKCIFPWHLDEQNKCSINYLHFGAPKQWYSIPSANTDQFLKILSKEPS--SNKENCPAFIRHQN :  89
Yjr119c: --------------KYCDHPMNLTNLPMAHNSLLPLFKRNISGMTIPWIYIGSLFSTFCWHMEDQYTLSANYQHEGDPKVWYSIPESGCTKFNDLLNDMSP--DLFIKQPDLLHQLV : 101
Ecm5 :   --------------KNILDQWNLDNIPLNELSLLQHLDLDMANFTRTTYDIGMLFSCQGWSVSDHFLPSIDFNHLGSTKLVYSIAPKDMEKFEALIARGKSEWDTIQSRPRYSTSDD : 103

         120       *       140       *       160       *       180       *       200       *       220       *
Jhd1 :   MELDAGD-----------------LFMI-PAGYIHAVYTPVDSLVFGGNFLTIRDLETHLKIVEIEK---------------------------------------------- : 165
Rph1 :   FLASP--KLLQENGIRCNEIVHHEGEFMITYPYGYHAGFNYGYNLAESVNFALEEWLPIGKKAGKCHC---------------------------------------------- : 163
Gis1 :   IITSP--DFLRKNNIKFNRVVQFQHEFIITFPYCMYSGFNYGYNFGESIEFILDQQAVVRKQPLKCGC---------------------------------------------- : 155
Yjr119c: TLISPYDPNFKKSGIPVYKAVQKPNEYIITFPKCYHAGFNTGYNFNEAVNFTIDFWLPYGFGAITDYK---------------------------------------------- : 169
Ecm5 :   ELKSFIETDFYKSFLDAEQSADYSNTGDNSKNSFPEDKIAGNTLHDGSQSDFIFEPNFILANGIKLYKTTQEQGSYIFKFPKAFTCSIGSGFYLSQNAKFAPSSWLRFSSEAAKWTS : 220
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FIGURE 1.3 | JmjC-domain-containing proteins in *Saccharomyces cerevisiae*. (**A**) JmjC-domain-containing proteins in *Saccharomyces cerevisiae*. The five budding yeast JmjC-domain-containing proteins and their domain architecture are illustrated. Jhd1 is a member of the JHDM1 family, which are histone demethylases with specificity for H3K36me2/1 (FANG *et al.* 2007; TSUKADA *et al.* 2006). Rph1 and Gis1 belong to the JHDM3/JMJD2 family, whose mammalian homologues have been identified as H3K9/36me3/2 demethylases (CLOOS *et al.* 2006; KLOSE *et al.* 2006b; WHETSTINE *et al.* 2006). Jhd2 and Ecm5 are members of the JARID family, whose orthologues in higher eukaryotes have been shown to histone demethylases specific for H3K4me3/2 (IWASE *et al.* 2007; KLOSE *et al.* 2007b; LEE *et al.* 2007b; SECOMBE *et al.* 2007; SEWARD *et al.* 2007; YAMANE *et al.* 2007). (**B**) Multiple sequence alignment of the JmjC domain of the five *Saccharomyces cerevisiae* JmjC proteins shows a high degree of homology among the predicted Fe(II)- (red) and α-KG (blue) binding sites of Rph1 and Yjr119c to the known histone demethylase Jhd1, suggesting that they likely function as histone demethylases. Substitution mutations within the JmjC domain of Gis1 and Ecm5 likely abrogate any histone demethylase activity.

Chapter Two

Demethylation of Histone H3K36 and H3K9 by Rph1: A Vestige of an H3K9 Methylation System in *Saccharomyces cerevisiae*?

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Histone methylation is an important posttranslational modification that contributes to chromatin-based processes including transcriptional regulation, DNA repair, and epigenetic inheritance. In the budding yeast *Saccharomyces cerevisiae*, histone lysine methylation occurs on histone H3 lysines 4, 36, and 79, and its deposition is coupled mainly to transcription. Until recently, histone methylation was considered to be irreversible, but the identification of histone demethylase enzymes has revealed that this modification can be dynamically regulated. In budding yeast, there are five proteins that contain the JmjC domain, a signature motif found in a large family of histone demethylases spanning many organisms. One JmjC-domain-containing protein in budding yeast, Jhd1, has recently been identified as being a histone demethylase that targets H3K36 modified in the di- and monomethyl state. Here, we identify a second JmjC-domain-containing histone demethylase, Rph1, which can specifically demethylate H3K36 tri- and dimethyl modification states. Surprisingly, Rph1 can remove H3K9 methylation, a histone modification not found in budding yeast chromatin. The capacity of Rph1 to demethylate H3K9 provides the first indication that *S. cerevisiae* may have once encoded an H3K9 methylation system and suggests that Rph1 is a functional vestige of this modification system.

Introduction

Posttranslational modification of histone molecules within chromatin contributes epigenetic information to the underlying DNA-based genetic code (KORNBERG and LORCH 1999). Recently, the histone lysine (K) methylation system has attracted a significant amount of interest due to its widespread roles in transcriptional regulation, DNA repair, and epigenetic inheritance (MARTIN and ZHANG 2005). In higher eukaryotes, histone lysine methylation occurs on histone H3K4, K9, K27, K36, and K79 and histone H4K20. In general, histone H3K4, K36, and K79

methylation is associated with actively transcribed genes, whereas H3K9, K27, and H4K20 methylation is associated with silenced regions (MARTIN and ZHANG 2005). Budding yeast has a less complex histone methylation system that encodes three histone lysine methyltransferase enzymes, Set1, Set2, and Dot1, which modify H3K4, K36, and K79, respectively (MILLAR and GRUNSTEIN 2006). Histone lysine methylation in budding yeast is tightly coupled to the process of transcription, and the deposition of these modifications occurs mainly during the initiation and elongation phases of RNA polymerase II-based transcription (MILLAR and GRUNSTEIN 2006). In particular, H3K36 methylation is tightly coupled to the process of active transcriptional elongation and forms an increasing concentration gradient from the 5' to the 3' end of the gene. H3K36 profiles are dictated by the preferential association of Set2 with the elongating form of RNA polymerase II, which is phosphorylated on serine 2 of the C-terminal domain (KROGAN *et al.* 2003b; LI *et al.* 2003; LI *et al.* 2002; SCHAFT *et al.* 2003; XIAO *et al.* 2003b). The functional outcome of histone methylation is often elicited through effector proteins that specifically recognize and interpret these histone modifications. In budding yeast, H3K36 methylation is recognized by the chromodomain protein Eaf3, which is a stable component of the Sin3 corepressor complex. Eaf3 acts as an effector protein by recruiting the Sin3 corepressor complex to the body of yeast genes, where it inhibits intragenic transcription (CARROZZA *et al.* 2005; JOSHI and STRUHL 2005; KEOGH *et al.* 2005). Under standard laboratory growth conditions, budding yeast lacking H3K36 methylation shows no obvious cellular defects. However, the widespread involvement of this modification in transcriptional elongation suggests that H3K36 methylation may have important roles in transcriptional fidelity under certain environmental or growth conditions.

Until recently histone methylation was considered to be a static modification, but the identification of histone demethylase enzymes has revealed that this modification can be

dynamically regulated (CLOOS *et al.* 2006; KLOSE *et al.* 2006a; KLOSE *et al.* 2006b; SHI *et al.* 2004; TSUKADA *et al.* 2006; WHETSTINE *et al.* 2006; YAMANE *et al.* 2006). Thus far, two histone demethylase enzyme families have been identified: the LSD1 family and the JmjC-domain-containing family. These enzymes are potentially important chromatin regulators, given their capacity to modify epigenetic information through the direct removal of histone lysine methylation marks. Functional characterization of existing histone demethylase enzymes has revealed that individual enzymes recognize specific lysine residues and can distinguish between the monomethylation (me1), dimethylation (me2), and trimethylation (me3) states of their target substrates (KLOSE *et al.* 2006b). The budding yeast genome is predicted to encode five JmjC-domain-containing proteins but has no apparent LSD1 homologue. JmjC-domain-containing proteins achieve histone demethylation by an oxidative mechanism requiring iron (Fe(II)) and α-ketoglutarate (α-KG) as cofactors and are capable of removing all three histone lysine methylation states (KLOSE *et al.* 2006b). Jhd1 is the only active JmjC-domain-containing histone demethylase identified in budding yeast, and it targets the demethylation of H3K36me2 and H3K36me1 (TSUKADA *et al.* 2006). Bioinformatic analysis indicates that other JmjC-domain-containing proteins in budding yeast may be enzymatically active based on the conservation of important cofactor binding residues and therefore may constitute novel histone demethylases (KLOSE *et al.* 2006a).

Here, we characterize a second budding yeast JmjC-domain-containing protein, Rph1, and reveal that it is an H3K36 demethylase capable of removing the trimethyl modification state. Biochemical analysis of Rph1 demonstrates that this enzyme is also capable of removing H3K9 methylation despite the fact that *S. cerevisiae* chromatin lacks this modification. These observations reveal that H3K36me3 is a reversible modification in budding yeast, and suggest

that Rph1-mediated demethylation of H3K9 may be a functional vestige of an extinct H3K9 methylation system in *S. cerevisiae*.

Materials and Methods

***Yeast strains and media*.** Yeast strains used for this study are listed in TABLE 2.1. All strains are isogenic to the BY4741 background, with the exception of those used for telomeric silencing assays, which are isogenic to the strain YCB647 (SMITH *et al.* 2000). Yeast transformations were performed using standard procedures (GIETZ and SCHIESTL 2007a; GIETZ and SCHIESTL 2007b). The *rph1Δ::natMX* strain was generated by homologous recombination using a PCR-amplified *natMX* knockout cassette (GOLDSTEIN and MCCUSKER 1999). Endogenous Rph1 was Flag-tagged by amplification of a p3Flag-*kanMX* cassette (GELBART *et al.* 2001) using primers A and B (sequences listed below) and introduced into BY4741 by homologous recombination.

A: CCGCAGGACGGGAAAGCGGCCATTAATCAACAGAGTACACCTTTAAACAGGGAACAAAAGCTGGAG

B: GCCTTCAAAATGAGAGATCTCGGTAAACAACTGGCAATGGTGAGTCACTATAGGGCGAATTGGGT

All yeast strains were maintained and cultured according to standard conditions on appropriate media (BURKE *et al.* 2000).

***Plasmid constructs*.** For recombinant protein expression, *RPH1* was PCR amplified from yeast genomic DNA isolated from BY4741 and cloned into the NcoI and NotI sites of pET28a (Novagen) encoding a C-terminal His tag. The H235A mutation in the predicted Fe(II) binding site was generated by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene). Deletion constructs were also generated by PCR and cloned into pET28a containing a C-terminal

His tag. For the expression of Rph1 in yeast, full-length Rph1 and Rph1 deletions were cloned into pAD4M (2 μm Amp^R *LEU2 ADH1* promoter/terminator) containing an N-terminal Flag tag. In all cases, the sequences of PCR-amplified clones were confirmed by sequencing.

***Recombinant protein purification*.** Recombinant protein was purified under native conditions according to the manufacturer's recommendations using nickel-nitrilotriacetic acid (Ni-NTA; QIAGEN) affinity chromatography. Briefly, recombinant Rph1 protein was expressed in BL21-DE3 *E. coli* by induction using 1 mM IPTG with incubation at 30°C for 3 hr. Cells were harvested and resuspended in lysis buffer [40 mM HEPES-OH (pH 7.9), 500 mM KCl, 10% glycerol, 1 mM DTT, 1X Complete protease inhibitor (Roche)]. Samples were sonicated six times at 40% amplitude for 30 sec (with alternating on and off pulses of 1 and 3 seconds, respectively). Cellular debris was pelleted using an SS-34 rotor at 15000 rpm for 20 min at 4°C. Incubated cleared cell lysate was incubated with pre-equilibrated Ni-NTA agarose beads (QIAGEN) for 2 hr at 4°C. Beads were washed in wash buffer [40 mM HEPES-OH (pH 7.9), 500 mM KCl, 10% glycerol, 1 mM DTT, 1X Complete protease inhibitor (Roche), 10 mM imidazole] three times for 10 min with rotation. Protein was batch eluted with elution buffer [40 mM HEPES-OH (pH 7.9), 500 mM KCl, 10% glycerol, 1 mM DTT, 1X Complete protease inhibitor (Roche), 200 mM imidazole]. Peak fractions were determined by SDS-PAGE analysis followed by Coomassie staining. Prior to use in *in vitro* histone demethylase assays, protein from the peak fraction(s) were pooled and dialyzed against BC100 [40 mM HEPES-OH (pH 7.9), 100 mM KCl, 10% glycerol, 1 mM PMSF].

***Histone demethylation assays*.** All histone substrates were radioactively labeled by performing histone methyltransferase reactions as described previously (KLOSE *et al.* 2006b; TSUKADA *et al.*

2006; TSUKADA and ZHANG 2006). Briefly, histone octomers or oligonucleosomes purified from HeLa cells as described previously (FANG *et al.* 2004) were incubated with different purified recombinant histone methyltransferases (namely, GST-Set7, CBP-Set2, and GST-Dot1) in HMT buffer [20 mM Tris-Cl (pH 8.0), 4 mM EDTA, 1 mM PMSF, 0.5 mM DTT, 0.03 mCi/mL *S*-adenosyl-L-[methyl-^{3}H]methionine (^{3}H-SAM) (Perkin Elmer)] for 2 hr at 30°C. GST- and CBP-fusion proteins were expressed in BL21-DE3 *E. coli* and purified on glutathione sepharose beads (Amersham) or calmodulin affinity resin (Stratagene), respectively, following manufacturer protocols. Labeled substrates were dialyzed against histone storage buffer [10 mM HEPES-OH (pH 7.5), 10 mM KCl, 0.2 mM PMSF, 10% glycerol] overnight at 4°C to remove unincorporated ^{3}H-SAM.

Histone demethylation, mass spectrometry assays, and histone Western blot analysis were carried out as described previously (TSUKADA *et al.* 2006). For *in vitro* formaldehyde-release histone demethylase assays, labeled histone substrates were incubated with purified recombinant proteins in histone demethylation buffer [50 mM HEPES-OH (pH 8.0), 70 μM $Fe(NH_4)_2(SO_4)_2$, 1 mM α-ketoglutarate, 2 mM ascorbate] at 37°C for 3 hr. Equal counts of labeled substrate were used for histone demethylation reactions. A modified NASH method was used for detection of released ^{3}H-labelled formaldehyde (KLEEBERG and KLINGER 1982), where following TCA precipitation, an equal volume of NASH reagent [3.89 M ammonium acetate, 0.1 M acetic acid, 0.2% 2,4-pentanedione] was added to the supernatant and incubated at 37°C for 50 min, thereby converting ^{3}H-labelled formaldehyde to radiolabeled 3,5-diacethyl-1,4-dihydrolutidine. Radioactive 3,5-diacethyl-1,4-dihydrolutidine was extracted with an equal volume of 1-pentanol, and was measured by scintillation counting.

For mass spectrometry analysis, H3K36 peptide substrates used encompasses amino acids 28 to 45 containing a trimethyl modification, amino acids 32 to 42 containing a dimethyl

modification, and amino acids 21 to 45 containing a monomethyl modification (Upstate Biotechnology). The H3K9me3 peptide substrate used in mass spectrometry analyses encompasses amino acids 1 to 18 of histone H3 (Upstate Biotechnology). Peptides were subjected to demethylation reactions in the presence or absence of purified recombinant Rph1. For detection of demethylation of peptide substrates, peptides in the reaction mixture were desalted on an RP micro-tip and MALDI-TOF mass spectrometry analysis was carried out as previously described (TSUKADA *et al.* 2006).

For Western blot analysis of histones following *in vitro* demethylase assays, histone octomers or oligonucleosomes purified from HeLa cells as described previously (FANG *et al.* 2004) were incubated in the presence or absence of recombinant Rph1 in histone demethylase buffer [50 mM HEPES-OH (pH 8.0), 70 μM $Fe(NH_4)_2(SO_4)_2$, 1 mM α-ketoglutarate, 2 mM ascorbate] at 37°C for 3 hr. Following the demethylation reaction, histone methylation levels were analyzed by Western blot analysis with modification specific antibodies using standard procedures. Briefly, demethylation reactions were quenched by addition of Laemmli sample buffer [60 mM Tris-Cl (pH 6.8), 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue]. After heating samples at 95°C for 5 min, histones were resolved on 15% SDS polyacrylamide gels, and proteins were transferred onto nitrocellulose membrane (0.2 μm, BIORAD) using a semi-dry transfer apparatus (HOEFER). The following antibodies were used at dilutions ranging from 1:200 to 1:1,000: α-H3K4me3 (Abcam, ab8580), α-H3K9me3 (Abcam, ab8898), α-H3K36me3 (Abcam, ab9050), α-H3K36me2 (Zhang lab), α-H3K36me1 (Abcam, ab9048), and α-H3K79me3/2 (Zhang lab).

***Size exclusion chromatography and sucrose gradient analysis*.** Whole-cell yeast extract or recombinant Rph1 (rRph1) was fractionated over a 24 mL Superose 6 size exclusion column

(Amersham) equilibrated with BC400 [40 mM HEPES-OH (pH 7.9), 400 mM KCl, 0.5 mM DTT, 10% glycerol, 0.2 mM PMSF] with the aid of an ÄKTA purifier (Amersham) at a flow rate of 0.2 mL/min, and 250 µL fractions were collected. Every other fraction was analyzed for Rph1 by Western blotting or Coomassie staining. Sucrose gradients were formed at 4°C in 13 mL SW40 tubes using a manual two-chamber gradient former. Chamber 1 was loaded with buffer A [300 mM KCl, 20 mM HEPES-OH (pH 7.9), 10% glycerol, 10 mM β-ME] containing 5% sucrose, and chamber 2 was loaded with buffer A containing 20% sucrose. Rph1 and protein molecular weight markers were applied to the 5 to 20% sucrose gradient and centrifuged at 40,000 rpm in an SW40 rotor for 19 hr at 4°C. Fractions (500 µL) were manually collected from the top of the gradient using a peristaltic pump fitted with a capillary tube. Each fraction was TCA precipitated and analyzed by SDS-PAGE analysis with Coomassie staining.

Native molecular weight and frictional coefficient calculations. To determine the native molecular weight (M_r) and frictional coefficient (f/f_0) of rRph1, the values obtained for radius and sedimentation in FIGURE 2.5 were applied to equations (1) and (2) (SIEGEL and MONTY 1966):

(1) $M_r = 6\pi\eta_{20,w} \bullet s_{20,w} \bullet R_S \bullet N/(1 - \rho_{20,w}\nu)$

(2) $f/f_0 = 6\pi\eta_{20,w} \bullet R_S/6\pi\eta_{20,w} \bullet (3\nu M_r/4\pi N)^{1/3}$

where R_S is Stoke's radius (cm), $s_{20,w}$ is the sedimentation velocity (S x 10^{-13}), $\eta_{20,w}$ is the viscosity of water at 20°C (0.01002 g·s^{-1} cm^{-1}), N is Avogadro's number (6.022 x 10^{23}·mol^{-1}), $\rho_{20,w}$ is the density of water at 20°C (0.9981 g·cm^3), and ν is the partial specific volume (used 0.725 cm^3/g).

***Transfection and immunofluorescence microscopy*.** NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin-streptomycin. For immunofluorescence, cells grown on coverslips in six-well plates were transfected with 2 to 6 μg of Flag-Rph1 expression plasmid using Fugene 6 transfection reagent (Roche). Cells were fixed 24 hr posttransfection for 20 min in 4% paraformaldehyde, washed three times with phosphate-buffered saline (PBS), and subsequently permeabilized for 20 min in 0.5% Triton X-100-PBS. Permeabilized cells were washed two times in PBS and blocked in 3% bovine serum albumin-PBS for 30 min. Cells were incubated with primary antibody in a humidified chamber for 1 to 3 h using histone modification antibodies at a dilution of 1:100 and the Flag monoclonal M2 antibody (Sigma, F3165) at a dilution of 1:1000. After primary antibody incubation, cells were washed three times and incubated with fluorescein isothiocyanate or rhodamine-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). Cells were washed twice with PBS, stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI), and mounted on glass slides in fluorescent mounting medium (DAKO). Slides were analyzed on an AxioSkop fluorescent microscope (Zeiss).

Results

***Rph1 is an H3K36me3 demethylase*.** We and others recently identified and characterized mammalian JHDM3/JMJD2 histone demethylases that target H3K9/36 methylation (CLOOS *et al.* 2006; FODOR *et al.* 2006; KLOSE *et al.* 2006b; WHETSTINE *et al.* 2006). These proteins contain N-terminal JmjN and JmjC domains that are required for enzymatic activity. Bioinformatic analysis has identified an *S. cerevisiae* protein, Rph1, which has a high level of similarity to the JHDM3/JMJD2 proteins within its JmjN and JmjC domains. Residues predicted to function as cofactor binding sites are completely conserved between the JmjC domain of Rph1 and the

JHDM3/JMJD2 proteins, suggesting that Rph1 could potentially encode a novel yeast histone demethylase (KLOSE *et al.* 2006a). Interestingly, very little similarity exists between mammalian JHDM3/JMJD2 proteins and Rph1 outside of the JmjN and JmjC domains, suggesting that Rph1 may have unique substrate specificity and function in yeast.

To test whether Rph1 is a histone demethylase, rRph1 or rRph1 with a replacement in a predicted iron binding residue (H235A) was used in a histone demethylase assay containing radioactively labeled methyl groups on histone H3 at positions K4, K36, and K79 (FIGURE 2.1, panel A). Histone demethylase activity was monitored by the release of the labeled reaction product formaldehyde. Demethylase activity was observed only when H3K36-labeled substrate was present in the reaction mixture, suggesting that Rph1 is an H3K36-specific histone demethylase (FIGURE 2.1, panel A). Mutation of a predicted iron-binding residue within Rph1 completely abolished enzymatic activity, verifying that Rph1 relies on the JmjC domain for catalysis. Because histone lysine methylation can occur in three modification states, we sought to identify which H3K36 modification states are targeted by Rph1. Rph1 was incubated with core histones or oligonucleosomes, and the resulting methylation states were analyzed by Western blotting using modification-specific antibodies (FIGURE 2.1, panel B). Rph1-mediated demethylation culminated in a reduction of H3K36me3 and an accumulation of H3K36me1 but did not affect H3K4me3 or H3K79me3 methylation. Interestingly, this property of Rph1 differs from that of mammalian JHDM3/JMJD2 proteins, which are incapable of efficiently demethylating oligonucleosomal substrates (KLOSE *et al.* 2006b).

Rph1 activity towards purified histone substrates clearly demonstrates that Rph1 is an H3K36 demethylase. To fully define Rph1 substrate specificity, mass spectrometry was used to analyze the modification state of histone H3 peptides containing K36me3, K36me2, and K36me1 following demethylation by Rph1 (FIGURE 2.1, panels C through G). In agreement with the Rph1-

mediate demethylation of H3K36me3 observed by histone Western blotting, mass spectrometric analysis revealed that Rph1 efficiently demethylates H3K36me3, leading to a processive reduction to the me2, me1, and me0 modification states (FIGURE 2.1, panel C). Rph1 is also capable of initiating demethylation on H3K36me2 substrates but is unable to demethylate the H3K36me1 modification state (FIGURE 2.1, panels D and E). Together, these data reveal the first yeast histone demethylase capable of removing the trimethyl modification state and demonstrate that Rph1 targets the demethylation of H3K36me3 and H3K36me2.

***Rph1 requires both the JmjN and JmjC domains to catalyze histone demethylation*.** The Rph1 protein has three curated protein domains including a JmjN domain, a JmjC domain, and a zinc finger (ZF) domain. Mutation of a predicted iron-binding site within the Rph1 JmjC domain abrogates demethylase activity, demonstrating that the JmjC domain is the catalytic core of the enzyme. Characterization of other JmjC-domain-containing proteins has revealed that additional domains can contribute to demethylase activity (FODOR *et al.* 2006; KLOSE *et al.* 2006b; TSUKADA *et al.* 2006; YAMANE *et al.* 2006). To understand which Rph1 domains are required for histone demethylation, a series of deletion proteins (FIGURE 2.2, panel A) were generated and analyzed for H3K36 demethylase activity using the formaldehyde release assay (FIGURE 2.2, panel B). A unique feature of Rph1 is its C-terminal ZF DNA binding domain, which is absent from the related mammalian JHDM3/JMJD2 histone demethylases (JANG *et al.* 1999). To determine whether this domain contributes to demethylase activity, the ZF was deleted, and the activity of the recombinant protein was analyzed by formaldehyde release (FIGURE 2.2, panel B). Removal of the ZF domain had no effect on enzymatic activity, suggesting that this domain may have alternative roles *in vivo*. In contrast, deletion of the JmjN domain completely abrogated H3K36 demethylase activity (FIGURE 2.2, panel B). Recently, the crystal structure of the human

JHDM3A/JMJD2A protein was solved, revealing that the JmjN domain folds into the JmjC domain, creating a single structural entity that is enzymatically active (CHEN *et al.* 2006). Given that Rph1 also relies on its JmjN domain for enzymatic activity, it seems likely that this domain contributes to the structure of the functional yeast enzyme. To determine whether the JmjN/JmjC domain alone is enzymatically active, a protein encompassing only these domains was generated and used in a histone demethylase assay. Although this protein showed a slight reduction in H3K36 demethylase activity, it was still capable of removing H3K36 methylation, demonstrating that the JmjN/JmjC domain is sufficient for demethylase activity (FIGURE 2.2, panel B). Together, these data show that Rph1 demethylase activity relies on the function of the JmjN and JmjC domains and indicate that the ZF domain may have alternate roles *in vivo*, perhaps involving protein targeting.

***Deletion of* RPH1 *causes no overt cellular phenotype*.** To analyze the role of Rph1 in the regulation of H3K36 *in vivo*, the *RPH1* locus was disrupted by homologous recombination, and the absence of the Rph1 transcript was verified by reverse transcription (RT)-PCR (FIGURE 2.3, panel A). The Rph1-deficient strain was morphologically wild-type, and analysis of H3K36 methylation by Western blot analysis with modification-specific antibodies revealed no global changes in H3K36 methylation (*data not shown*). Given that H3K36 methylation has been previously linked to transcriptional elongation (KIZER *et al.* 2005; KROGAN *et al.* 2003b), we tested whether deletion of *RPH1* causes sensitivity to mycophenolic acid (MPA), a drug that affects transcriptional elongation. Results shown in FIGURE 2.3 (panel B) indicate that the deletion of *RPH1* does not confer sensitivity to MPA, nor does it cause defects in telomeric silencing (FIGURE 2.3, panel C). In addition, we have tested a number of conditions used for phenotypic analysis TABLE 2.2 and observed no apparent phenotype. Yeast Jhd1 is also an H3K36 demethylase, but

in contrast to Rph1, it specifically demethylates H3K36me2/1 modification states. To examine whether there are synthetic effects in yeast lacking both Rph1 and Jhd1, a double mutant strain was generated and subjected to the same phenotypic analysis as the Rph1-deficient strain TABLE 2.2. The double mutant strain failed to display any synthetic effects and grew normally under all conditions tested. Together, these data indicate that Rph1 and Jhd1 do not play an essential global role in regulating cellular processes including transcription, DNA replication, and heterochromatin function. This does not, however, rule out the possibility that Rph1 or Jhd1 contributes to these functions in a more subtle manner not realized using standard phenotypic analyses.

***Rph1 can demethylate H3K36* in vivo.** To verify that Rph1 can target H3K36 demethylation *in vivo*, Flag-tagged Rph1 was overexpressed in wild-type cells (FIGURE 2.4, panels A and B). Interestingly, the overexpression of Rph1 resulted in a severe inhibition of cell growth, suggesting that elevated levels of Rph1 have a detrimental effect on cell function (FIGURE 2.4, panel C). To determine if the growth defect was a result of Rph1 demethylase activity, a catalytically inactive Rph1 was overexpressed, and growth was analyzed (FIGURE 2.4, panels A through C). Like the wild-type Rph1 protein, the overexpression of the mutant protein resulted in a growth defect, indicating that the effect of Rph1 on cell growth is independent of demethylase activity (FIGURE 2.4, panel C). Rph1 has previously been shown to function as a transcriptional repressor, suggesting that the growth defect may be related to the silencing of genes involved in cell division or other growth-related pathways. The slow growth and low levels of protein expression in cells expressing full-length Rph1 made it impossible to reproducibly observe changes in H3K36 methylation. To try to separate the growth suppression and catalytic activities of Rph1, a protein lacking the C-terminal ZF domain was overexpressed

(FIGURE 2.4, panels A and B). Deletion of the ZF domain completely abrogated the growth defect, indicating that the DNA binding ZF is important for growth suppression, perhaps functioning as a targeting mechanism for Rph1-mediated repression (JANG *et al.* 1999) (FIGURE 2.4, panel C). Cells expressing Rph1 that lack the ZF domain grew normally and expressed high levels of protein, making it possible to analyze the H3K36 methylation levels by Western blotting using modification-specific antibodies (FIGURE 2.4, panel D). Consistent with the observation that Rph1 is an H3K36me3 demethylase *in vitro*, the overexpression of Rph1 lacking the ZF caused a decrease in H3K36me3 methylation levels *in vivo* (FIGURE 2.4, panel D, compare lanes 1 and 2). Demethylase activity was dependent on an intact JmjC domain, as a point mutation in the catalytic domain abrogated this effect (FIGURE 2.4, panel D). In agreement with domain-mapping studies *in vitro*, the overexpression of the JmjN/JmjC domain alone was sufficient to catalyze H3K36me3 demethylation, and this function relied on an intact JmjC domain (FIGURE 2.4, panel D, lanes 4 and 5). Together, these data reveal that Rph1 functions to demethylate H3K36 *in vivo* and that elevated levels of Rph1 lead to growth defects that are independent of demethylase activity.

***Rph1 is not stably associated with other proteins* in vivo.** Many chromatin remodeling and chromatin-modifying enzymes are found in high-molecular-weight complexes containing auxiliary proteins that are required to regulate enzymatic function and target the enzyme to defined genomic regions (CAIRNS 2005; CAO *et al.* 2002; CARROZZA *et al.* 2005; JOSHI and STRUHL 2005; KEOGH *et al.* 2005; KUZMICHEV *et al.* 2002; LI *et al.* 2006; MULLER *et al.* 2002; WANG *et al.* 2003). To identify potential Rph1 functional protein partners, we performed TAP-tag purification, which failed to reveal any stable associated proteins (*data not shown*). To verify that Rph1 is not a component of a high-molecular-weight protein complex, extract from a Flag-

tagged Rph1 strain (FIGURE 2.5, panel A) was fractionated by size exclusion chromatography (FIGURE 2.5, panel B). Rph1-containing fractions were identified by Western blot analysis using a Flag-specific antibody (FIGURE 2.5, panel B). Rph1 eluted from the size exclusion column with an apparent native molecular mass of greater than 440 kDa, which is much larger than its theoretical molecular mass of 90.2 kDa based on the amino acid composition (FIGURE 2.5, panel B). Rph1 affinity purification failed to reveal associated proteins, but size exclusion analysis suggests that the native molecular weight of Rph1 is larger than that expected for Rph1 alone. To determine whether the high apparent native molecular weight of Rph1 in size exclusion fractionation was due to an association with other proteins, rRph1 was separated over the same size exclusion column and analyzed by Coomassie staining (FIGURE 2.5, panel C). Surprisingly, the recombinant protein also eluted from the size exclusion column with a native molecular mass of greater than 440 kDa (FIGURE 2.5, panel C). This observation indicates that the high apparent native molecular weight of Rph1 in yeast extracts is not due to additional stably associated proteins but instead is an intrinsic property of Rph1 alone. Given that size exclusion chromatography separates proteins based on radius and not molecular weight, the aberrant size of Rph1 in these experiments could be due to an abnormally elongated Rph1 molecule or the result of a homogenous multimeric Rph1 complex. Over four decades ago, Siegel and Monty derived a series of formulae that combine biophysical properties obtained from size exclusion chromatography and sedimentation analysis to accurately determine the native molecular weight of proteins and protein complexes (SIEGEL and MONTY 1966). Using those formulae, the experimentally determined radius and sedimentation coefficient can be exploited to determine whether a given protein species has an abnormal elution profile due to a highly elongated shape or multimerization. The Stokes radius of rRph1 calculated from size exclusion chromatography was ~6.77 nm (FIGURE 2.5, panel C). To determine the sedimentation coefficient, rRph1 was

analyzed by sucrose gradient sedimentation. The sedimentation coefficient ($S_{20,w}$) of rRph1 calculated from the sucrose gradient was ~12.76 S (FIGURE 2.5, panel D). By applying values obtained from the size exclusion and sedimentation analysis to the Siegel and Monty formulas, the derived native molecular mass of Rph1 was calculated to be 355.43 kDa, and the frictional ratio (f/f_0) was 1.45. This analysis suggests that Rph1 is not an elongated molecule but instead consists of four 90.2 kDa (theoretical mass) Rph1 subunits. It is surprising that Rph1 does not form a stable heterogeneous protein complex in budding yeast given that many other chromatin-modifying enzymes are found in high-molecular-weight complexes that have accessory proteins involved in targeting the enzymatic activity to chromatin. One explanation for the apparent absence of a stable Rph1 complex could be the intrinsic ability of Rph1 to directly bind DNA through its C-terminal ZF domain (JANG *et al.* 1999). The DNA binding properties of Rph1 may allow it to function independently of associated factors in recognizing target sites in chromatin and permit more transient interactions with additional protein factors while antagonizing H3K36 methylation.

Rph1 demethylates H3K9 despite the absence of this modification in budding yeast. In *S. cerevisiae*, histone lysine methylation is limited to positions 4, 36, and 79 of histone H3. Interestingly, the JHDM3/JMJD2 proteins in mammals, which are related to Rph1, are capable of removing both H3K36 and H3K9 methylation (CLOOS *et al.* 2006; FODOR *et al.* 2006; KLOSE *et al.* 2006b; WHETSTINE *et al.* 2006). An H3K9 methylation system is absent from budding yeast, suggesting that the capacity of the mammalian JHDM3/JMJD2 demethylase enzymes to target H3K9 methylation may have been adaptively acquired during the evolution of a more complex chromatin modification system. To analyze whether Rph1 specifically catalyzes H3K36 demethylation, the recombinant enzyme was incubated with histone substrates radioactively

labeled on H3K9, and demethylase activity was monitored by formaldehyde release (FIGURE 2.6, panel A). Surprisingly, Rph1 efficiently demethylated the H3K9-modified substrate requiring both the JmjN/JmjC domain but not the ZF motif for enzymatic activity (FIGURE 2.6, panel A). To verify the Rph1 H3K9 demethylase activity observed by formaldehyde release, Rph1 was incubated with histone substrates, and H3K9 methylation was assessed by Western blot analysis using modification-specific antibodies (FIGURE 2.6, panel B). Like its mammalian counterparts, Rph1 also targets the demethylation of H3K9me3, resulting in an accumulation of the monomethyl state. In contrast to the mammalian JHDM3 enzymes, Rph1 is capable of demethylating both core histone and oligonucleosomal substrates (FIGURE 2.6, panel B) (KLOSE *et al.* 2006b). The capacity of Rph1 to demethylate H3K9me3 was also demonstrated by incubating Rph1 with an H3K9me3 peptide substrate and analyzing the resulting modification states by mass spectrometry (FIGURE 2.6, panels C and D). Rph1 demethylated the H3K9me3 substrate, resulting in the accumulation of di- and monomethyl methyl peptides (FIGURE 2.6, panels C and D).

The surprising observation that Rph1 demethylates both H3K9 and H3K36 methylation indicates that the H3K9 demethylase activity of mammalian JHDM3/JMJD2 enzymes is not simply a feature acquired through adaptation but is likely a feature of the ancestral enzyme. To determine whether Rph1 can remove H3K36 and H3K9 methylation in cellular chromatin containing both of these modifications, an expression vector was generated to overexpress Flag-tagged Rph1 in mammalian cells. Consistent with a role in counteracting histone methylation, Rph1 localized predominantly to the nucleus when expressed in mouse NIH 3T3 cells (FIGURE 2.6, panels E and F). In cells expressing Rph1, both H3K36me3 methylation and H3K9me3 methylation were dramatically reduced, as assessed by immunofluorescence analysis using modification-specific antibodies (FIGURE 2.6, panels E and F, top). Demethylation by Rph1 in

mammalian cells was dependent on an intact JmjC domain, as mutation in the catalytic domain abolished demethylase activity (FIGURE 2.6, panels E and F, bottom). This effect on H3K9me3 and H3K36me3 methylation was specific, as other histone methylation marks associated with silencing in mammals (including H3K27me3 and H4K20me3) were unaffected (FIGURE 2.6, panel G). These observations strongly suggest that the bifunctional substrate specificity of the mammalian JHDM3 enzymes is not an acquired feature, but instead is inherited from the ancestral form of the protein. Furthermore, this suggests that the budding yeast genome may have encoded an H3K9 methylation system that was lost at some point during evolution of the current budding yeast chromatin modification system. The fact that Rph1 has the capacity to target H3K9 methylation may represent a functional vestige of this H3K9 modification system in budding yeast. The activity of Rph1 towards H3K9 methylation has presumably been retained in the absence of this modification through selective pressure to preserve the structurally linked H3K36 demethylase activity.

Discussion

The identification of demethylase enzymes has revealed that histone methylation can be dynamically regulated in a manner similar to that of histone acetylation and phosphorylation. In *S. cerevisiae*, the enzymes that place histone methylation marks are well-characterized and coordinate mainly the addition of these modifications during the process of active transcription (MILLAR and GRUNSTEIN 2006). Previously, only one histone demethylase enzyme, Jhd1, was identified in budding yeast. Jhd1 is a JmjC-domain-containing protein that catalyzes the demethylation of H3K36me2 and H3K36me1 modification states (TSUKADA *et al.* 2006). Given that Jhd1 does not target H3K36me3 in yeast, it remained possible that this methylation state was irreversible.

Here, we identify Rph1 as being a histone demethylase with activity towards histone H3K36me3 and H3K36me2 modification states. Deletion of *RPH1* does not affect global histone H3K36 methylation profiles, and deletion strains are viable, displaying no obvious morphological or cellular defects. This observation is not surprising given that deletion of *SET2*, the sole H3K36 methyltransferase in budding yeast, causes no obvious cellular defects and has subtle effects on gene expression. The overexpression of Rph1 leads to a cellular growth defect, but this property appears to be independent of H3K36 demethylase activity and instead relies on the C-terminal ZF DNA binding domain. It remains possible that the growth defect in Rph1-overexpressing cells is due to demethylase-independent repression of growth-related genes through the ZF DNA binding domain. The overexpression of the Rph1 JmjN/JmjC domains alone is sufficient to mediate the demethylation of H3K36, verifying that this portion of the protein is catalytically competent *in vivo*. In contrast to many other chromatin-modifying enzymes, Rph1 does not stably associate with other proteins, but instead forms a homogenous complex comprised of four Rph1 subunits. Often, chromatin remodeling complexes rely on associated protein factors for enzyme targeting, but the fact that Rph1 has an intrinsic DNA binding domain may alleviate the requirement for genomic targeting by auxiliary protein factors in some instances. Removal of the ZF relieves growth defects in cells overexpressing Rph1, supporting the argument that this domain contributes to protein function and perhaps genomic targeting *in vivo*. Additional functional analyses will be required to define specific genomic targets of Rph1 and to understand how Rph1-mediated demethylation contributes to transcriptional regulation by Rph1.

The two characterized budding yeast histone demethylase enzymes, Jhd1 and Rph1, both target H3K36 methylation. Two of the three remaining JmjC-domain-containing proteins, Gis1 and Ecm5, have mutations in cofactor binding residues that ablate demethylase activity (Y.

Tsukada, K. E. Gardner, and Y. Zhang, *unpublished data*). The remaining protein, Yjr119C, is an H3K4 demethylase that catalyzes the removal of the H3K4me3 modification state (*our unpublished data*). Therefore, it appears that JmjC-domain-containing proteins in budding yeast target the removal of H3K4 and H3K36 methylation but not H3K79 methylation. H3K4 and H3K36 methylation are placed by SET-domain-containing histone methyltransferases. In contrast, H3K79 methylation is catalyzed by Dot1, which does not have a SET domain. The inability of JmjC-domain-containing proteins to remove H3K79 methylation strikingly parallels the fact that a unique enzyme is required to place this modification. Perhaps H3K79 methylation is also removed by a novel class of demethylase enzymes with unique enzymatic properties. Further biochemical and genetic analyses of H3K79 methylation in budding yeast will be instrumental in determining whether this modification is dynamically regulated and provide insight into potentially novel enzymes involved in metabolizing this modification.

The JmjC-domain-containing histone demethylase enzymes characterized thus far have a very define substrate specificity towards the lysine modification site and state. The catalytic domain of Rph1 is homologous to the mammalian JHDM3/JMJD2 enzymes, which target both H3K36 and H3K9 demethylation. The capacity of mammalian enzymes to target H3K9 methylation, a modification that is absent from budding yeast chromatin, may have been adaptively evolved in the presence of enzymes that place this modification. Surprisingly, the characterization of Rph1 substrate specificity revealed that Rph1 is also capable of demethylating H3K9 *in vitro* as well as on mammalian chromatin *in vivo*. This property of Rph1 is not simply due to promiscuous substrate specificity, as Rph1 does not affect other yeast or mammalian histone methylation sites. The capacity of Rph1 to demethylate this modification suggests that an H3K9 methylation system may have once existed in budding yeast. Despite the fact that H3K9 methylation is no longer found in budding yeast chromatin, the enzymatic activity

of Rph1 towards this modification may have been inadvertently retained due to its bifunctional requirement as a regulator of H3K36 methylation. Other components of the H3K9 methylation system, including the H3K9 methyltransferase, may have been lost or become functionally inactive.

No SET-domain-containing protein has been shown to modify H3K9 in budding yeast. The SET-domain-containing protein Set3 is a structurally integral component of a high-molecular-weight histone deacetylase complex (PIJNAPPEL *et al.* 2001) that, much like Set2, is targeted to the body of active genes, where it regulates chromatin modifications (WANG *et al.* 2002). Deletion of *SET2* in a strain lacking any component of the Set3 complex results in synthetic growth defects, suggesting that these factors contribute to similar processes (KROGAN *et al.* 2002b). It has recently been demonstrated that in addition to H3K36 methylation, H3K9 methylation is targeted to the body of actively transcribed genes in mammalian cells (VAKOC *et al.* 2005; VAKOC *et al.* 2006), and at least one mammalian histone deacetylase complex also contains H3K9 methyltransferase activity (SHI *et al.* 2003). No histone methyltransferase activity has been identified for the budding yeast Set3 complex, and residues within the SET domain that are required for methyltransferase activity are substituted. The role of this complex in the transcribed regions of yeast genes raises the possibility that Set3 may have once played a role analogous to that of the methyltransferases that place H3K9 methylation in the body of mammalian genes. During the evolution of the yeast chromatin modification system, a loss of selective pressure for H3K9 methylation could have potentially allowed components of this system to functionally deteriorate, while an intact H3K9 methylation system in higher eukaryotes was retained. Perhaps Set3 remains as a relic of this modification system due to its essential structural role in the assembly of the Set3 protein complex and its role in histone deacetylation. It will be interesting to determine whether the SET domain of Set3 can be

replaced with the SET domain from an active H3K9 methyltransferase to recapitulate H3K9 methylation profiles in budding yeast that are found in the body of transcribed genes in mammals. The revelation that Rph1 can demethylate H3K9 provides the first evidence for the possibility of an extinct H3K9 methylation system in budding yeast and suggests that Rph1 may represent a functional vestige of this system.

Acknowledgements

We thank Henrik Dohlman and Chris Brandl for kindly providing reagents. We are grateful to Brian Strahl for providing the BY4741, *set2Δ*, *spt4Δ*, *rtf1Δ*, *snf2Δ*, *spt7Δ*, *htz1Δ*, and *sir2Δ* strains. We thank Emma Klose and Nara Lee for critically reading this manuscript and many thoughtful discussions.

TABLE 2.1 | Yeast strains table

Strain	Genotype	Reference/Source
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	B. Strahl
rph1Δ	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rph1Δ::natMX*	This study
jhd1Δ	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 jhd1Δ::kanMX*	Open Biosystems
rph1Δ jhd1Δ	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rph1Δ::natMX jhd1Δ::kanMX*	This study
set2Δ	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 set2Δ::kanMX*	B. Strahl
spt4Δ	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 spt4Δ::kanMX*	B. Strahl
rtf1Δ	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 rtf1Δ::kanMX*	B. Strahl
snf2Δ	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 snf2Δ::kanMX*	B. Strahl
spt7Δ	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 spt7Δ::kanMX*	B. Strahl
htz1Δ	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 htz1Δ::kanMX*	B. Strahl
sir2Δ	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 sir2Δ::kanMX*	B. Strahl
GY73	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 RPH1-3Flag::kanMX*	This study
YCB647	*MATα his3Δ200 leu2Δ1::TRP1 lys2Δ202 trp1Δ63 ura3-52 ADH4::TEL::URA3*	(SMITH *et al.* 2000)
GY90	*MATα his3Δ200 leu2Δ1::TRP1 lys2Δ202 trp1Δ63 ura3-52 ADH4::TEL::URA3 sir2Δ*	B. Strahl
GY83	*MATα his3Δ200 leu2Δ1::TRP1 lys2Δ202 trp1Δ63 ura3-52 ADH4::TEL::URA3 rph1Δ::natMX*	This study

TABLE 2.2 |Phenotype analysis of the *rph1Δ* strain

Phenotype	Functional implication (HAMPSEY 1997)	Control (Reference)
Slow growth	General protein defects indicating important genes	
Heat sensitivity	General protein defects indicating important genes	*spt4Δ* (BASRAI *et al.* 1996)
Mycophenolic acid (MPA) sensitivity	Transcriptional elongation	*rtf1Δ* (DESMOUCELLES *et al.* 2002)
Galactose fermentation	Transcriptional activation	*snf2Δ* (NEIGEBORN and CARLSON 1984)
Raffinose fermentation	Transcriptional derepression	*snf2Δ* (NEIGEBORN and CARLSON 1984)
Inositol auxotrophy	Inositol biosynthesis; transcriptional activation	*spt7Δ* (PATTON-VOGT and HENRY 1998)
Hydroxyurea sensitivity	DNA replication	*htz1Δ* (MIZUGUCHI *et al.* 2004)
Caffeine sensitivity	Mitogen-activated protein (MAP) kinase pathway; chromatin remodeling	*htz1Δ* (MIZUGUCHI *et al.* 2004)
Telomeric silencing defect	Heterochromatin silencing	*sir2Δ* (SMITH *et al.* 2000)

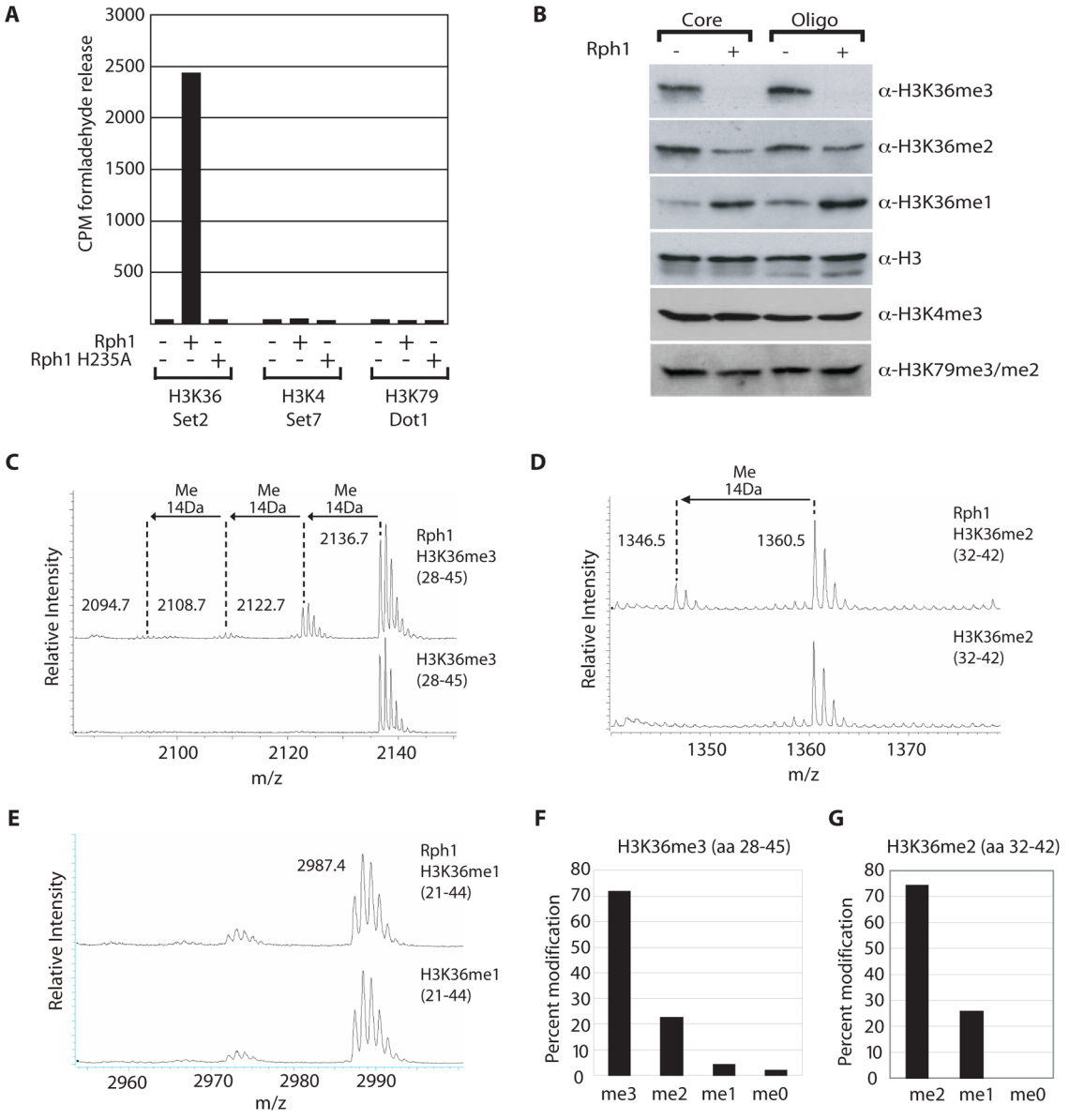

FIGURE 2.1 | Rph1 is an H3K36 demethylase capable of removing trimethyl lysine. (**A**) Labeled histone substrates corresponding to known histone methylation sites in budding yeast were incubated with wild-type rRph1 or rRph1 H235A. Set7, Set2, and Dot1 methyltransferase enzymes were used to label histone substrates on histone H3 lysine residues 4, 36, and 79, respectively. Histone demethylase activity was monitored by the release of labeled formaldehyde. Wild-type Rph1, but not the mutant Rph1 H235A, specifically demethylates H3K36-labeled substrate. CPM, counts per minute. (**B**) Core histones (Core) and oligonucleosomes (Oligo) were incubated with Rph1, and histone methylation levels were analyzed by Western blotting using H3K36, H3K4, and H3K79 methylation-specific antibodies. Rph1 demethylates H3K36me3 and H3K36me2, resulting in an accumulation of H3K36me1. (**C to E**) H3K36me3, H3K36me2, and H3K36me1 peptides were incubated with Rph1 in demethylase assays followed by mass spectrometry analysis. Rph1 specifically demethylates the H3K36me3 and H3K36me2 modification states. (**F and G**) Bar graphs representing the level of

each modification state following Rph1-mediated demethylation of H3K36me3 and H3K36me2 peptides. aa, amino acids.

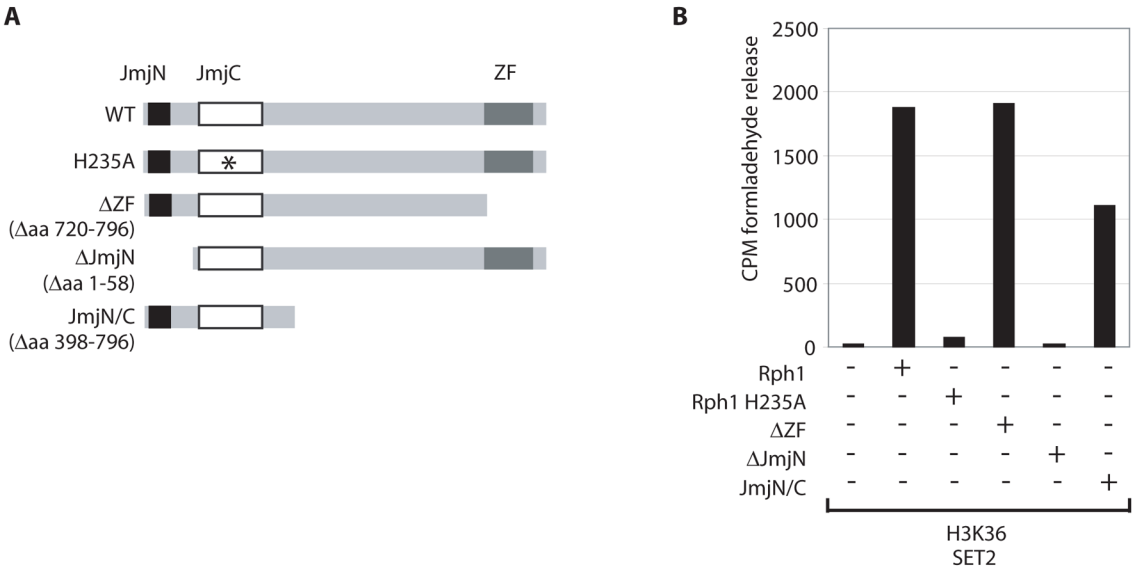

FIGURE 2.2 | Rph1 requires the JmjN/JmjC domain but not the ZF domain for demethylase activity. (**A**) Schematic representation of Rph1 indicating the three curated domains within Rph1. The JmjN, JmjC, and ZF domains were individually deleted or mutated to examine the domain requirements and to map the smallest catalytically active fragment of Rph1. WT, wild-type; aa, amino acids. (**B**) The mutant Rph1 proteins displayed in **A** were used in a histone demethylase assay containing H3K36-labeled substrate, and histone demethylase activity was monitored by formaldehyde release. The JmjN and JmjC domains of Rph1 are sufficient for H3K36 demethylation. CPM, counts per minute.

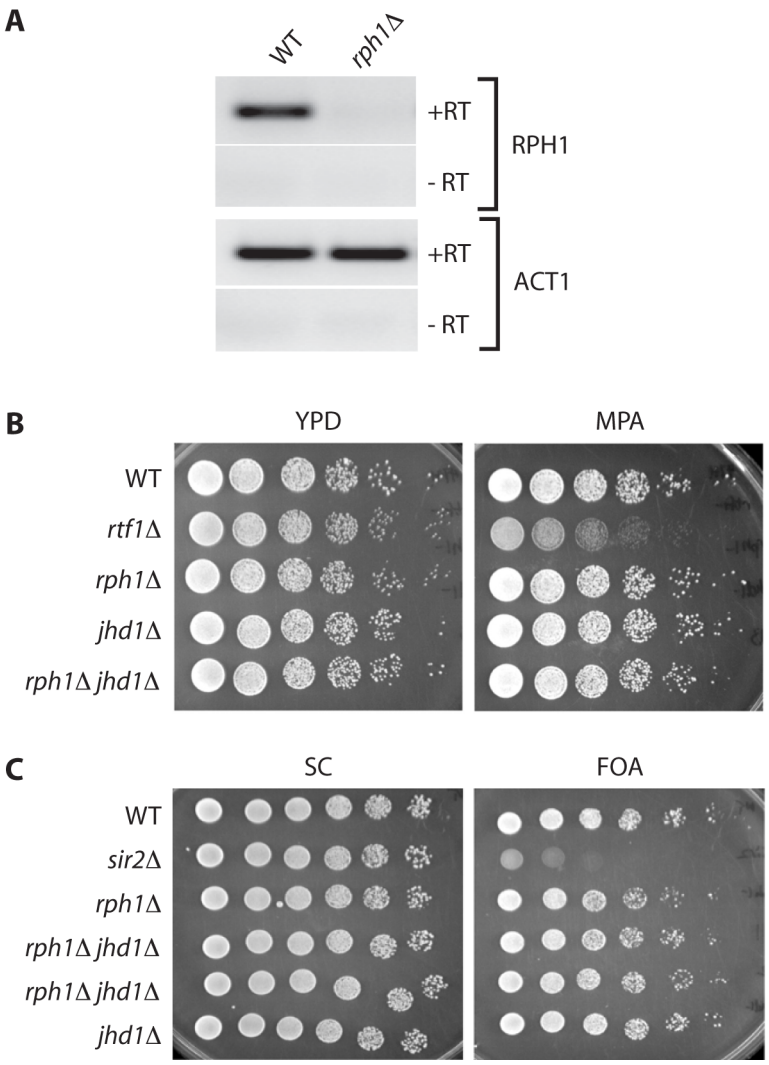

FIGURE 2.3 | Deletion of *RPH1* causes no overt phenotype. (**A**) The *RPH1* gene was disrupted by homologous recombination, and loss of the transcript was verified by RT-PCR. Shown are *RPH1* (top) and actin (*ACT1*, bottom) RT-PCR products. (**B**) Wild-type (WT), *rph1Δ*, *jhd1Δ*, *rph1Δ* jhd1Δ, and *rtf1Δ* strains were spotted onto yeast extract-peptone-dextrose (YPD) and a YPD plate containing 50 µg/mL mycophenolic acid (MPA) plates in five-fold serial dilutions. Growth was analyzed following incubation at 30°C for 2 days. (**C**) Wild-type (WT) *sir2Δ*, *rph1Δ*, *rph1Δ jhd1Δ*, and *jhd1Δ* strains were spotted onto synthetic complete (SC) and SC containing 50 mg/mL 5-fluoroorotic acid (FOA) plates in five-fold serial dilutions. Growth was analyzed following incubation at 30°C for 2 days.

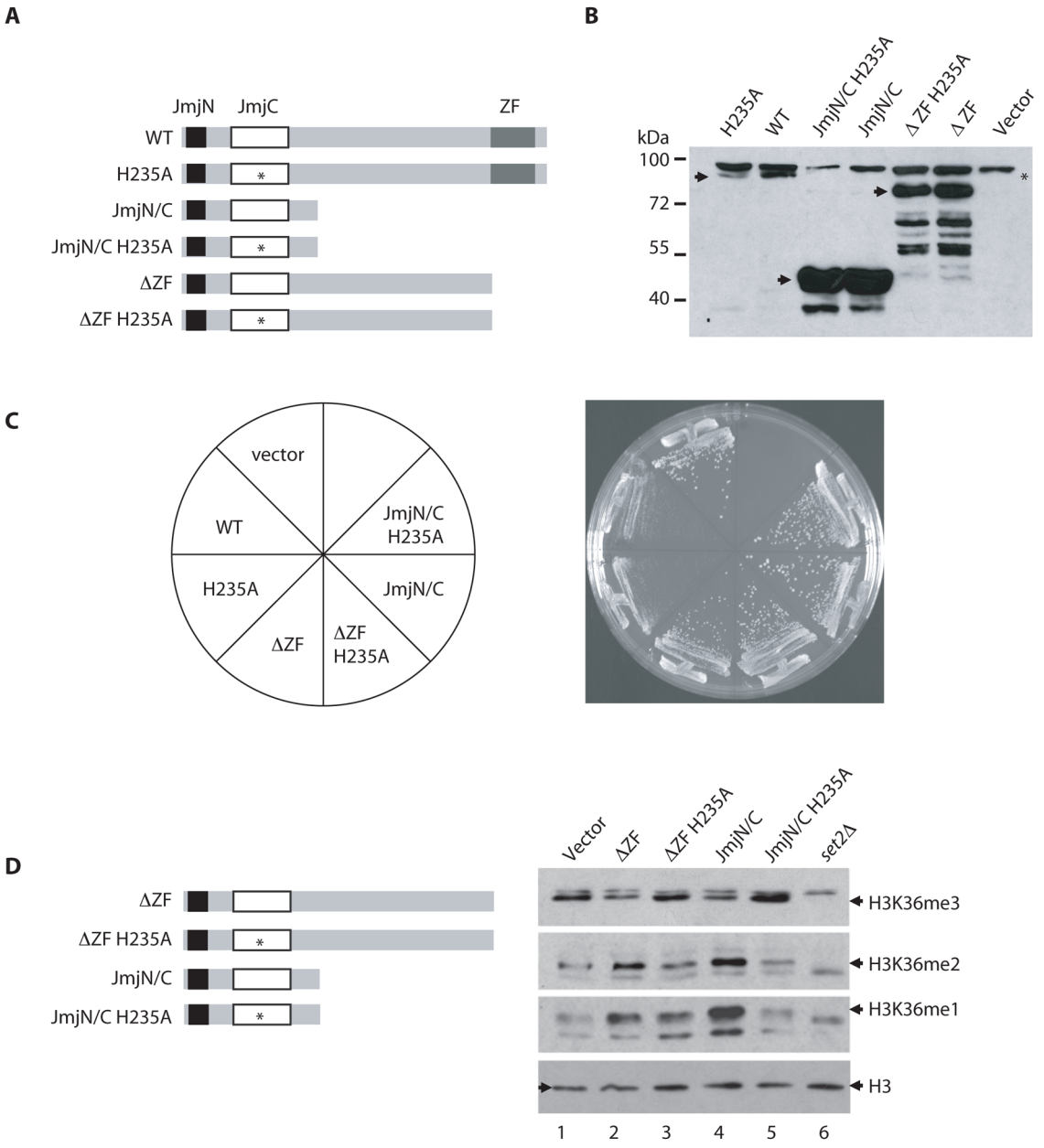

FIGURE 2.4 | Rph1 demethylates H3K36 *in vivo*. (**A**) Schematic representation of Flag-tagged Rph1 and Rph1 H235A yeast overexpression constructs. (**B**) Western blot analysis of yeast whole cell extract (WCE) expressing Flag-Rph1 proteins. The asterisk (*) denotes a cross-reactive and in the yeast WCE, and the arrows indicate overexpressed Rph1 proteins. (**C**) Yeast strains overexpressing Rph1 and Rph1 H235A constructs displayed in **A** were analyzed for growth (right). The left panel indicates the identity of each strain. Full-length Rph1 causes a severe defect in growth that is not alleviated by mutation of the catalytic domain. Deletion of the ZF domain rescues the growth defect, but in all cases, mutation of the catalytic domain has no effect on cell growth. (**D**) H3K36 methylation levels were analyzed in cells overexpressing Rph1 ΔZF and Rph1 JmjN/JmjC proteins (schematic representation on the left) using H3K36 modification-specific antibodies (arrow indicates that H3K36 modification-specific band). The overexpression of both proteins causes a reduction in the level of H3K36me3 and an increase in

the levels of H3K36me2 and H3K36me1. Mutation of the catalytic domain abolishes this effect, verifying that demethylation was a direct consequence of Rph1 catalytic function. The specificity of the H3K36 signal was verified by Western blot analysis using a *set2Δ* strain that lacks all H3K36 methylation states. WT, wild-type.

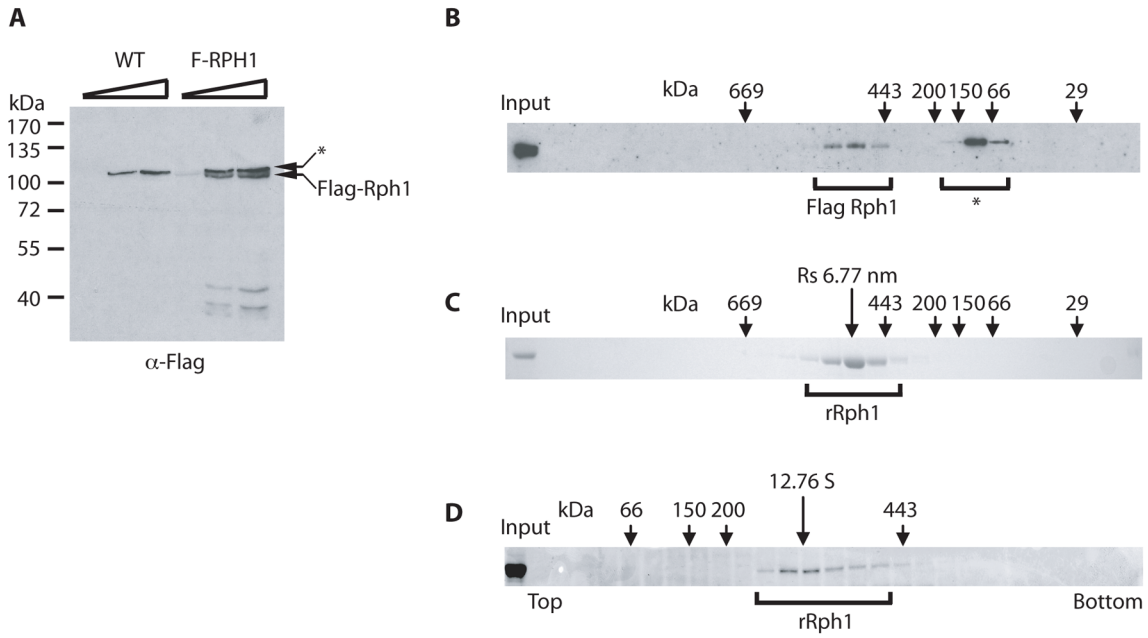

FIGURE 2.5 | Rph1 is not stably associated with other proteins in yeast extracts. (**A**) Endogenous Rph1 was Flag-tagged, and the wild-type (WT) and Flag-tagged Rph1 strains were analyzed by Western blot analysis using a Flag-specific antibody. A signal corresponding to Flag-Rph1 was evident only in the tagged strain. The asterisk (*) indicates a cross-reactive band observed in budding yeast whole cell extract (WCE). (**B**) Flag-Rph1 yeast WCE was fractionated by size exclusion chromatography, and the Rph1-containing fractions were identified by Western blotting using a Flag-antibody. The asterisk (*) indicates a cross-reacting band found in yeast WCE. Size exclusion chromatography molecular mass markers are indicated above the panel. Rph1 elutes from the size exclusion column with an apparent molecular mass of greater than 440 kDa. (**C**) Recombinant Rph1 (rRph1) was fractionated using the same size exclusion chromatography conditions as those employed for the Flag-Rph1 WCE, and the Rph1-containing fractions were identified by Coomassie staining. Size exclusion chromatography molecular mass markers are indicated above the panel, and the calculated radius of Rph1 is given above in nm. rRph1 elutes from the size exclusion column at the same position as endogenous Rph1. (**D**) rRph1 was fractionated over a 5-20% sucrose gradient, and the Rph1-containing fractions were identified by Coomassie staining. Molecular mass standards are indicated above the panel, and the calculated sedimentation coefficient in *S* is given above.

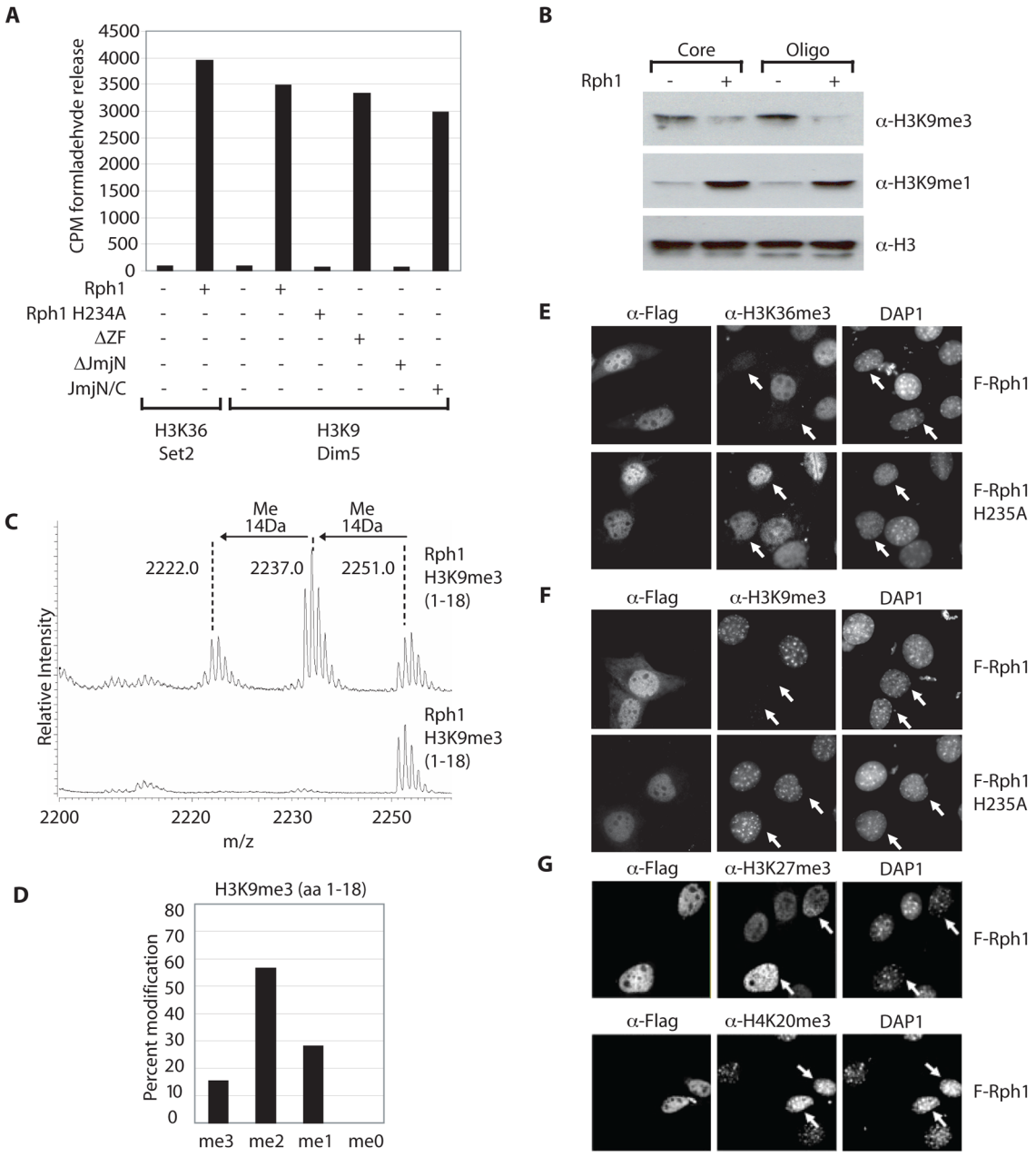

FIGURE 2.6 | Rph1 removes H3K9 methylation both *in vitro* and *in vivo*. (**A**) Histone substrates were radioactively labeled on H3K36 and H3K9 and incubated with the Rph1 proteins detailed in Figure 2.2 (panel A). Demethylase activity was monitored by the release of radioactive formaldehyde. Rph1 efficiently demethylates both H3K36 and H3K9 substrates requiring an intact JmjN/JmjC domain for enzymatic activity. CPM, counts per minute. (**B**) Core histones (Core) and oligonucleosomes (Oligo) were incubated with Rph1, and histone methylation levels were analyzed by Western blotting with H3K9 modification-specific antibodies. Rph1 demethylates H3K9me3, resulting in an accumulation of H3K9me1. (**C**) An H3K9me3 peptide was incubated with Rph1, and the resulting modification state was analyzed by mass spectrometry. Rph1 can demethylate H3K9me3, leading to an accumulation of H3K9me2 and H3K9me1 modification states. (**D**) Bar graph representing the percentage of each modification

state after Rph1-mediated demethylation of the H3K9me3 peptide. (**E and F**) Flag-tagged Rph1 and Rph1 H235A were expressed in NIH 3T3 cells, and the levels of H3K36me3 (**E**) and H3K9me3 (**F**) were analyzed by indirect immunofluorescence using histone methylation-specific antibodies. Rph1 localizes to the nucleus and demethylates both H3K36me3 and H3K9me3 (top panels). Demethylase activity requires an intact JmjC domain, as a mutation of the catalytic domain abrogated this effect (bottom panels). (**G**) Expression of Flag-tagged Rph1 in NIH 3T3 cells does not cause demethylation of other repressive histone methylation marks, including H3K27me3 (top) or H4K20 (bottom), as assessed by indirect immunofluorescence with methylation-specific antibodies.

Chapter Three

Identification of Lysine 37 of Histone H2B as a Novel Site of Methylation

Recent technological advancements have allowed for highly-sophisticated mass spectrometry-based studies of the histone code, which predicts that combinations of post-translational modifications (PTMs) on histone proteins result in defined biological outcomes mediated by effector proteins that recognize such marks. While significant progress has been made in the identification and characterization of histone PTMs, a full appreciation of the complexity of the histone code will require a complete understanding of all the modifications that putatively contribute to it. Here, using the top-down mass spectrometry approach for identifying PTMs on full-length histones, we report that lysine 37 of histone H2B is dimethylated in the budding yeast *Saccharomyces cerevisiae*. By generating a modification-specific antibody and yeast strains that harbor mutations in the putative site of methylation, we provide evidence that this mark exist *in vivo*. Importantly, we show that this lysine residue is highly conserved through evolution, and provide evidence that this methylation event also occurs in higher eukaryotes. By identifying a novel site of histone methylation, this study adds to our overall understanding of the complex number of histone modifications that contribute to chromatin function.

Introduction

In eukaryotic cells, DNA is packaged in the form of chromatin. Approximately 147 base pairs of DNA wrap around an octomer composed of two H2A-H2B dimers and one H3-H4 tetramer to form nucleosomes, the fundamental repeating unit of chromatin (KORNBERG and LORCH 1999; LUGER *et al.* 1997). Because nucleosomes are organized into progressively higher-ordered structures, significant chromatin remodeling is necessary for the numerous DNA-templated processes that must occur for normal cellular function, such as transcription, DNA replication, DNA repair, and chromosome segregation.

One means by which alterations to chromatin structure is accomplished is through post-translational modifications (PTMs) of the histone proteins. The core histones are largely globular, with the exception of unstructured N-terminal tails that protrude from the surface of the core particle. Although numerous PTMs have been shown to occur on residues located on the histone tails (KOUZARIDES 2007), it is becoming increasingly evident that residues within the globular domain are also subject to modifications (CAMPOS and REINBERG 2009; FREITAS *et al.* 2004; MERSFELDER and PARTHUN 2006). The type of PTMs demonstrated to occur on histone proteins include acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, proline isomerization, citrullination, butyrylation, propionylation and glycosylation (CHEN *et al.* 2007; KOUZARIDES 2007; SAKABE *et al.* 2010). While the functional significance of some of the aforementioned modifications remains to be elucidated, it is well established that some of the histone PTMs function by at least one of the following mechanisms: (1) disruption of nucleosomal contacts between histones and their associated DNA or between histones in contiguous nucleosomes, or (2) recruitment of non-histone proteins (CAMPOS and REINBERG 2009; KOUZARIDES 2007). Acetylation of lysine residues is the best-characterized modification shown to affect higher-order chromatin structure, where this mark neutralizes the basic charge of the residue on which it occurs, thereby inhibiting histone-histone or histone-DNA interaction and thus chromatin compaction (HONG *et al.* 1993; SHOGREN-KNAAK *et al.* 2006; WOLFFE and HAYES 1999). With regard to the other means by which histone PTMs can function, the recruitment of non-histone proteins is facilitated by the ability of specialized domains to recognize and bind to defined marks (TAVERNA *et al.* 2007a). For example, methylation of specific lysine residues in a defined state (mono-, di-, or trimethyl) can serve as a binding platform for effector proteins containing one of the following types of methyl-binding domains: chromodomain, tudor

domain, PHD finger, MBT, Ankyrin repeat, PWWP domain and WD40 repeats (COLLINS *et al.* 2008; TAVERNA *et al.* 2007a; VEZZOLI *et al.* 2010).

The complexity of the number and diverse types of PTMs has led to the hypothesis of a "histone code" (JENUWEIN and ALLIS 2001; STRAHL and ALLIS 2000), which posits that combinatorial patterns of histone PTMs lead to defined biological outcomes brought about by the recruitment of effector proteins necessary for function in DNA-templated processes. For example, TAF1 (the largest subunit of the TFIID complex which is involved in initiating the assembly of transcriptional machinery) contains a double bromodomain that preferentially binds to multiply acetylated histone H4 (JACOBSON *et al.* 2000), and itself can function as a histone acetyltransferase (MIZZEN *et al.* 1996). There are numerous other examples of how defined combinations of histone modifications positively or negatively affect recruitment of specific proteins (AGALIOTI *et al.* 2002; FISCHLE *et al.* 2005; SHI *et al.* 2006; TAVERNA *et al.* 2006; WYSOCKA *et al.* 2006; ZIPPO *et al.* 2009). Despite the identification of numerous histone PTMs to date, it is likely that other modifications still await discovery. Thus, of immediate importance in deciphering the histone code is the need for identifying all the PTMs that are present on histones, so that subsequent studies can be completed to determine the combinatorial patterns in which such modifications exist on physiological substrates and what the functional outcomes of such combinations are.

In recent history, mass spectrometry (MS) has widely been used as the primary method to identify histone PTMs. MS studies have commonly employed the bottom-up approach, in which short peptides derived from proteolytic cleavage of reverse-phase HPLC (RP-HPLC)-purified histones are analyzed by MS with peptide mass fingerprinting (PMF) or a combination of liquid chromatography (LC) and tandem MS (MS/MS) using electron transfer or collision-induced dissociation methods (ETD and CID, respectively) (GARCIA *et al.* 2007d). While this

technique is a highly effective means by which to determine the molecular mass (by MS-PMF) or the sequence of a protein (by LC-MS/MS), it is limited in that incomplete sequence coverage of the protein of interest often occurs, and proteins with multiple cleavage sites (including the histone core proteins, which are rich in lysine and arginine residues) result in peptide segments that are too small for effective retention and/or detection (BORCHERS *et al.* 2006; GARCIA *et al.* 2007b; HAN and BORCHERS 2010; PESAVENTO *et al.* 2008; ZHANG *et al.* 2002). More recently, advances in MS have led to the development of the top-down approach as a complementary method to bottom-up analysis as a highly useful means by which to identify PTMs on histones (BOYNE *et al.* 2006; KELLEHER 2004; PESAVENTO *et al.* 2004; SIUTI *et al.* 2006; TAVERNA *et al.* 2007b; THOMAS *et al.* 2006; ZUBAREV *et al.* 1998). Full-length proteins are analyzed with top-down MS, as samples are infused into the mass spectrometer by electrospray ionization (ESI), allowing for MS/MS fragmentation via ETD or electron capture dissociation (ECD) of intact proteins (GARCIA *et al.* 2007c). A major advantage of top-down MS is that combinatorial patterns of modifications that exist on a single histone molecule can be identified (UEBERHEIDE and MOLLAH 2007), which is particularly valuable in outlining the global landscape of PTMs on histone proteins.

In this study, we sought to use top-down MS to analyze the global landscape of PTMs on histone H2B. From this analysis, we identified lysine 37 of histone H2B (H2BK37) as a novel site of methylation in the budding yeast *Saccharomyces cerevisiae*, and that this modification exists in the dimethyl state. We generated an antibody specific for dimethylated H2BK37 (H2BK37me2), with which we were able to confirm that this mark does in fact occur *in vivo*. Though our candidate approach to identify the methyltransferase responsible for placing this mark and phenotypic analysis to reveal a biological function did not offer conclusive results, we provide evidence that this modification is evolutionarily conserved supporting its overall

importance as a novel histone modification. Furthermore, these results demonstrate that despite the numerous rounds of previous MS analysis, additional series of MS analyses employing recent technological advancements are necessary for continued identification of novel sites of modifications to generate a more complete atlas of the factors that putatively function in the context of the histone code.

Materials and Methods

Yeast strains and DNA constructs. A list of yeast strains used for these studies can be found in TABLE 3.1. Plasmids harboring wild-type or mutant histone H2B were introduced into yeast H2A-H2B shuffle strains using standard transformation (GIETZ and SCHIESTL 2007b) and shuffling (BOEKE *et al.* 1987) protocols.

The plasmids pZS145 (*HTA1*-Flag-*HTB1 CEN HIS3*) and pZS146 (*HTA1*-Flag-*htb1* (*K123R*) *CEN HIS3*) were isolated from the strains YZS276 and YZS277, respectively, obtained from Z.W. Sun (SUN and ALLIS 2002). The plasmids pKG1 (*HTA1*-Flag-*htb1* (*K37R*) *CEN HIS3*) and pKG2 (*HTA1*-Flag-*htb1* (*K37A*) *CEN HIS3*) were derived from site-directed mutagenesis of pZS145 (SUN and ALLIS 2002) using the QuikChange II Site-Directed Mutagenesis kit (Stratagene). The accuracy of all constructs was verified by DNA sequencing.

Histone acid extraction. Histones were extracted from yeast nuclei using a standard acid extraction method (EDMONDSON *et al.* 1996). Briefly, 250 mL cultures were grown at 30˚C to an OD_{600} approximately equal to 1.5. Cells were collected by centrifugation at 2700 x *g* for 5 minutes, washed once with sterile water, and collected again by centrifugation. Cells were resuspended in 7.5 mL Solution 1 [0.1 mM Tris-Cl (pH 9.4), 10 mM DTT], and then incubated at 30˚C for 15 minutes with shaking at 100 rpm. Cells were collected by centrifugation at 2700 x *g*

for 5 minutes, washed in 15 mL Solution 2 [1.2 M sorbitol, 20 mM HEPES-OH (pH 7.4)], and pelleted again. Cells were resuspended in 15 mL Solution 2 containing Zymolyase 20T at a final concentration of 0.2 mg/mL, and were then incubated at 30°C with shaking at 100 rpm until spheroplasting was greater than 90% (as determined by measuring the OD_{600} of 10 µL sample in 1 mL 1% SDS; typically 45-50 minutes), at which point 15 mL ice-cold Solution 3 [1.2 M sorbitol, 20 mM PIPES-OH (pH 6.8), 1 mM $MgCl_2$] was added. Cells were pelleted again at 1300 x *g* for 5 minutes 4°C. Pellets were resuspended in 7.5 mL ice-cold Solution 4 [250 mM sucrose, 60 mM KCl, 14 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 15 mM MES (pH 6.6), 1 mM PMSF, 0.8% TritonX-100], incubated on ice for 20 minutes, and spun at 1700 x g for 5 minutes at 4°C. Nuclei isolation in Solution 4 was carried out a total of three times. Nuclei were washed three times in 12.5 mL Wash 1 [10 mM Tris-Cl (pH 8.0), 0.5% NP-40, 75 mM NaCl, 1 mM PMSF] for 15 minutes on ice for the first two washes, and 5 minutes on ice for the third wash, followed by two washes in 12.5 mL Wash 2 [10 mM Tris-Cl (pH 8.0), 400 mM NaCl, 1 mM PMSF] for 10 minutes on ice for the first wash, and centrifuged immediately following the second resuspension. Histones were extracted in 1.5 mL 0.4 N H_2SO_4 with incubation on ice for 30 minutes, with occasional vortexing. Debris was pelleted by centrifugation at 10,000 x *g*. Histone proteins were precipitated from the supernatant by addition of 100% TCA to a final concentration of 20% with incubation on ice for 30 minutes. Histone proteins were pelleted at 15,000 x *g*. Pellets were washed once with acetone containing 1% HCl, and once with acetone. After being air-dried, histone proteins were resuspended in 300 µL 10 mM Tris-Cl (pH 8.0).

***Reverse-phase HPLC purification of histone proteins*.** Following sulfuric acid extraction, histones derived from the strain YMP001 were subject to RP-HPLC isolation. Gradient conditions used for histone isolation were adapted from conditions previously described (STRAHL

et al. 1999). Briefly, proteins from sulfuric acid extracts were injected onto a Zorbex C-18 column with a pore size of 3.5 μm using an Agilent 1100 series RP-HPLC (Agilent, Santa Clara CA). The column was washed and prepared using the following method: 5-35% Acetonitrile (CH_3CN) with 0.1% Trifluoroacetic acid (TFA) for 5 minutes followed by 35% CH_3CN/0.1% TFA for 10 minutes. Histones were separated using the following gradient: 35%-60% CH_3CN/0.1%TFA for 30 minutes (WATERBORG 2000). Protein elution was monitored by UV absorption at 220 nm. Fractions containing histone H2B were determined by Western blot analysis using an α-H2B antibody (Active Motif, Cat. No. 39237).

μESI-FTICR-MS analysis

MS Conditions. Acquisition of MS spectra was performed using a hybrid Qe-Fourier Transform Ion Cyclotron Resonance - Mass Spectrometer, equipped with a 12.0 Tesla actively shielded magnet (Apex Qe-FTICR-MS, 12.0 T AS, Bruker Daltonics, Billerica, MA, USA), and an Apollo II microelectrospray (μESI) source. The voltages on μESI spray capillary, spray shield, capillary exit, deflector, ion funnel and skimmer were set at +4.2 kV, +3.6 kV, +340 V, +310 V, +185 V and +25 V, respectively. The temperature of the μESI source was maintained at 120˚C. Desolvation was carried out using a nebulization gas flow (2.0 bar) and a countercurrent drying gas flow (4.0 L/s). Histone H2B samples were prepared by resuspending lyophilized RP-HPLC fractions containing H2B in a mixture of acetonitrile/water/acetic acid (49.0:49.0:2.0 v/v/v) at a concentration of 0.1-0.2 μg/μL, directly infused with a syringe pump (Harvard Apparatus, Holliston, MA, USA) and a 100-μL syringe (Hamilton, Reno, NV, USA), and electrosprayed at an infusion flow rate of 90 μL/hr. Before transfer, ion packets were accumulated inside the collision cell for a duration of 0.5-1.0 seconds. 100 MS scans per spectrum were acquired in the ICR cell with a resolution of 580,000 at m/z 400 Da.

MS/MS Conditions. FTICR-ECD MS/MS method was employed to fragment histone H2B. Precursor ions were isolated with a quadrupole (Q1) and subjected to ICR cell directly. The isolation window width was 2.0 Da. Low energy electrons were generated by the heated hollow dispenser cathode with a bias voltage of -2.5 V. ECD lens voltage was set at +15.0 V. The electrons, produced by the hollow dispenser cathode (operated at 1.7 A), were pulsed into the ICR cell with a length of 3.0 ms, which led to fragmentation of the ions that were already trapped in the ICR cell. To maximize the ion population before irradiation, the ICR cell was filled with 1-5 iterations of ion accumulation from the external collision cell (BORCHERS *et al.* 2006). 100 MS/MS scans per spectrum were acquired with a resolution of 580,000 at m/z 400 Da.

α-H2BK37me2 antibody production and antibody affinity purification. A synthetic peptide containing H2B sequence from 33 to 41, in which lysine 37 was dimethylated, was conjugated to keyhole limpet hemocyanin via a C-terminal cysteine in the peptide and was used to immunize rabbits (Pocono Rabbit Farm and Laboratory Inc.). The α-H2BK37me2 antibody was affinity purified from serum. Briefly, equilibrated Affigel-10 (BIORAD) was incubated with the peptide SKARKme2ETYS-C (where me2 is dimethyl lysine) in PBS for 2 hr at 4˚C. Unbound peptide was removed, and the peptide-bound resin was blocked with 0.2 M ethanolamine (pH 8.0) for 2 hr at 4˚C. After washing with 1 M NaCl and PBS, the blocked peptide-bound resin was incubated with serum for 3 hr at room temperature with rotation. The flow-through was collected, and the resin was washed with 0.5 M NaCl followed by PBS. Antibody was eluted with 0.1 M glycine (pH 3.0) at one-half column volume/fraction, and 1/10 (v/v) 1 M Tris-Cl (pH 8.0) was added to neutralize the pH. Purity of antibody fractions were analyzed on 12% SDS-polyacrylamide gels followed by Coomassie-staining, allowing for pooling of peak antibody fractions.

IgG was purified from pre-immune serum. Briefly, Protein A beads (GE Healthcare) pre-equilibrated with Tris-salt buffer [100 mM Tris-Cl (pH 7.95), 135 mM NaCl] were incubated with pre-immune serum for 2 hr at room temperature with rotation. The flow-through was collected, and the column was washed with Tris-salt buffer, followed by 10 mM Tris-Cl (pH 7.95). IgG was eluted with 0.1 M glycine (pH 3.0) at one-half column volume/fraction, and 1/10 (v/v) 1 M Tris-Cl (pH 8.0) was added to neutralize the pH. Purity of IgG fractions were analyzed on 12% SDS-polyacrylamide gels followed by Coomassie-staining, allowing for pooling of peak IgG fractions.

Western blot analysis and peptide competition assay. Histone samples were run on 15% SDS-polyacrylamide gels, which were transferred to PVDF membranes (Pall Corporation) using a semi-dry apparatus (Hoefer) and Towbin buffer. Membranes were blotted using standard techniques, and probed with the antibodies at the following dilutions: α-H3 (Active Motif, Cat. No. 39163; 1:5000), α-H2BK37me2 (PRF&L, generated in this study; 1:2000), α-H3K4me3 (Active Motif, Cat. No. 39159; 1:10,000), α-H3K36me3 (Abcam, Cat. No. ab9050; 1:2000), α-H3K79me3 (Abcam, Cat. No. ab2621; 1:2000), or α-H2B (Active Motif, Cat. No. 39237; 1:10,000).

For peptide competition assays to demonstrate the specificity of purified α-H2BK37me2 antibody for H2BK37me2, purified IgG or α-H2BK37me2 antibody was pre-incubated with no peptide, a H2K37 peptide (SKARKETYS-C) or a H2K37me2 peptide (SKARKme2ETYS-C, where me2 is dimethyl lysine) at a final peptide concentration of 0.1 μg/mL for 1.5 hr at room temperature prior to incubation of PVDF membranes with primary antibody followed by standard Western blot analysis.

***RNA isolation, microarray and RT-qPCR mRNA analyses*.** Yeast cultures were grown at 30˚C in YPD simultaneously in triplicate to an OD_{600} of approximately 1.0. Ten OD_{600} units of cells were collected, washed once with water, and pellets were flash frozen in liquid nitrogen. Total RNA was isolated using the hot acidic phenol-chloroform method (COLLART and OLIVIERO 2001). Briefly, cell pellets were resuspended in 400 µL TES solution [10 mM Tris-Cl (pH 7.5), 10 mM EDTA, 0.5% SDS], to which 400 µL acidic phenol-chloroform (Ambion) was added. Samples were vortexed vigorously, incubated at 65˚C for 1 hour with occasional vortexing, and then placed on ice for 5 min. The aqueous layer was back-extracted once with acidic phenol-chloroform and once with chloroform. Following back-extraction with chloroform, RNA was precipitated using a standard ethanol precipitation protocol, and resuspended in RNase-free water. RNA was cleaned up using an RNeasy Mini Kit (QIAGEN), and RNA quality was determined using an Agilent Bioanalyzer.

Biotinylated-cRNA was generated using the MessageAmp™II-Biotin Enhanced Kit (Ambion) and was hybridized to Yeast Genome 2.0 arrays (Affymetrix), following manufacturer's protocol. Briefly, hybridizations were completed for 16 hr at 45˚C at 60 rpm in a GeneChip Hybridization Oven 640. Arrays were washed and stained using the GeneChip Fluidics Station 450, and were scanned with the GeneChip Scanner 3000 7G Plus Scanner with Autoloader. Microarray hybridization and analysis was completed at the University of North Carolina at Chapel Hill Functional Genomics Core Facility.

For real-time quantitative PCR (qPCR) gene expression analysis, following treatment of isolated RNA with DNA-*free* (Ambion) and RNA clean-up using an RNeasy Mini Kit (QIAGEN), first-strand cDNA was generated from total RNA using the Improm-II Reverse Transcription System (Promega). PCR reactions using 1/20 of total cDNA as template were completed using primers specific to the indicated genes. Primers used are as follows: *ACT1* Forward:

GAGGTTGCTGCTTTGGTTATTGA, Reverse: ACCGGCTTTACACATACCAGAAC. *AQR1* 5' Forward: GCTTTGAGGCAGTTGGAAAA, 5' Reverse: CACCGCTAACTGTGGGAGAT; *AQR1* 3' Forward: TGGGTTCCTTCTTCACAGGT, 3' Reverse: CTCTGCGTCTTGTGGAATCA. *FMP43* 5' Forward: ATTAGCGACGGCACTGATTT, 5' Reverse: CAGTGCAACCCAGGAAAAA; *FMP43* 3' Forward: GGATACGGAACGGTGATTCT, 3' Reverse: TCATCGATGTGGATGCAGTT. PCR reactions were carried out in triplicate for qPCR analysis using SYBR GreenER qPCR master mix (Invitrogen) and the Applied Biosystems 7900HT Fast Real-Time PCR system.

***Microarray data*.** All microarray data is MIAME compliant. Raw data generated from these studies have been deposited into the MIAME compliant database Gene Expression Omnibus (NCBI, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO series accession number GSE24380.

***Phenotypic spotting assays*.** To assay for growth in phenotypic spotting assays, five-fold serial dilutions of saturated overnight yeast cultures grown in YPD medium, or in synthetic complete medium supplemented as appropriate for plasmid selection, were plated onto appropriate media at a starting OD_{600} of 0.5. Growth on plates was imaged after 2-4 days of incubation at 30˚C, unless temperature is otherwise indicated.

Results

H2B is dimethylated at lysine 37. To date, only three lysine residues have been well-characterized as sites of methylation in budding yeast (namely lysines 4, 36, and 79 of histone H3) (MILLAR and GRUNSTEIN 2006). In higher eukaryotes, methylation is known to also occur on histone H3 at lysine residues 9 and 27 and histone H4 at lysine 20 (MARTIN and ZHANG 2005). To

begin to address whether histone methylation occurs on other sites in budding yeast, as well as to acquire a more comprehensive atlas of histone PTMs, we sought to use MS analysis to identify novel histone modifications. Given recent advancements in MS technology, it is now possible to use the top-down MS approach to analyze intact histone proteins, thereby allowing for more precise delineation and quantification of the complex modified forms in which the histones exist (GARCIA *et al.* 2007c). We initially performed our top-down MS studies on histone H2B, as this histone has more recently been shown to be monomethylated at lysine 5 in humans (BARSKI *et al.* 2007; WANG *et al.* 2008), and we were interested in determining whether this modification is conserved or if alternative sites of methylation exist in budding yeast.

According to its amino acid sequence, the theoretical monoisotopic mass ($[M+H]^+$) of yeast histone H2B is 14113.6056 Da. Using a 12 Tesla Bruker Daltonics μESI-FTICR-MS with ultrahigh mass accuracy and resolution, exact mass measurement of the protein was performed to validate sample preparation of histone H2B following isolation from yeast nuclei and RP-HPLC purification. The experimental monoisotopic mass of one of the major peaks (peak 2) was at 14113.6028 Da, extremely close to the theoretical value (mass error < 1 ppm) (FIGURE 3.1, panel A). Patterns of PTMs of yeast histone H2B were also mapped by exact mass measurement. The PTM site(s) on each form was further identified and characterized based on exact masses and sequence information from MS and MS/MS experiments. Relative abundances of modified forms were obtained by integrating the four most abundant isotopic peaks in three different charge states of MS spectra and taking their sum (TABLE 3.2).

With a mass of 14141.6352 Da, the second strongest peak (peak 4) exactly matched the theoretical monoisotopic mass of yeast histone H2B with two methyl marks (mass error < 1 ppm). To identify the modification site(s), the precursor ion corresponding to the modified protein (m/z 1415.9 Da, 10+ charge state) was isolated for top-down experiments using μESI-

FTICR-MS with ECD (FIGURE 3.1, panel B, upper). Inspection of the c and z fragment ions derived from the ECD MS/MS spectrum revealed +28 Da mass shifts of c_{37} to c_{49} ions, indicating that lysine 37 is dimethylated (FIGURE 3.1, panel B, lower). As indicated in TABLE 3.2, the relative abundance of dimethylated lysine 37 on histone H2B is over 25.7% in all yeast protein isoforms. Other PTMs (e.g., sites of acetylation and methylation) could be identified based on ECD MS/MS experiments. However, with the exception of N-terminal acetylation at serine 1 (*data not shown*), which has previously been identified (DELANGE *et al.* 1969; PESAVENTO *et al.* 2004; SONG *et al.* 2003), additional PTMs could not be conclusively assigned.

The finding that lysine 37 of histone H2B is dimethylated is in agreement with recently published MS results from a study surveying for sites of lysine propionylation and butylyration (Zhang et al. 2009). However, very little is known about this lysine residue. Physically, lysine 37 of histone H2B is located between the DNA gyres of the nucleosome structure (FIGURE 3.1, panel C). A previous study surveying the role of the N-terminal domain of histone H2B in transcription on a genome-wide level demonstrated that residues 30-37 of histone H2B are necessary and sufficient for the repression of a subset of genes in the budding yeast genome, and subsequently termed this region the H2B repression (HBR) domain (PARRA *et al.* 2006). This study posited a model by which the changes in gene expression that are observed upon deletion of the HBR could be due to elimination of yet to be identified PTMs that function in repression, and specifically suggest lysine 37 as a potential site of methylation (PARRA *et al.* 2006).

To validate the finding that H2B is dimethylated on lysine 37 in budding yeast, we first raised an antibody specific for this modified state in rabbit. Western blot analysis of acid-extracted wild-type histones using crude serum compared to pre-immune serum demonstrated that this mark exists *in vivo* (*data not shown*). To further corroborate this finding and characterize this novel mark, α-H2BK37me2 antibody was affinity purified from crude serum

and peptide competition analysis was completed using acid-extracted wild-type H2B, H2B K37A, and H2B K123R mutant histone samples. Where affinity purified α-H2BK37me2 antibody shows a clear signal in histone samples containing wild-type H2B, mutation of lysine 37 to a non-modifiable alanine (K37A) abrogates this signal (FIGURE 3.2, panel A, No peptide controls: left column, upper panels). Mutant H2B harboring a K123R mutation was used as a control to demonstrate specificity of this antibody for lysine 37. As a further measure of control, we showed that H2BK37me2 was not detectable in Western blot analysis using IgG purified from pre-immune serum (FIGURE 3.2, panel A, lower). The affinity purified antibody is specific for dimethylation of lysine 37, as pre-incubation of the α-H2BK37me2 antibody with a dimethylated H2BK37 peptide resulted in a loss of signal in all three histone samples, but preincubation with an unmodified H2BK37 peptide did not alter reactivity (FIGURE 3.2, panel A, middle and right columns, upper panels). Altogether, these data support the *in vivo* existence of dimethylation of histone H2B on lysine 37 and the generation of an antibody that is capable of specifically recognizing this modification.

Given that mutation of lysine 123 of histone H2B results in a loss of H2B monoubiquitylation at this site as well as a loss of methylation of histone H3 on lysines 4 and 79 (BRIGGS *et al.* 2002; DOVER *et al.* 2002; NAKANISHI *et al.* 2009; NG *et al.* 2002b; SUN and ALLIS 2002), we sought to determine whether crosstalk existed between histone H2B lysine 37 methylation and other known sites of histone methylation in budding yeast. Western blot analysis, using acid-extracted histones from wild-type H2B and H2B K37A mutant strains, showed that the loss of H3K37 methylation did not disrupt H3K4, H3K36 or H3K79 methylation (FIGURE 3.2, panel B). In contrast, and as a control, the H2B K123R mutant resulted in a loss of both H3K4 and H3K79 methylation, in agreement with previously published results (FIGURE 3.2, panel B, and (NAKANISHI *et al.* 2009)). Finally, the H2B K123R mutation does not disrupt H2BK37 methylation (FIGURE 3.2,

panel B). Together, these results suggest that dimethylation of H2BK37 is neither affected by H2B K123 ubiquitylation nor affects the ability of additional lysine residues to be methylated.

Elucidating the enzymes that place and remove H2BK37 methylation. We next sought to identify the putative histone methyltransferase responsible for placing this mark. To this end, a candidate screen in which acid-extracted histones from individual deletion strains from the Yeast Knockout Collection (Open Biosystems) were analyzed by Western blot analysis using our α-H2BK37me2 antibody (FIGURE 3.3, panel A). Included in the list of candidates were: the budding yeast SET-domain containing proteins; the histone lysine methyltransferase Dot1; known non-histone lysine methyltransferases; known yeast arginine methyltransferases (specific for both histone and non-histone substrates); and putative methyltransferases (TABLE 3.3). The SET domain is the catalytic domain of all identified histone lysine methyltransferases to date, with the exception of Dot1 (DILLON *et al.* 2005). To date, there are 12 proteins in budding yeast that harbor a SET domain (including Set1 through Set7, Rkm1 through Rkm3, and Ctm1) (PETROSSIAN and CLARKE 2009a). Of these proteins, only Set1 and Set2 have been demonstrated to function as histone lysine methyltransferases, and are specific for histone H3 lysine residues 4 and 36, respectively (BRIGGS *et al.* 2001; JENUWEIN *et al.* 1998; ROGUEV *et al.* 2001; STRAHL *et al.* 2002). Methylation of histone H3 at lysine 79 is catalyzed by Dot1, which is structurally unrelated to the other identified methyltransferases, as it lacks a SET domain altogether (NG *et al.* 2002a; SAWADA *et al.* 2004). In addition to histone lysine methyltransferases, budding yeast enzymes from the SET domain family that are capable of methylating non-histone substrates on lysine residues (namely, Ctm1, Rkm1, Rkm2, and Rkm3; (POLEVODA *et al.* 2000; PORRAS-YAKUSHI *et al.* 2006; PORRAS-YAKUSHI *et al.* 2005; WEBB *et al.* 2008)) were also tested in this screen. As arginine methylation is also known to occur in budding yeast, it is possible that enzymes

responsible for such modification on arginine residues could demonstrate substrate promiscuity, and thus the known arginine methyltransferases Hmt1, Rmt2, and Hsl7 (GARY *et al.* 1996; LEE *et al.* 2000; NIEWMIERZYCKA and CLARKE 1999) were also included in this screen. Finally, a number of annotated proteins (of both known and unknown function) predicted to function as methyltransferases based on structural predictions were also screened for activity toward histone H2B lysine 37, including the following: Trm12, Mtq1, Ylr137w, Ynl092w, Mni1, Ybr271w, Tae1, Ymr209c, Ylr063w, Ybr141c, Crg1, Yjr129c, and See1 (KALHOR *et al.* 2005; KATZ *et al.* 2003; NIEWMIERZYCKA and CLARKE 1999; PETROSSIAN and CLARKE 2009b; POLEVODA *et al.* 2006; WEBB *et al.* 2010).

We predicted that deletion of the responsible histone methyltransferase would result in a loss of signal in Western blot analysis using the α-H2BK37me2 antibody, as is observed in a parallel manner with Western blot analysis of samples derived from strains harboring individual deletions of the other known histone methyltransferases and the antibodies specific for their respective substrates. Unfortunately, all candidates screened to date (TABLE 3.3) did not give insight into the identity of the responsible methyltransferase. A loss of H2BK37me2 signal by Western blot analysis was not detected upon deletion of the individual candidates, as was observed for the control H2B K37R and H2B K37A mutants compared to their isogenic strain expressing wild-type H2B (FIGURE 3.3, panel A, bottom). This could be due functional redundancy amongst methyltransferases, which would be masked by single gene deletions. This, however, seems unlikely, as histone methyltransferases are typically highly specific for both the lysine residue that they target as well as the degree to which they can methylate their respective substrate (SHILATIFARD 2006; XIAO *et al.* 2003a). Alternatively, another class of yet to be identified histone methyltransferases or a methyltransferase that is essential for viability could facilitate placement of this mark, in which case a candidate screen of non-essential ORFs

would fail to reveal the responsible enzyme and rather an unbiased approach would have to be employed to identify the catalytic enzyme.

Recently, the JmjC domain has been identified as the catalytic domain of a family of histone demethylases (KLOSE *et al.* 2006a; TSUKADA *et al.* 2006). There are five JmjC-domain-containing proteins in budding yeast: Jhd1, Rph1, Gis1, Jhd2, and Ecm5 (KLOSE *et al.* 2007a). Jhd1, Rph1, and Jhd2 have all been demonstrated to possess histone demethylase activity, with specificity for H3K36me2/1, H3K36me3/2, and H3K4me3/2, respectively (FANG *et al.* 2007; KIM and BURATOWSKI 2007; KLOSE *et al.* 2007a; LIANG *et al.* 2007; SEWARD *et al.* 2007; TSUKADA *et al.* 2006). We also tried a candidate approach using deletion analysis of the five JmjC-domain-containing proteins to identify a putative demethylase for this mark. Again, acid-extracted histones were analyzed by Western blot analysis using the α-H2BK37me2 antibody, with wild-type H2B and H2B K37A mutant histones serving as controls (FIGURE 3.3, panel B). We anticipated that deletion of the putative demethylase would result in an increase in the total H2BK37me2, but deletion of the individual JmjC-domain-containing proteins did not show global changes in the level of H2BK37me2. This was not entirely surprising, as individual deletion of demethylases such as Jhd1 or Rph1 fails to show global changes in the levels of their target substrates (FANG *et al.* 2007; KLOSE *et al.* 2007a). Collectively, both the methyltransferase and demethylase enzymes specifically responsible for placing and removing dimethyl marks on H2BK37 remain to be identified.

Mutation of H2BK37 leads to no overt cellular phenotype. In parallel to identifying enzymes that catalyze the placement and removal of this methylation event, we sought to define the biological function of this mark. To this end, a number of phenotypic assays were completed using a series of strains harboring wild-type H2B, H2B K37A, H2B K37R, or H2B K123R mutant

histones (in most cases, except where specifically noted, the H2B K123R mutant strain was included as a positive control). General growth at various temperatures and on various types of complete media was assessed, but both the H2B K37R and H2B K37A strains failed to show differential growth as compared to the isogenic wild-type strain. This was in contrast to the H2B K123R strain, which exhibited a slow growth phenotype at all of the temperatures and various medias assessed (*data not shown*). Examination of growth under anaerobic conditions, as well as following release from stationary phase, also failed to show a difference between the K37 mutant and wild-type histone strains (*data not shown*). Mutation of lysine 37 to either arginine or alanine also did not affect the ability of yeast cells to properly sporulate as compared to an isogenic strain expressing wild-type H2B (*data not shown*). We next posited that H2BK37me2 might be cell-cycle regulated, and therefore synchronized wild-type cells in G2/M with nocodazole and harvested cells at defined points along the cell cycle following nocodazole release. Western blot analysis of these cells at various stages of the cell cycle failed to reveal an enrichment and/or depletion of H2BK37me2 at any defined cell cycle stage (as compared to known cell-cycle regulated marks such as phosphorylation of histone H3 on serine 10 and threonine 45, which occur during mitosis and S-phase, respectively (BAKER *et al.* 2010; HSU *et al.* 2000)) (*data not shown*).

We also performed assays to screen for phenotypes related to DNA replication and repair. To that end, wild-type H2B and the H2B K37 mutant strains were spotted on media containing the agents hydroxyurea (HU, an agent which blocks replication leading to replication fork collapse) or methyl methanesulfonate (MMS, an alkylating agent that causes DNA lesions and ultimately DNA strand breaks). However lysine 37 mutations in histone H2B did not alter cellular growth compared to an isogenic wild-type parent on media containing 0.05% MMS (*data not shown*) or 100 mM HU (FIGURE 3.4, panel A), where cells bearing a H2B K123R

mutation were sensitive to both. Moreover, to assess the ability of lysine 37 mutant strains to carry out replication, plasmid maintenance assays were completed, where the ability of a cell to replicate a reporter plasmid containing a single origin of replication and a selectable marker is measured (HOGAN and KOSHLAND 1992). Mutation of lysine 37 on histone H2B to either arginine or alanine did not affect the ability of yeast strains to faithfully replicate the reporter plasmid as compared to isogenic wild-type cells (*data not shown*). Taken together, the results from these screening assays suggest that histone H2B lysine 37 does not have a significant role in DNA replication or repair.

As methylation of both lysine 4 and 79 of histone H3 have been previously demonstrated to be necessary for proper telomeric silencing (KROGAN *et al.* 2002; NG *et al.* 2002a; NG *et al.* 2002b; SUN and ALLIS 2002), we next sought to determine if mutation of lysine 37 would also result in loss of telomeric silencing. To that end, H2B K37R and H2B K37A mutations were introduced into a histone H2A-H2B shuffle strain engineered to assay for defects in telomeric silencing, where expression of *URA3*, located at the left-end telomere of chromosome VII (*URA3*-TEL), is used as a readout for proper silencing (SUN and ALLIS 2002). If telomeric silencing properly occurs, the *URA3* gene is silenced, and cells grow normally on media containing 5-fluoroortic (5-FOA), an agent that is toxic only to cells that express *URA3*. Introduction of H2B K37R and H2B K37A mutations in *URA3*-TEL strains results in comparable growth on 5-FOA-containing media to the isogenic *URA3*-TEL strain expressing wild-type H2B (FIGURE 3.4, panel B). This is in direct contrast to cells expressing H2B K123R or cells deleted of *SIR2*, which both fail to grow on media containing 5-FOA due to improper silencing of the *URA3* gene (FIGURE 3.4, panel B), in agreement with previously published results (SUN and ALLIS 2002). Together, these data suggest that lysine 37 of histone H2B is not essential for gene silencing in yeast.

Several assays to test for transcriptional defects were also employed. Spotting assays on media containing 6-azauracil (6-AU) or mycophenolic acid (MPA), which both deplete intracellular levels of nucleotides leading to altered cellular viability when combined with mutations that affect transcriptional elongation, were completed. In both cases, strains with mutant H2B K37R or K37A grew comparably to cells with wild-type H2B, where an H2B K123R mutation resulted in a slow growth phenotype (*data not shown*). Transcription induction was also assessed by measuring the induction of *GAL1* and *GAL10* transcripts in wild-type H2B and H2B K37 mutant strains. However, gene expression analysis by reverse-transcription quantitative PCR (RT-qPCR) revealed that mutation of lysine 37 on histone H2B does not alter induction of either *GAL1* or *GAL10*, as compared to wild-type cells, supporting that this residue does not significantly contribute to transcriptional induction of these genes. Finally, we were curious to see how mutation in H2B K37 would behave in combination with mutant *SPT16*, a member of the FACT histone chaperone complex that promotes transcription elongation (BELOTSERKOVSKAYA *et al.* 2003; BISWAS *et al.* 2006; MASON and STRUHL 2003; SAUNDERS *et al.* 2003). Previous results have shown that the growth phenotype observed upon inactivation of *SPT16* is enhanced and suppressed by mutations in lysine residues 4 and 36 of histone H3, respectively, suggesting that FACT function is dependent upon H3K4 methylation and is opposed by H3K36 methylation (BISWAS *et al.* 2006). We therefore introduced lysine 37 mutations into a histone H2A-H2B shuffle-strain containing a temperature-sensitive allele of *SPT16* (*spt16-197*), and cellular growth was assessed at range of temperatures. However, this analysis failed to reveal a combinatorial effect between mutation of lysine 37 on histone H2B and inactivation of *SPT16*, as H2B K37R/A *spt16-197* double mutant strains grew comparably to isogenic *spt16-197* containing wild-type H2B (FIGURE 3.4, panel C). This is in direct opposition to a H2B K123R *spt16-197* double mutant strain, which demonstrated a synthetic effect upon inactivation of the FACT allele.

These data together substantiate that methylation of lysine 37 does not appear to play a major role in transcription, as mutation of this histone residue results in no overt phenotype in all transcription-based assays completed to date.

Finally, given that Parra *et al* presented a model by which gene expression changes observed upon deletion of the HBR domain could be a consequence of eliminating a modified form of this domain (PARRA *et al.* 2006), we sought to address whether methylation lysine 37 of H2B in particular functions in transcriptional regulation on a genomic level. To this end, gene expression changes upon mutation of lysine 37 were assessed by microarray analysis. Comparison of gene expression changes in cells expressing wild-type H2B versus a H2B K37A mutant revealed that lysine 37 does not appear to function significantly in genome-wide transcription regulation, as only 20 genes showed differential gene expression using a cutoff of a two-fold difference in expression (where two genes were upregulated (TABLE 3.4) and 18 genes were downregulated (TABLE 3.5) in a H2B K37A mutant relative to the isogenic wild-type strain). RT-qPCR analysis was able to recapitulate the microarray results of genes shown to be up- or downregulated in a H2B K37A mutant strain relative to the isogenic parent strain (FIGURE 3.5 and *data not shown*), thus validating the microarray results. However, the lack of a significant number of genes showing differential expression between wild-type and H2B K37A mutant strains indicates overall that H2BK37me2 alone does not play a major role in regulation of transcription on a genome-wide level in budding yeast.

Methylation of H2BK37 is conserved in higher eukaryotes. Sequence alignment of histone H2B from *Saccharomyces cerevisiae* against multiple species reveals that lysine 37 is conserved along evolution, despite lower sequence similarity of surrounding amino acid residues (FIGURE 3.6, panel A). To determine if we could detect the presence of methylated lysine 37 in higher

eukaryotes, we performed Western blot analysis comparing oligonucleosomes isolated from chicken erythrocyte nuclei and core histones from HeLa cell nuclei to yeast histones. Western blot analysis using the α-H2BK37me2 antibody revealed that this mark is indeed conserved in higher eukaryotes (Figure 3.6, panel B), as a comparable species is observed in both the chicken and human histone samples as to histones extracted from yeast harboring wild-type, but not the K37A mutant, H2B. The presence of a discernable signal in samples derived from higher eukaryotic species suggests that, despite the lack of an obvious cellular phenotype in yeast to date, this mark is likely to be biologically important since it was retained during evolution.

Discussion

To date, only six lysines residues have been identified and characterized as sites of histone methylation (namely, lysines 4, 9, 27, 36 and 79 of histone H3, and lysine 20 of histone H4) (MARTIN and ZHANG 2005). Recently, a comprehensive study employing LC-ESI MS/MS to identify PTMs of histones associated with each phase of the yeast cell cycle revealed that lysine 111 of histone H2B is also a site of histone methylation (UNNIKRISHNAN *et al.* 2010), in agreement with additional previously publishes results (ZHANG *et al.* 2009). Phenotypic analyses have revealed that mutation of this lysine residue confers sensitivity to the DNA-damaging agent MMS and renders telomeric silencing defective (KYRISS *et al.* 2010), supporting the importance of this lysine residue and its methylation in chromatin function. Trimethylation of lysine 64 on histone H3 has been shown to be enriched at pericentric heterochromatin in human and mice samples, and is dynamically regulated during early development, supporting a function for this modification in the reprogramming process involved in germ cell development (DAUJAT *et al.* 2009). Additionally, methylation of histone H3 at lysine 122 has recently been reported in mice (COCKLIN and WANG 2003), and genome-wide localization patterns of methylation of lysine 5 on

histone H2B have been reported in humans (BARSKI *et al.* 2007; WANG *et al.* 2008). However, the latter two sites of histone methylation are largely uncharacterized at present. It is likely that additional sites of histone lysine methylation remain to be identified, and that much remains to be discovered with regard to the complexity of histone methylation and how this PTM in particular contributes to the histone code and cellular function. That additional sites of modifications critical for normal cellular function remain to be identified thereby necessitates further investigations directed toward elucidating a complete atlas of histone PTMs.

In this manuscript, we reveal the utility of top-down MS analysis in the identification of novel histone PTMs, and report that lysine 37 of histone H2B is dimethylated in budding yeast. We also provide evidence that this modification is evolutionarily conserved. Much remains to be determined with respect to the placement and removal, regulation and biological function(s) of this mark. For example, a candidate screen employing all known lysine methyltransferases in budding yeast (both specific for histone and non-histone substrates) has revealed that the methyltransferase responsible for placement of this mark does not fall into the category of one of the previously identified methyltransferases. This suggests that either multiple methyltransferases function redundantly to methylate H2BK37, or that a novel class of methyltransferases capable of placing this mark exists. Using a similar candidate approach to screen known histone demethylases for specificity for this mark also failed to expose a demethylase specific for this mark. Given that deletion of known JmjC-domain-containing demethylases does not result in global changes in the levels of histone modifications that they have been shown to target (FANG *et al.* 2007; KLOSE *et al.* 2007a), it is likely that identification of the demethylase responsible for removal of lysine 37 methylation cannot be revealed by deletion analysis. Alternatively, multiple demethylases could be functionally redundant in the removal of this mark, thus making deletion analysis a less ideal assay for identification of the

enzyme responsible for erasing methylation at H2BK37. It is also possible that a family of enzymes other than JmjC-domain-containing histone demethylases exists that is responsible for removal of this mark, as well as others (for example, a demethylase specific for H3K79 remains to be identified), or that there simply is not a demethylase for this mark.

Saccharomyces cerevisiae provides an advantageous genetic system for studying the functional consequence of loss of a specific amino acid residue (a feat that cannot be readily accomplished in higher eukaryotes (KOUZARIDES 2007)), thus prompting us to carry out phenotypic analysis in budding yeast. As MS analysis has revealed that H2BK37 dimethylation is a relatively abundant modification, we reasoned that mutation of lysine 37 would likely cause pleiotropic effects. However, all assays screened to date have failed to reveal a functional phenotype when lysine 37 is changed to either arginine or alanine. It is possible that this modification could function redundantly with another histone modification, in which case combinatorial mutations would be necessary to reveal the functional significance of these marks. Thus, further studies will have to be completed to determine the biological significance of this mark in chromatin.

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hybridization. We acknowledge members of the labs of Jean Cook (University of North Carolina at Chapel Hill) and Scott Briggs (Purdue University) for assisting with DNA plasmid maintenance and anaerobic growth phenotypic assays, respectively.

TABLE 3.1 | Yeast strains

Strain	Genotype	Reference/Source
FY406	*MAT***a** *(hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63* [pSAB6 (*HTA1-HTB1, URA3*)]	(HIRSCHHORN *et al.* 1995)
YKG001	*MAT***a** *(hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63* [pZS145 (*HTA1-Flag-HTB1 CEN HIS3*)]	(NAKANISHI *et al.* 2009)
YKG002	*MAT***a** *(hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63* [pZS146 (*HTA1-Flag-htb1 (K123R) CEN HIS3*)]	(NAKANISHI *et al.* 2009)
YKG006	*MAT***a** *(hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63* [pKG1 (*HTA1-Flag-htb1 (K37R) CEN HIS3*)]	This study
YKG007	*MAT***a** *(hta1-htb1)Δ::LEU2 (hta2-htb2)Δ::TRP1 leu2Δ1 ura3-52 lys2Δ1 lys2-128δ his3Δ200 trp1Δ63* [pKG2 (*HTA1-Flag-htb1 (K37A) CEN HIS3*)]	This study
YZS272	*MAT***a** *ura3-1 leu2-3,112 ade2-1 trp1-1 his3-11,15 can1-100 (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ* [pZS144 *(HTA1-Flag-HTB1 CEN TRP1)*] *URA3-TEL*	(SUN and ALLIS 2002)
YKG027	*MAT***a** *ura3-1 leu2-3,112 ade2-1 trp1-1 his3-11,15 can1-100 (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ* [pZS145 *(HTA1-Flag-HTB1 CEN HIS3)*] *URA3-TEL*	This study
YKG028	*MAT***a** *ura3-1 leu2-3,112 ade2-1 trp1-1 his3-11,15 can1-100 (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ* [pKG1 *(HTA1-Flag-htb1 (K37R) CEN HIS3)*] *URA3-TEL*	This study
YKG029	*MAT***a** *ura3-1 leu2-3,112 ade2-1 trp1-1 his3-11,15 can1-100 (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ* [pKG2 *(HTA1-Flag-htb1 (K37A) CEN HIS3)*] *URA3-TEL*	This study
YZS274	*MAT***a** *ura3-1 leu2-3,112 ade2-1 trp1-1 his3-11,15 can1-100 (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ* [pZS146 *(HTA1-Flag-htb1 (K123R) CEN HIS3)*] *URA3-TEL*	(SUN and ALLIS 2002)
YZS275	*MAT***a** *ura3-1 leu2-3,112 ade2-1 trp1-1 his3-11,15 can1-100 (hta1-htb1)Δ::LEU2 (hta2-htb2)Δ* [pZS145 *(HTA1-Flag-HTB1 CEN HIS3)*] *URA3-TEL sir2Δ::TRP1*	(SUN and ALLIS 2002)
YZS276	*MAT***a** *(hta1-htb1)Δ::LEU2 (hta2-htb2)Δ leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can 1-100* [pZS145 *(HTA1-Flag-HTB1 CEN HIS3)*]	(SUN and ALLIS 2002)
YZS277	*MAT***a** *(hta1-htb1)Δ::LEU2 (hta2-htb2)Δ leu2-3,112 his3-11,15 trp1-1 ura3-1 ade2-1 can 1-100* [pZS146 *(HTA1-Flag-htb1 (K123R) CEN HIS3)*]	(SUN and ALLIS 2002)

Y131	*MAT***a** *(hta1-htb1)Δ::LEU2 (hta2-htb2)Δ leu2-3,112 trp1-1 ura3-1 ade2-1 can1-100 his3-11,15* [pRS426 (*HTA1-HTB1 URA3 2 μm*)]	(ROBZYK *et al.* 2000)
YCH278	*MAT***a** *(hta1-htb1)Δ::LEU2 (hta2-htb2)Δ leu2-3,112 trp1-1 ura3-1 ade2-1 can1-100 his3-11,15 spt16:kanMX* [pRS426 *(HTA-HTB URA3 2 μm)*] [pBM46-*spt16-197*]	(FLEMING *et al.* 2008)
YKG031	*MAT***a** *(hta1-htb1)Δ::LEU2 (hta2-htb2)Δ leu2-3,112 trp1-1 ura3-1 ade2-1 can1-100 his3-11,15 spt16:kanMX* [pZS145 *(HTA1-Flag-HTB1 CEN HIS3)*] [pBM46-*spt16-197*]	This study
YKG032	*MAT***a** *(hta1-htb1)Δ::LEU2 (hta2-htb2)Δ leu2-3,112 trp1-1 ura3-1 ade2-1 can1-100 his3-11,15 spt16:kanMX* [pZS146 *(HTA1-Flag-htb1 (K123R) CEN HIS3)*] [pBM46-*spt16-197*]	This study
YKG033	*MAT***a** *(hta1-htb1)Δ::LEU2 (hta2-htb2)Δ leu2-3,112 trp1-1 ura3-1 ade2-1 can1-100 his3-11,15 spt16:kanMX* [pKG1 *(HTA1-Flag-htb1 (K37R) CEN HIS3)*] [pBM46-*spt16-197*]	This study
YKG034	*MAT***a** (*hta1-htb1)Δ::LEU2 (hta2-htb2)Δ leu2-3,112 trp1-1 ura3-1 ade2-1 can1-100 his3-11,15 spt16:kanMX* [pKG2 *(HTA1-Flag-htb1 (K37A) CEN HIS3)*] [pBM46-*spt16-197*]	This study
YMP001	*MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 rad5-535 HTZ1::myc/7xHis*	This study
YBC63	*MATα lys2-128 leu2Δ ura3-52 trp1Δ63 his3Δ200*	(SCHLICHTER and CAIRNS 2005)
YBC1236	*MATα lys2-128 leu2Δ ura3-52 trp1Δ63 his3Δ200 set1Δ::HIS3MX6*	(SCHLICHTER and CAIRNS 2005)
DY2390 (W303)	*MATα ade2 can1 his3 leu2 lys2 trp1 ura3*	(WATSON *et al.* 2000)
YAR005	*MATα ade2 can1 his3 leu2 lys2 trp1 ura3 rph1Δ::kanMX*	This study
YAR007	*MATα ade2 can1 his3 leu2 lys2 trp1 ura3 jhd1Δ::kanMX*	This study
YAR009	*MATα ade2 can1 his3 leu2 lys2 trp1 ura3 gis1Δ::kanMX*	This study
YAR011	*MATα ade2 can1 his3 leu2 lys2 trp1 ura3 jhd2Δ::kanMX*	This study
YAR013	*MATα ade2 can1 his3 leu2 lys2 trp1 ura3 ecm5Δ::kanMX*	This study
YNL037	*MATα ade2 can1 his3 leu2 lys2 trp1 ura3 dot1Δ::kanMX*	This study
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*	Open Biosystems
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Open Biosystems

The following deletion strains used for candidate screening are from the Yeast Knockout Collection in the BY4741 background (Open Biosystems): *crg1Δ::kanMX*, *ctm1Δ::kanMX*, *htm1Δ::kanMX*, *mni1Δ::kanMX*, *mtq1Δ::kanMX*, *rkm1Δ::kanMX*, *rkm2Δ::kanMX*, *rkm3Δ::kanMX*, *rmt2Δ::kanMX*, *see1Δ::kanMX*, *set2Δ::kanMX*, *set3Δ::kanMX*, *set4Δ::kanMX*, *set5Δ::kanMX*, *set6Δ::kanMX*, *set7Δ::kanMX*, *tae1Δ::kanMX*, *trm12Δ::kanMX*, *ybr141cΔ::kanMX*, *ybr271wΔ::kanMX*, *yjr129cΔ::kanMX*, *ylr063wΔ::kanMX*, *ylr137wΔ::kanMX*, *ymr209cΔ::kanMX*, *ynl092wΔ::kanMX*. The following deletion strain used for candidate screening is from the Yeast Knockout Collection in the BY4742 background (Open Biosystems): *hsl7Δ::kanMX*.

TABLE 3.2 | Yeast histone H2B patterns of PTMs

Yeast H2B PTM	Relative Abundance* (%)
H2B-H_2O	7.0
H2B	12.8
Me**	5.5
Me2K37	25.7
Ac-αS1	29.9
Ac-αS1+Me**	7.3
Ac-αS1+2Me**	4.7
Ac-αS1+3Me/Ac**	4.6
Ac-αS1+4Me/Ac**	2.4

* RSD = ± 1.0%.

** PTM sites cannot be assigned.

TABLE 3.3 | Candidates screened for putative H2BK37me2 histone methyltransferase activity

Candidate	Annotated SGD description(s)
CRG1	Putative S-adenosylmethionine-dependent methyltransferase; mediates cantharidin resistance
CTM1	Cytochrome c lysine methyltransferase; trimethylates residue 72 of apo-cytochrome c (Cyc1p) in the cytosol; not required for normal respiratory growth
DOT1	Nucleosomal histone H3-Lys79 methylase; methylation is required for telomeric silencing, meiotic checkpoint control, and DNA damage response
HMT1	Nuclear SAM-dependent mono- and asymmetric arginine dimethylating methyltransferase that modifies hnRNPs, including Npl3p and Hrp1p, affecting their activity and nuclear export; methylates U1 snRNP protein Snp1p and ribosomal protein Rps2p
HSL7	Protein arginine N-methyltransferase that exhibits septin and Hsl1p-dependent bud neck localization and periodic Hsl1p-dependent phosphorylation; required along with Hsl1p for bud neck recruitment, phosphorylation, and degradation of Swe1p
MNI1	AdoMet-dependent methyltransferase involved in a novel 3-methylhistidine modification of ribosomal protein Rpl3p; seven beta-strand MTase family member; null mutant exhibits a weak vacuolar protein sorting defect and caspofungin resistance
MTQ1	S-adenosylmethionine-dependent methyltransferase; methylates translational release factor Mrf1p
RKM1	SET-domain lysine-N-methyltransferase, catalyzes the formation of dimethyllysine residues on the large ribosomal subunit protein L23a (RPL23A and RPL23B)
RKM2	Ribosomal protein lysine methyltransferase, responsible for trimethylation of the lysine residue at position 3 of Rpl12Ap and Rpl12Bp
RKM3	Ribosomal lysine methyltransferase specific for monomethylation of Rpl42ap and Rpl42bp (lysine 40); nuclear SET domain containing protein
RMT2	Arginine N5 methyltransferase; methylates ribosomal protein Rpl12 (L12) on Arg67
SEE1	Probable lysine methyltransferase involved in the dimethylation of eEF1A (Tef1p/Tef2p); sequence similarity to S-adenosylmethionine-dependent methyltransferases of the seven beta-strand family; role in vesicular transport
SET1	Histone methyltransferase, subunit of the COMPASS (Set1C) complex which methylates histone H3 on lysine 4; required in transcriptional silencing near telomeres and at the silent mating type loci; contains a SET domain
SET2	Histone methyltransferase with a role in transcriptional elongation, methylates a lysine residue of histone H3; associates with the C-terminal domain of Rpo21p; histone methylation activity is regulated by phosphorylation status of Rpo21p
SET3	Defining member of the SET3 histone deacetylase complex which is a meiosis-specific repressor of sporulation genes; necessary for efficient transcription by RNAPII; one of

	two yeast proteins that contains both SET and PHD domains
SET4	Protein of unknown function, contains a SET domain
SET5	Zinc-finger protein of unknown function, contains one canonical and two unusual fingers in unusual arrangements; deletion enhances replication of positive-strand RNA virus
SET6	SET domain protein of unknown function; deletion heterozygote is sensitive to compounds that target ergosterol biosynthesis, may be involved in compound availability
SET7/RKM4	Ribosomal lysine methyltransferase specific for monomethylation of Rpl42ap and Rpl42bp (lysine 55); nuclear SET-domain containing protein
TAE1	AdoMet-dependent proline methyltransferase; catalyzes the dimethylation of ribosomal proteins Rpl12 and Rps25 at N-terminal proline residues; has a role in protein synthesis; fusion protein localizes to the cytoplasm
TRM12	S-adenosylmethionine-dependent methyltransferase of the seven beta-strand family; required for wybutosine formation in phenylalanine-accepting tRNA
YBR141C	Putative S-adenosylmethionine-dependent methyltransferase; GFP-fusion protein localizes to the nucleolus
YBR271W	Putative S-adenosylmethionine-dependent methyltransferase of the seven beta-strand family; GFP-fusion protein localizes to the cytoplasm; predicted to be involved in ribosome biogenesis
YJR129C	Putative protein of unknown function; predicted S-adenosylmethionine-dependent methyltransferase of the seven beta-strand family; GFP-fusion protein localizes to the cytoplasm
YLR063W	Putative S-adenosylmethionine-dependent methyltransferase; GFP-fusion protein localizes to the cytoplasm
YLR137W	Putative S-adenosylmethionine-dependent methyltransferase
YMR209C	Putative S-adenosylmethionine-dependent methyltransferase
YNL092W	Putative S-adenosylmethionine-dependent methyltransferase of the seven beta-strand family

TABLE 3.4 | Genes that are upregulated at least two-fold in H2B K37A mutant cells

Candidate	Name	Fold Change*	Annotated SGD description(s)
YNL065W	*AQR1*	2.50	Plasma membrane multidrug transporter of the major facilitator superfamily, confers resistance to short-chain monocarboxylic acids and quinidine; involved in the excretion of excess amino acids
YGR079W	---	2.30	Putative protein of unknown function; YGR079W is not an essential gene

*K37A:WT

TABLE 3.5 | Genes that are downregulated at least two-fold in H2B K37A mutant cells

Candidate	Name	Fold Change*	Annotated SGD description(s)
YHR209W	*CRG1*	0.49	Putative S-adenosylmethionine-dependent methyltransferase; mediates cantharidin resistance
YKL163W	*PIR3*	0.49	O-glycosylated covalently-bound cell wall protein required for cell wall stability; expression is cell cycle regulated, peaking in M/G1 and also subject to regulation by the cell integrity pathway
YEL011W	*GLC3*	0.48	Glycogen branching enzyme, involved in glycogen accumulation; GFP-fusion protein localizes to the cytoplasm in a punctate pattern
YOR028C	*CIN5*	0.48	Basic leucine zipper (bZIP) transcription factor of the yAP-1 family, mediates pleiotropic drug resistance and salt tolerance; nuclearly localized under oxidative stress and sequestered in the cytoplasm by Lot6p under reducing conditions
YHR184W	*SSP1*	0.48	Protein involved in the control of meiotic nuclear division and coordination of meiosis with spore formation; transcription is induced midway through meiosis
YMR101C	*SRT1*	0.48	Cis-prenyltransferase involved in synthesis of long-chain dolichols (19-22 isoprene units; as opposed to Rer2p which synthesizes shorter-chain dolichols); localizes to lipid bodies; transcription is induced during stationary phase
YBR147W	*RTC2*	0.47	Protein of unknown function; identified in a screen for mutants with decreased levels of rDNA transcription; detected in highly purified mitochondria; null mutant suppresses *cdc13-1*; similar to a G-protein coupled receptor from *S. pombe*
YDL169C	*UGX2*	0.47	Protein of unknown function, transcript accumulates in response to any combination of stress conditions
YDL079C	*MRK1*	0.47	Glycogen synthase kinase 3 (GSK-3) homolog; one of four GSK-3 homologs in *S. cerevisiae* that function to activate Msn2p-dependent transcription of stress responsive genes and that function in protein degradation
YMR206W	---	0.46	Putative protein of unknown function; YMR206W is not an essential gene
YGL158W	*RCK1*	0.45	Protein kinase involved in the response to oxidative stress; identified as suppressor of *S. pombe* cell cycle checkpoint mutations
YOR178C	*GAC1*	0.45	Regulatory subunit for Glc7p type-1 protein phosphatase (PP1), tethers Glc7p to Gsy2p glycogen synthase, binds Hsf1p heat

			shock transcription factor, required for induction of some HSF-regulated genes under heat shock
YFL052W	*ROP1*	0.44	Putative zinc cluster protein that contains a DNA binding domain; null mutant sensitive to calcofluor white, low osmolarity and heat, suggesting a role for YFL052Wp in cell wall integrity
YDR277C	*MTH1*	0.44	Negative regulator of the glucose-sensing signal transduction pathway, required for repression of transcription by Rgt1p; interacts with Rgt1p and the Snf3p and Rgt2p glucose sensors; phosphorylated by Yck1p, triggering Mth1p degradation
YGR243W	*FMP43*	0.43	Putative protein of unknown function; expression regulated by osmotic and alkaline stresses; the authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies
YBR299W	*MAL32*	0.43	Maltase (alpha-D-glucosidase), inducible protein involved in maltose catabolism; encoded in the MAL3 complex locus; functional in genomic reference strain S288C; hydrolyzes the disaccharides maltose, turanose, maltotriose, and sucrose
YMR280C	*CAT8*	0.42	Zinc cluster transcriptional activator necessary for derepression of a variety of genes under non-fermentative growth conditions, active after diauxic shift, binds carbon source responsive elements
YIL057C	*RGI2*	0.37	Putative protein of unknown function; expression induced under carbon limitation and repressed under high glucose

*K37A:WT

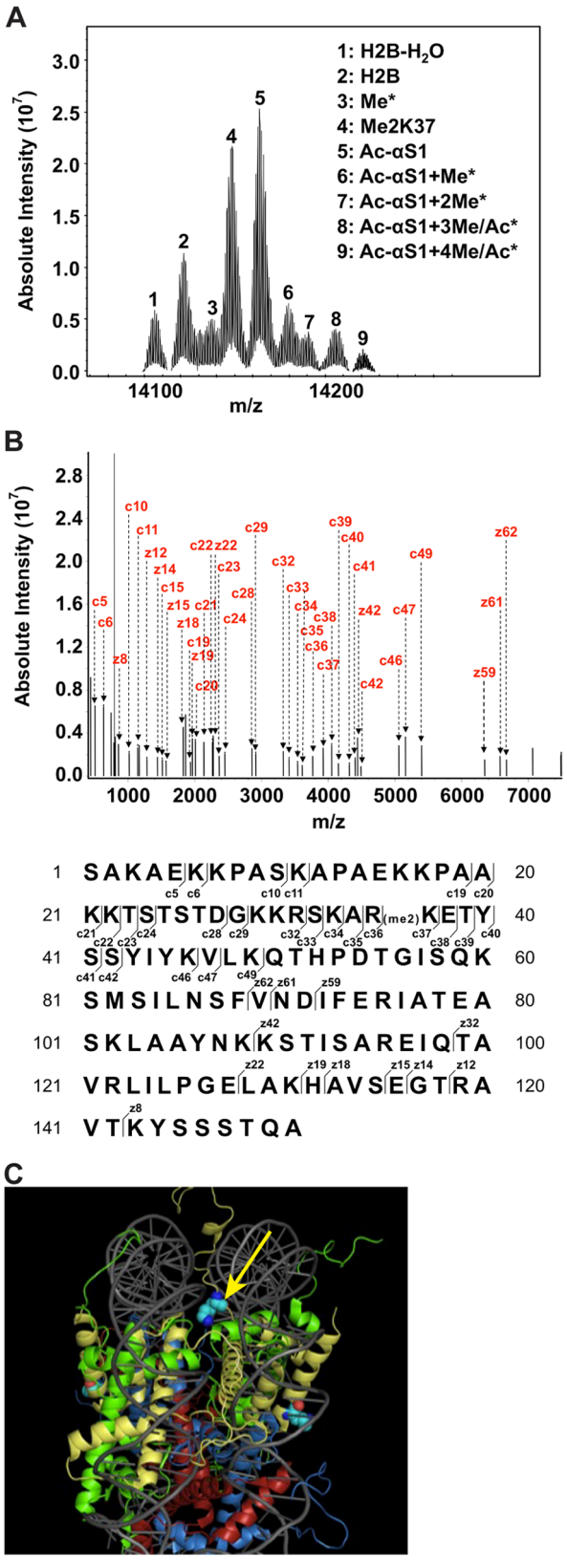
A
Absolute Intensity (10^7)
m/z
1: H2B-H2O
2: H2B
3: Me*
4: Me2K37
5: Ac-αS1
6: Ac-αS1+Me*
7: Ac-αS1+2Me*
8: Ac-αS1+3Me/Ac*
9: Ac-αS1+4Me/Ac*
B
Absolute Intensity (10^7)
m/z
1 SAKAEKKPASKAPAEKKPAA 20
21 KKTSTSTDGKKRSKAR(me2)KETY 40
41 SSYIYKVLKQTHPDTGISQK 60
81 SMSILNSFVNDIFERIATEA 80
101 SKLAAYNKKSTISAREIQTA 100
121 VRLILPGELAKHAVSEGTRA 120
141 VTKYSSSTQA
C

FIGURE 3.1 | Top-down mass spectrometry (MS) analysis reveals histone H2B is dimethylated at lysine 37. (**A**) Top-down µESI-FTICR-MS analysis of yeast histone H2B. Shown is a mass spectrum of H2B revealing multiply modified forms of this histone, as indicated by peaks numbered 1-9. Each peak was analyzed by top-down µESI-FTICR-MS/MS analysis and modifications identified are denoted in the legend. Asterisks indicate PTMs that were not assigned. 100 scans per spectrum were acquired in the ICR cell with a resolution of 580,000 at m/z 400 Da. (**B**) Top-down µESI-FTICR-MS/MS analysis of peak 4. ECD MS/MS spectrum of histone H2B with two methyl marks (precursor: m/z 1415.9 Da, 10+ charge state) reveals lysine 37 is dimethylated. N-terminal (c ions) and C-terminal (z ions) fragment ions are assigned and shown in the upper panel. Lower panel denotes the ions in the sequence. Unassigned ions are either internal fragment ions or electronic noise. 100 scans per spectrum were acquired in the ICR cell with a resolution of 580,000 at m/z 400 Da. (**C**) Lysine 37 of H2B is located within the DNA gyres in the nucleosomal structure. Histones H2A, H2B, H3 and H4 are shaded green, yellow, red, and blue, respectively. The DNA backbone is colored gray. The yellow arrow points to the location of lysine 37 of histone H2B. The nucleosomal representation was generated using open-source PyMOL software (PyMOL 0.99rev10, DeLan Scientific LCC) with structural data taken from (DAVEY *et al.* 2002) (PDB file 1kx5).

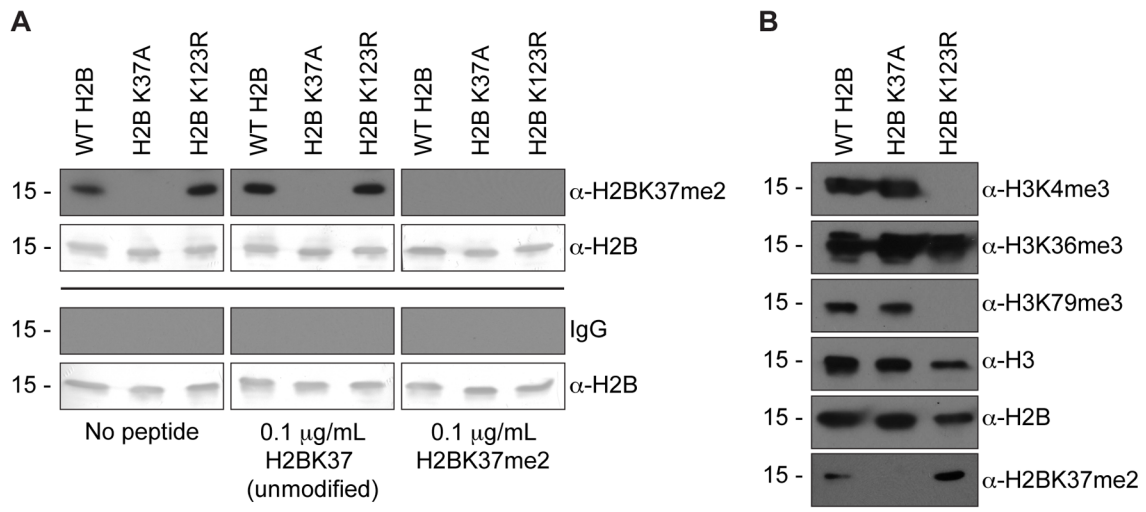

FIGURE 3.2 | α-H2BK37me2 antibody is specific for dimethylated lysine 37 on histone H2B. (**A**) A polyclonal antibody was purified from antiserum raised by immunizing rabbits with the peptide SKARKme2ETYS-C, where me2 is dimethyl lysine. Peptide competition assay demonstrates specificity of purified α-H2BK37me2 antibody for dimethyl lysine 37 of histone H2B. Western blot analysis was completed using acid-extracted histones from strains harboring wild-type Flag-H2B (YKG001), Flag-H2B K37A (YKG007), and Flag-H2B K123R (YKG002), demonstrating that dimethylation of lysine 37 on histone H2B occurs *in vivo*, as the antibody is able to recognize this modification in wild-type and H2B K123R-derived histone samples, but not histones extracted from the Flag-H2B strain harboring a K37A mutation (No peptide controls: left column, upper panels). Preincubation of the purified antibody with H2K37me2 peptide resulted in a loss of the ~15 kDa band in all three histone samples, whereas preincubation with unmodified H2BK37 peptide did not alter the reactivity (middle and right columns, upper panels). H2BK37me2 signal was not detectable in Western blot analysis using IgG purified from pre-immune serum (lower panels). All blots were stripped and reprobed with an α-H2B antibody to demonstrate equal loading. (**B**) Western blot analysis using modification specific antibodies indicates that mutation of lysine 37 on histone H2B does not affect methylation at other known sites of methylation in budding yeast, including histone H3 lysines 4, 36, and 79. A H2B K123R mutation abrogates methylation at H3K4 and H3K79, in agreement with previously published results (NAKANISHI *et al.* 2009), but does not affect H2BK37 methylation.

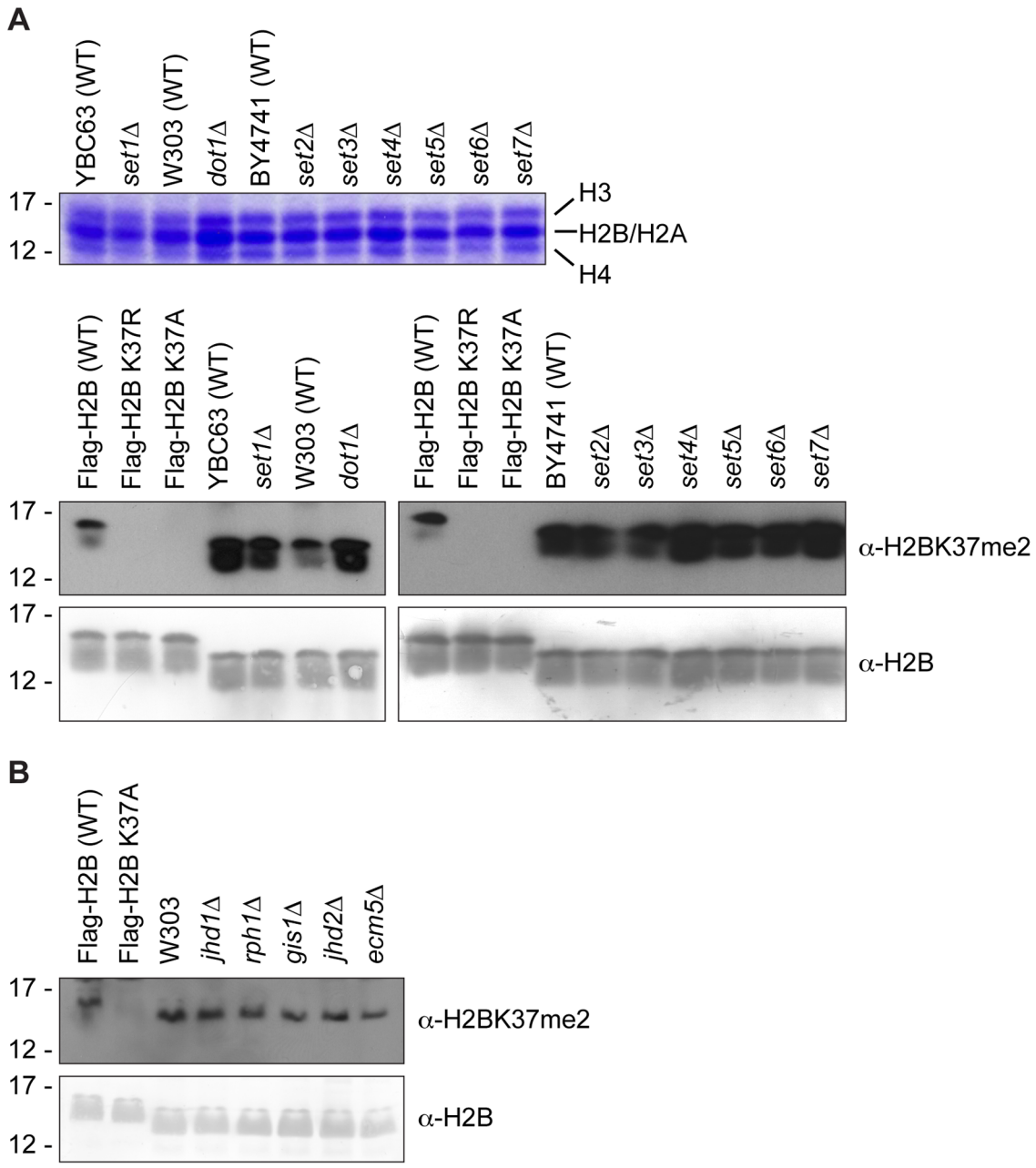

FIGURE 3.3 | Candidate approach by Western blot analysis does not reveal the methyltransferase and demethylase responsible for H2B lysine 37 methylation. (**A**) Following validation of correct deletion of the ORF of interest and replacement with *kanMX* by genomic PCR (*data not shown*), histones were acid-extracted from candidates from the Yeast Knockout Collection (Open Biosystems), and putative histone methyltransferase activity was tested by Western blot analysis using the purified α-H2BK37me2 antibody. A Coomassie-stained gel illustrating a representative purification of histones is shown in upper panel, and representative Western blots results from the candidate screen are shown below. The blots were first probed with the α-H2BK37me2 antibody (upper) and then striped and reprobed with an α-H2B antibody (lower) to demonstrate equal loading. Histones derived from strains harboring wild-type Flag-H2B (YKG001) and Flag-H2B K37R (YKG006) or K37A (YKG007) were loaded on all gels

to demonstrate loss-of-signal upon mutation of lysine 37, thereby serving as a control for antibody specificity. The presence of a Flag-tag on histone H2B results in the slight shift in electrophoretic mobility observed in the control strains, as compared to untagged H2B species in the candidate deletion strains. Deletion of candidate genes did not reveal a putative H2BK37me2 histone methyltransferase by Western blot analysis. (**B**) Histones were acid-extracted from the five JmjC-domain-containing protein deletions in *Saccharomyces cerevisiae*, and putative histone demethylase activity was analyzed by Western blot analysis using the purified α-H2BK37me2 antibody. Shown are Western blot results from the candidate screen, in which the blots were first probed with the α-H2BK37me2 antibody (upper) and then striped and reprobed with an α-H2B antibody (lower) to demonstrate equal loading. Again, histones derived from strains harboring wild-type Flag-H2B (YKG001) and Flag-H2B K37A (YKG007) were used as a control for antibody specificity, and the presence of a Flag-tag on histone H2B results in the slight shift in electrophoretic mobility observed in the control strains, as compared to untagged H2B species in the candidate deletion strains. Deletion of each individual candidate did not result in an enhanced signal, suggesting that none of these candidates function as the histone demethylase for H2BK37me2.

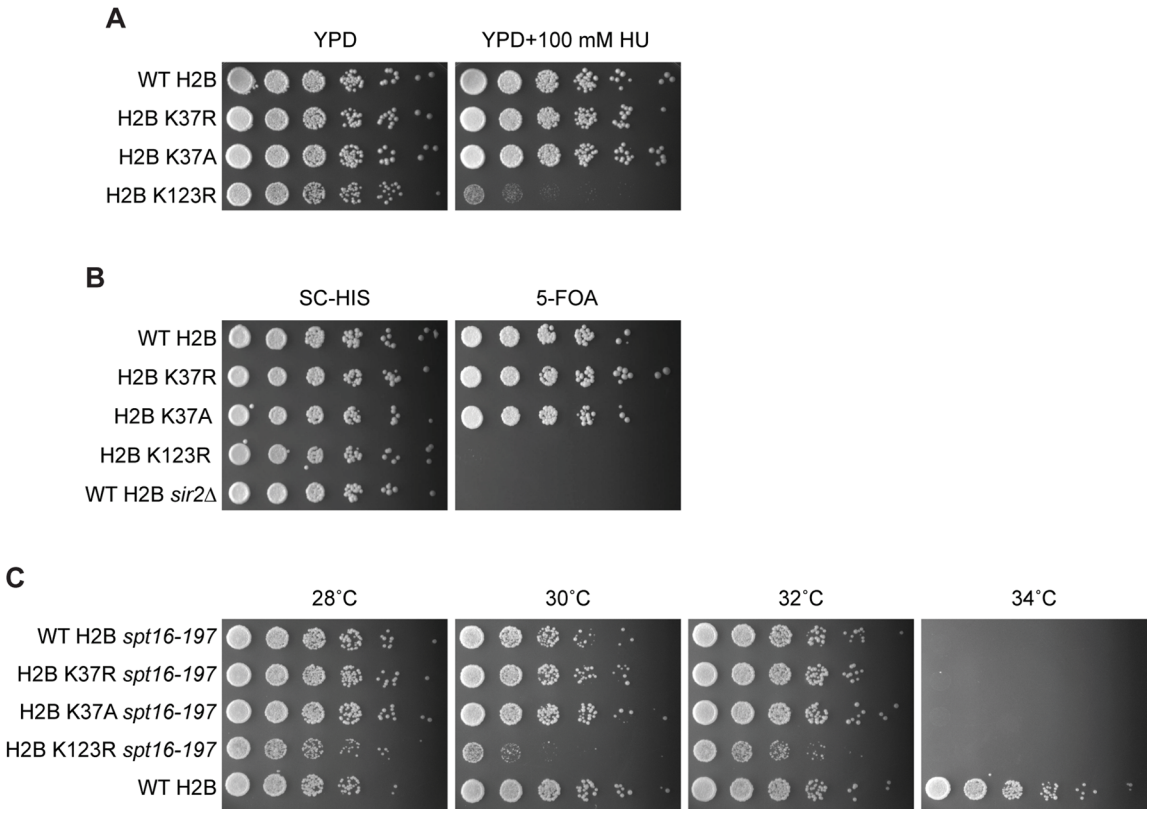

FIGURE 3.4 | Phenotypic analysis of strains harboring H2B K37R/A mutations. (**A**) Phenotypic spotting assays indicate that cells harboring mutations at lysine 37 in histone H2B to arginine (YKG006) or alanine (YKG007) do not show sensitivity to YPD media containing 100 mM hydroxyurea (HU; a DNA damaging agent that leads to replication fork collapse), as is observed in an H2B K123R mutant strain (YKG002) (DAI *et al.* 2010), but rather grow similarly to yeast containing wild-type H2B (YKG001). (**B**) Telomeric silencing assay demonstrates that reporter strains harboring H2B K37R and H2B K37A mutations (YKG028 and YKG029, respectively) exhibit normal silencing like that observed for reporter strains expressing wild-type H2B (YKG027), but not strains that express an H2B K123R mutation (YZS274) or are deleted for *SIR2* (YZS275), which have known defects in telomeric silencing (SUN and ALLIS 2002). Growth on SC-HIS serves as a plating control, as all strains express H2B-containing plasmids carrying a *HIS3* auxotrophic marker. (**C**) Introduction of H2B K37R or K37A mutations (YKG033 and YKG034, respectively) into strains containing a temperature-sensitive allele of *SPT16* (*spt16-197*) does not affect cellular growth at the semi- and non-permissive temperatures (32˚C and 34˚C, respectively), as cells grow at a similar rate to those harboring wild-type H2B (YKG031). Introduction of an H2B K123R mutation (YKG032) exacerbates growth in the *spt16-197* background at the semi-permissive temperature, in agreement with previously published results (FLEMING *et al.* 2008). The isogenic parental strain Y131 expressing wild-type *SPT16* grows phenotypically normal at the non-permissive temperature for the *spt16-197* strain.

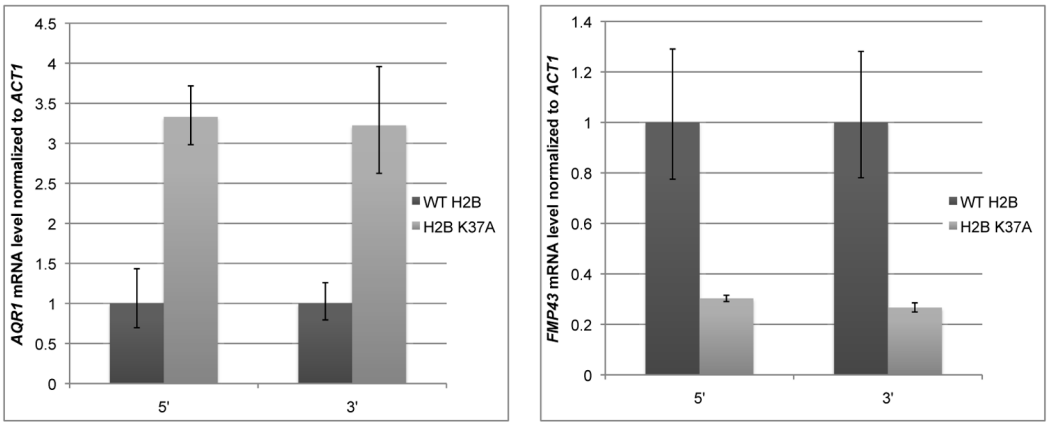

FIGURE 3.5 | RT-qPCR analysis recapitulates microarray results of gene expression changes upon mutation of H2B lysine 37. Yeast cells harboring wild-type H2B (YKG001) or a H2B K37A mutation (YKG007) were grown to mid-log phase, and RNA samples were isolated. The expression of genes identified as up- or downregulated upon mutation of lysine 37 by microarray analysis was verified by RT-quantitative real time PCR analysis (RT-qPCR) using primers designed against the 5' or 3' end of the open reading frame. Representative RT-qPCR analysis is shown for *AQR1* and *FMP43*, which were up- and downregulated, respectively, in yeast cells harboring the H2B K37A mutation relative to wild-type H2B according to microarray analysis. Gene expression was normalized against actin (*ACT1*).

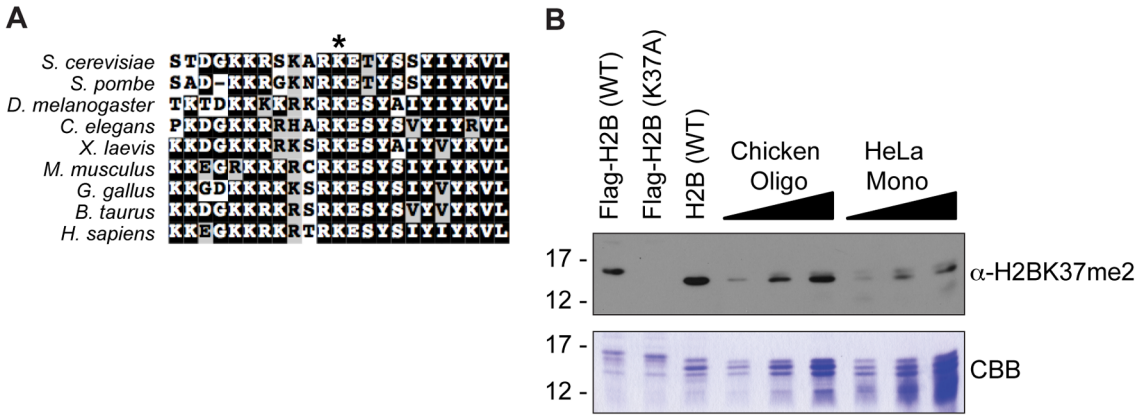

FIGURE 3.6 | Methylation of lysine 37 of histone H2B is conserved. (**A**) Multiple sequence alignment of histone H2B from different species reveals that budding yeast histone H2B lysine 37 is conserved from yeast to humans. Sequence alignment was completed using ClustalX (LARKIN *et al.* 2007). NCBI accession numbers are as follows: *Saccharomyces cerevisiae*: NP_010510.1; *Schizosaccharomyces pombe*: NP_588181.1; *Drosophila melanogaster*: NP_724342.1; *Caenorhabditis elegans*: NP_507031.1; *Xenopus laevis*: NP_001086753.1; *Mus musculus*: NP_783594.1; *Gallus gallus*: CAA40537.1; *Bos taurus*: DAA31692.1; *Homo sapiens*: NP_733759.1. Asterisk (*) denotes position of conserved lysine residue. (**B**) Increasing amounts of oligonucleosomes purified from chicken erythrocyte nuclei and mononucleosomes isolated from HeLa cell nuclei were run against histones extracted from yeast strains harboring wild-type Flag-H2B (YKG001), Flag-H2B K37A (YKG007), and wild-type H2B (untagged) (BY4742), as shown by Coomassie brilliant blue (CBB) staining of histone proteins electrophoresed on 15% SDS-polyacrylamide gels (lower panel). An equivalent loading of histone substrate was used for Western blot analysis using purified α-H2BK37me2 antibody (upper panel). Similar signals are detected for chicken- and human-derived histone substrates to that observed for yeast harboring wild-type H2B (either tagged or untagged), but not yeast H2B with an K37A mutation, thus demonstrating that dimethylation of histone H2B lysine 37 is conserved among species.

Chapter Four

OPERating ON chromatin, a Colorful Language where Context Matters

Histones, the fundamental packaging elements of eukaryotic DNA, are highly decorated with a diverse set of post-translational modifications (PTMs) that are recognized to govern the structure and function of chromatin. Ten years ago, we put forward the histone code hypothesis, which provided a model to explain how single and/or combinatorial PTMs on histones regulate the diverse activities associated with chromatin (e.g. gene transcription). At that time, there was a limited understanding of both the number of PTMs that occur on histones as well as the proteins that place, remove and interpret them. Since the conception of this hypothesis, the field has witnessed an unprecedented advance in our understanding of the enzymes that contribute to the establishment of histone PTMs, as well as the diverse effector proteins that bind them. While debate continues as to whether histone PTMs truly constitute a strict "code", it is becoming clear that PTMs on histone proteins function in elaborate combinations to regulate the many activities associated with chromatin. In this special issue, we celebrate the 50th anniversary of the landmark publication of the *lac* operon with a review that provides a current view of the histone code hypothesis, the lessons we have learned over the last decade, and the technologies that will drive our understanding of histone PTMs forward in the future.

> "Small changes modifying the distribution in time and space of the same structures are sufficient to affect deeply the form, the functioning, and the behavior of the final product…. It is always a matter of using the same elements, of adjusting them, of altering here or there, of arranging various combinations to produce new objects of increasing complexity. It is always a matter of tinkering."
>
> – François Jacob, "Evolution and Tinkering" (*Science* 1977)

The adult animal was in actuality the final product that François Jacob was referring to in this eloquent statement taken from his article "Evolution and Tinkering" (JACOB 1977). Yet, as chromatin biologists, we delight in the applicability of Jacob's quote regarding the plasticity of a

single template to the chromatin landscape. However, François Jacob is not best known for his theories on how patterns of gene expression affect evolution, but rather for his seminal work with Jacques Monod establishing the basis of the *lac* operon. In celebration of the 50th anniversary of François Jacob and Jacques Monod's landmark publication on the *lac* operon (JACOB and MONOD 1961), we are honored to contribute this piece in which we reflect on how several of the scientific themes put forward by Jacob and Monod in their historic work are widely applicable to topics as diverse as chromatin biology and the histone code hypothesis.

In simplistic terms, an operon is a functional genomic unit comprised of a cluster of genes that is controlled by a single regulatory element or promoter (JACOB *et al.* 1960). Complementary genetic and biochemical studies revealed that the basic principle underlying the *lac* operon is that the coordinated expression of the genes necessary to metabolize lactose is under the control of the *lac* repressor protein and activator protein CAP, which negatively and positively control transcription of the *lac* operon, respectively (JACOB and MONOD 1961). From the pioneering studies on the *lac* operon completed by Jacob and Monod, we now know that there are three major types of regulatory DNA sequences that function in the control of gene expression in prokaryotes: (1) promoter sequences to which RNA polymerase binds; (2) operator sequences to which transcriptional repressors bind; and (3) positive control elements to which transcriptional activator proteins bind (STRUHL 1999). While the *lac* operon provides a simple yet elegant mechanism by which gene expression is controlled in prokaryotes, it is unreasonable to think that such a system would adequately provide a means by which efficient regulation of gene expression could occur in eukaryotes, where DNA must be highly compacted to fit within the confines of the nuclear space. The need for differential patterns of gene expression to specify diverse types of tissues from a single genome in multicellular organisms also calls for the existence of additional regulatory mechanisms. For example, cellular identity must be faithfully

maintained through cell divisions for a lifetime, despite differentiation occurring earlier during embryonic development. The plasticity of cellular differentiation and the stability of cellular memory are thought to represent *epigenetic* phenomena wherein inherited changes in phenotype occur independently of changes in the underlying DNA sequence and without the need for *trans*-factors that establish the initial programs of coordinated gene regulation. Hence, while the historic work of Jacob and Monod reveals an elegant mechanism for prokaryotic gene regulation, it is clear that more sophisticated means of gene regulation involving components that do more than engage the DNA template alone are necessary for processes such as cellular memory in multicellular eukaryotes.

Based on many insightful studies on chromosome structure, we know that in eukaryotes, DNA is assembled on a histone scaffold to form chromatin (KORNBERG and LORCH 1999). The nucleosome core particle, or fundamental repeating unit of chromatin, consists of approximately 147 base pairs of DNA wrapped around an octamer containing one tetramer of histones H3 and H4 (two copies each) and two histone H2A-H2B dimers (KORNBERG 1974; KORNBERG and LORCH 1999; LUGER *et al.* 1997; OUDET *et al.* 1975). Nucleosomes are packaged into progressively higher-order structures to ultimately form chromosomes. Chromatin structure largely affects DNA-templated processes such as transcription, thus necessitating that access to DNA be tightly controlled to allow factors that function in such processes to make appropriate contacts with the DNA template itself (KORNBERG and LORCH 1999; WOLFFE and HAYES 1999). Post-translational modifications (PTMs) to the histone proteins themselves can significantly affect the levels of chromatin compaction by creating generally condensed "heterochromatic" or more open "euchromatic" regions, and therefore provide a means by which rapid and localized access to DNA can be accomplished (BERGER 2007; KOUZARIDES 2007). Additionally, other well-studied mechanisms, such as ATP-dependent chromatin remodeling and the exchange of primary

sequence histone variants, introduce meaningful variation into the chromatin polymer, "tinkering" in such a way that one relatively stable genome can give rise to the demands of multicellular development (CLAPIER and CAIRNS 2009; HO and CRABTREE 2010; TALBERT and HENIKOFF 2010).

The "histone code hypothesis": the first ten years. In 2000, we proposed what has commonly come to be referred to as the 'histone code hypothesis', which, in its original form, posits that "multiple histone modifications, acting in a combinatorial or sequential fashion on one or multiple histone tails, specify unique downstream functions" (STRAHL and ALLIS 2000). Parallels to François Jacob's quote from "Evolution and Tinkering" are readily apparent. The same fixed set of amino acids that make up the histone proteins have the potential of being post-translationally modified within the chromatin template, where distinct spatiotemporal patterns of modifications ultimately shape functional outcome. One of the more striking phenomena predicted by such a code is that subtle variations to the same template can result in vastly different outcomes, especially in the context of regulation of gene expression.

At the time that we proposed the histone code hypothesis, we had a limited understanding of the true breadth of the number and type of PTMs that exist on histone residues either on the unstructured N-terminal tails that protrude from the nucleosomal surface or within the structured globular domains. Acetylation and phosphorylation were the best-characterized modifications at that time, with multiple sites and several of the enzymes responsible for their placement and removal having been identified. However, investigations on the dynamics of histone methylation were in their infancy. Only a handful of sites modified by methylation were known, and the function of histone methylation was largely unclear, primarily because the enzyme systems responsible for the steady-state balance of methyl marks (histone

methyltransferases and demethylases) were not yet identified and the intricacies associated with a modification that could exist in multiple states (mono-, di-, or trimethyl) complicated studies. Insight into other modifications was even more rudimentary. Today, we know that a number of PTMs exist, including acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP-ribosylation, proline isomerization, citrullination, butyrylation, propionylation, and glycosylation (TABLE 4.1) (CHEN *et al.* 2007; KOUZARIDES 2007; SAKABE *et al.* 2010). Numerous studies using both biochemical and genetic approaches have revealed many of the enzymes that are responsible for placement or removal of these modifications on specific amino acid residues on histones as well as non-histone proteins. While the functional significance of some of these modifications remains to be determined, the collective field of chromatin biologists has made great strides toward identifying the biological consequence of others. For example, modifications can disturb contacts between histones in contiguous nucleosomes or histones with DNA, resulting in alteration of higher-order chromatin structure. Specifically, acetylation of lysine residues on histone tails neutralizes the basic charge of the residue on which it occurs, thereby disrupting histone contacts with other histones and/or DNA and in turn chromatin compaction (WOLFFE and HAYES 1999). While it had been known that histone modifications such as methylation did not disrupt nucleosomal contacts by altering the charge of the modified residue, we now know that specialized domains within effector proteins facilitate recognition and binding to methyl marks in a defined state on specific residues to mediate downstream effects. Domains characterized thus far as being able to bind to methylated residues include chromodomains, tudor domains, PHD fingers, MBT domains, Ankyrin repeats, PWWP domains, HEAT domains and WD40 repeats (TABLE 4.1) (COLLINS *et al.* 2008; LIU *et al.* 2010; TAVERNA *et al.* 2007a; VEZZOLI *et al.* 2010; WANG *et al.* 2009). Other domains that recognize and bind to specifically modified histone forms have also been characterized. For instance, where

bromodomains can bind to acetylated lysine residues, 14-3-3, BRCT, and BIR domains can bind to phosphorylated threonine and serine residues (TABLE 4.1) (KELLY *et al.* 2010; TAVERNA *et al.* 2007a).

The chromatin-modifying enzymes that facilitate alterations to the chromatin landscape by placing, removing, or interpreting modifications to establish variable states have been more recently come to be generally referred to as writers, erasers, and readers, respectively, of the histone code (FIGURE 4.1). Returning to the idea of tinkering with chromatin, we are now in a position to appreciate the true potential of a "toolkit" (LIM and PAWSON 2010) of writers, erasers, and readers of the histone code in the establishment of proper spatiotemporal patterns of modifications necessary for cellular identity and function. At defined points, writers place marks on defined histone residues, which are in turn interpreted by readers harboring specialized domains that facilitate recognition and binding to the specific mark of interest to drive the progression of a specific biological phenomenon. At a time when such signaling needs to be terminated, erasers are recruited to their defined target(s) to remove the mark, thereby ending the associated functional outcome of the previously defined reader. Admittedly, the situation is made vastly more complicated by the fact that particular amino acid residues can house more than one type of modification (this is largely true for lysine residues, which can be methylated, acetylated, ubiquitylated, or sumoylated), and that some enzymes can write, erase, or read more than one modification. Moreover, one mark can often recruit multiple effector proteins (RUTHENBURG *et al.* 2007a; SIMS and REINBERG 2006). Such complications, however, support the general notion of tinkering with combinatorial pattern of PTMs to control proper recruitment of effector proteins or complexes in which they reside.

We appreciate that the "histone code hypothesis", as originally articulated by us in 2000, evolved into an influential review on the function(s) of covalent histone modifications.

We acknowledge that this hypothesis, and extensions of it, rest heavily on the foundation of many biologists and biochemists who were dedicated to the general view that chromatin was going to be much more than a passive way to package the genome. However, because of the rapid pace of research in chromatin biology and the complexity associated with chromatin modifications such as those mentioned above, we must continually refine how we define the histone code. In fact, the mere existence of a code in the first place has been a point of contention (SMITH and SHILATIFARD 2010). Beyond discussions in the field as to whether a strict histone code truly exists, there is also debate over whether it is most appropriate to define it as "code" in which definite combinations lead to an absolute outcome (as exemplified by the genetic code). Some see it more in terms of a "language", where complex combinatorial patterns of modifications form words that ultimately give rise to a vocabulary of histone crosstalk (LEE *et al.* 2010). Others yet prefer to think of it more specifically in terms of an "epigenetic code" that is defined by combinations of histone PTMs which are predictive of, and necessary for, expression patterns of differentiation and developmental-specific genes (TURNER 2007). On the other hand, it has been argued that histone modifications are not truly "epigenetic", as the nature of their heritability (a requisite condition to be defined in the classical sense of epigenetic) is questionable (PTASHNE 2007), thereby disputing the appropriateness of an "epigenetic code". At some point, the question of how exactly to define the histone code becomes somewhat rhetorical, as at their very essence, all definitions ultimately seem to convey the same fundamental principle that histone PTMs act in concert to elicit downstream biological outcomes. Here we reflect on the many forms the 'histone code hypothesis' has come to take since the time of its inception a decade ago, and suggest that individual definitions may not be mutually exclusive of one another, but are perhaps instead complementary.

Transcribing the "histone code": chicken or egg? Although applicable to a diverse set of cellular processes, the histone code is most commonly considered in the context of transcription regulation. Within this realm, there has been much debate as to whether a putative code formed by combinatorial modifications can formally regulate transcription itself or rather, if patterns of modifications are generally associated with a particular transcriptional state. On one side is the argument that genes are not necessarily regulated by chromatin modifications *per se*, but rather are regulated by specific DNA-binding proteins that recruit activating and repressive complexes to genomic loci to modulate transcriptional activity. According to this line of reasoning, the histone-modifying machinery is recruited by canonical transcriptional activators and repressors (as would be defined in the classical sense by Jacob and Monod), and the placement of modifications by these enzymes then contributes to transcription by creating a more or less permissive chromatin environment for the further recruitment of downstream factors that regulate transcription. In support of this idea, it has long been known that histone acetylation is associated with active genes (ALLFREY *et al.* 1964), and functions to facilitate the disruption of higher-order chromatin structure prior to gene activation (WOLFFE and HAYES 1999). Thus, one would argue that it is the action of the activators that directly determine transcriptional output, and that the targeting of acetylation to histones via activators that bind to specific upstream activating sequences functions to make the chromatin environment more permissive for transcriptional regulation (STRUHL 1998; WOLFFE and HAYES 1999). In an analogous fashion, binding of transcriptional repressors to upstream repressive sequences facilitates recruitment of histone deacetylase (HDAC) enzymes to chromatin, which in turn remove acetyl marks to contribute to transcriptional repression through chromatin compaction (KATAN-KHAYKOVICH and STRUHL 2002; PAZIN and KADONAGA 1997; STRUHL 1998). By this argument,

modifications are thereby associated with gene activation and/or silencing (much like RNA polymerase II (RNAPII) is associated with active genes), but do not formally regulate transcription itself. An extension of this position would be that chromatin modifications themselves do not intrinsically regulate gene expression alone because an element of targeting or recruitment is necessary (in other words, how do the enzymes know where to place the marks?). Once set, PTMs putatively function in transcriptional regulation by promoting or excluding the binding of elements that directly function in regulation (i.e., activators and/or repressors) to such regions.

One counterargument that could be made in response to the aforementioned view of the histone code whereby chromatin-modifying machinery is recruited by transcriptional activators or repressors would be that histone modifications are a prerequisite for recruitment of certain elements of the transcriptional machinery. For example, two TBP-associated factor (TAF) subunits of the transcription factor complex TFIID have been shown to bind directly to histone PTMs, which would suggest that modification of histone proteins is necessary for binding of the transcriptional machinery. The double bromodomain of Taf1, the largest subunit of TFIID, binds preferentially to diacetylated histone H4 (JACOBSON *et al.* 2000). Taf3 harbors a PHD finger that is selective for binding to trimethylated lysine 4 of histone H3 (H3K4me3), and loss of this chromatin mark results in reduced TFIID association with and transcriptional activity from certain promoters (VERMEULEN *et al.* 2007), providing support for the role of histone PTMs as a requisite component in the recruitment of transcription factors.

Despite the seemingly opposite lines of reasoning regarding the role of histone modifications in transcriptional regulation, we maintain that the nature of the histone code may not necessarily be as clear-cut as histone PTMs functioning solely as a consequence of or prerequisite for recruitment of the canonical transcriptional machinery. It is likely that both

arguments hold true in their own rights with respect to transcription (as well as other DNA-templated processes), and that possibly no absolute rule exists favoring either position over the other, thereby necessitating examination of such codes on an individual basis. It is, therefore, perhaps more judicious to focus our discussion on the histone code in the context of how it more generally contributes to the physical organization of eukaryotic genomes. Three major principles have developed during the evolution of the histone code hypothesis over the past ten years: (1) interactions between histone modifications are not limited to a single tail; (2) a single mark can recruit more than one protein; and (3) proteins acting alone or in the context of a macromolecular complex can contain multiple domains to facilitate binding to chromatin (FIGURE 4.2). At the time when the histone code hypothesis was put forward, we had a relatively limited scope of the existent histone PTMs, the combinations in which they exist, and how they affect downstream functionality. That marks located in close proximity to one another often times exhibit functional interplay was demonstrated by examples such as phosphorylation of serine 10 of histone H3 (H3S10ph) reducing the affinity of the chromodomain of heterochromatin protein 1 (HP1) for di- and trimethylated lysine 9 of histone H3 (FISCHLE *et al.* 2005). At present, the chromatin field continually refines our understanding of how individual modifications affect placement of another, especially in the context of how modifications on one histone tail affects placement of marks and recruitment of effector proteins on other tails. A clear example of this idea is provided by studies that have demonstrated that a signal cascade in which 14-3-3 is recruited to the enhancer of FOSL1 by binding to H3S10ph and itself subsequently recruits the histone acetyltransferase MOF, which acetylates histone H4 on lysine 16 (H4K16ac) to create a doubly-modified H3S10ph/H4K16ac nucleosome (ZIPPO *et al.* 2009). These PTMs then function as a platform for the bromodomain-containing protein BRD4 (which in turn recruits the positive transcription factor b (P-TEFb)) to activate transcription elongation, thus providing an elegant

example of the numerous intricacies associated with interactions between multiple histone PTMs across multiple tails (ZIPPO *et al.* 2009). It is becoming increasingly clear that modifications that work together to form a putative code are not limited to a single histone tail, but are likely to span multiple tails within one nucleosome, between adjacent nucleosomes, or between non-adjacent nucleosomes that are physically located in close proximity to one another due to higher-order chromatin structure. Examples of histone crosstalk continue to evolve, and many more are likely to surface from future work, thereby shedding light on the growing complexity associated with the many permutations of a histone code.

As alluded to above, modifications of histone residues in defined states can serve as platforms for binding of more than one effector protein. For example, multiple proteins (including JMJD2A, Rag2, BPTF, Ing2 and Taf3) have all been demonstrated to bind to H3K4me3 (HUANG *et al.* 2006; LI *et al.* 2006; MATTHEWS *et al.* 2007; PENA *et al.* 2006; SHI *et al.* 2006; VERMEULEN *et al.* 2007; WYSOCKA *et al.* 2006). Such promiscuity by a defined mark for multiple readers indicates that secondary levels of specification must exist. One possible explanation is that one protein can harbor multiple domains that cooperatively facilitate recognition and binding to chromatin (RUTHENBURG *et al.* 2007b). For example, Tsai *et al* have recently shown that the tandem PHD finger and bromodomain of the protein TRIM24, a co-activator of oestrogen receptor α (ERα), bind combinatorially to unmodified H3K4 and acetylated H3K23 to facilitate chromatin recognition and contribute to ERα-mediated transcription activation (TSAI *et al.* 2010). Alternatively, more than one histone PTM (or the recognition of unmodified histone residues with modified ones) can function in concert to form a recognition code for a single protein with multiple chromatin-binding domains or multiple proteins within a chromatin-associated complex (OLIVER and DENU 2010). One example of this type of nucleosomal interaction is provided by the Rpd3S histone deacetylase complex, which stably interacts with

H3K36 methylated nucleosomes via recognition of H3K36 methylation by the chromodomain-containing subunit Eaf3 and H3 recognition by a PHD finger within in Rco1 subunit of this same complex (LI *et al.* 2007b). Thus, it is becoming increasingly clear that the one mark:one reader (or writer or eraser in certain instances) ratio does not allow for generation of enough physically distinct relationships to sufficiently impart the degree of information necessary to mediate diverse outcomes, supporting the existence of numerous levels of complexity built into the histone code. Such complexity would allow multiple ways to tinker with the same chromatin landscape to promote diverse biological outcomes.

Tinkering the "histone code hypothesis" in years to come. The key question that remains then, is perhaps not one of mulling over how to best define the histone code, but rather, what form will the histone code hypothesis take over the years to come? Given the rapidity of chromatin-based research and the prominent role of chromatin in numerous DNA-based processes, research in the years to come is likely to continue along the same fruitful path of discovery that it has witnessed in the past ten years, demonstrating additional levels of complexity by which intrinsic cellular machines tinker with the chromatin template. While studies aimed at identifying additional writers and erasers of the histone code as well as novel marks remain ever important, investigations elucidating how chromatin marks act in concert to recruit readers are of equal significance. Technological advancements and new methodologies have significantly progressed our efforts in both areas of study, and are expected to continue to do so well into the future (VOIGT and REINBERG 2010; YOUNG *et al.* 2010). Histone PTMs have traditionally been identified by metabolic labeling, microsequencing, the generation of immunological reagents, and more recently, mass spectrometry (MS) (GARCIA *et al.* 2007c). Advancements in MS technology include the recently developed top-down methodology, which analyzes intact

proteins samples (as opposed to the more canonical bottom-up approach where proteins are fragmented prior to analysis). Because proteins are analyzed at the whole-molecule level, top-down MS allows for identification of combinatorial patterns of modifications that exist within one histone protein (SIUTI and KELLEHER 2007). For example, top-down MS analysis has now been completed on all three human histone H3 variants (H3.1, H3.2, and H3.3), revealing complex patterns of modified H3 forms (GARCIA *et al.* 2007b; THOMAS *et al.* 2006). Additionally, analysis of asynchronously grown HeLa cells treated with the HDAC inhibitor sodium butyrate has revealed a surprising and complex number of combinatorially-modified species of histone H3.2 and H4 (GARCIA *et al.* 2007b; YOUNG *et al.* 2009). Though still in its infancy, studies such as these have made it readily apparent that top-down MS analysis will be a highly utilized technique in future studies to decipher how combinatorial patterns of histone modifications contribute to the regulation of diverse biological processes (YOUNG *et al.* 2010).

Identification of the histone marks themselves and the combinatorial patterns in which they exist is not enough to understand functional consequences of their placement. The availability of modification-specific antibodies has allowed for immunoprecipitation of DNA fragments associated with a particular mark by chromatin immunoprecipitation (ChIP). It should be formally noted that one major limitation to be kept in mind when designing and/or interpreting experiments involving ChIP is the requirement for a high-quality antibody that can specifically recognize a defined modification state (e.g., a dimethylated but not trimethylated lysine residue). Moreover, as neighboring modifications may unpredictably impact antibody specificity, it is becoming increasingly clear that rigorous validation of antibody quality is essential for any ChIP-based analysis to effectively provide insight into the location of a particular modification in a defined state (BOCK *et al.* 2011; EGELHOFER *et al.* 2011; FUCHS *et al.* 2011).

Early approaches for studying chromatin-modifications on a genome-wide level utilized ChIP combined with DNA microarray analysis (ChIP-chip). More recently, ChIP coupled with next-generation sequencing technology (ChIP-seq) has provided considerable insight into the function of histone PTMs, allowing for the identification of genome-wide patterns of specific modifications as well as transcription factors and the machinery responsible for modifying the chromatin landscape under defined biological conditions (SCHONES and ZHAO 2008). Early ChIP-seq analyses mapping histone modifications in $CD4^+$ T cells or mouse embryonic stem (mES) cells revealed a number of findings (BARSKI *et al.* 2007; MIKKELSEN *et al.* 2007; WANG *et al.* 2008). For instance, a comparative ChIP-seq analysis of mES, neural progenitor and embryonic fibroblasts confirmed the existence of bivalent domains characterized by the co-localization of H3K4 and H3K27 trimethylation that function in cellular plasticity and commitment to a defined lineage (BERNSTEIN *et al.* 2006; MIKKELSEN *et al.* 2007). However, how widespread bivalent domains occur in various developmental contexts remains unclear and is under active investigation. Genome-wide association studies derived from ChIP-seq analyses completed to date have led many to see the histone code less in terms of as sets of definite combinations that produce an absolute outcome, but rather, more as patterns of modifications that when in combination tend to favor a specified outcome. In that vein, the ramifications of the histone code are correlative rather than causal in that combinatorial patterns provide a bias for a specific outcome rather than serve as an absolute mark of one. ChIP-seq analyses has, for example, revealed that in general, higher levels of H3K9me1 and H2BK5me1 in the 5' end, H3K27me1 distributed throughout, and H3K36me3 in the 3' end of a transcribed region mark actively transcribed regions (BARSKI *et al.* 2007). Furthermore, another study found that there is a combinatorial pattern of methylation and acetylation events on histone tails that are co-associated with each other on a significant fraction of genes within the human genome (WANG *et al.* 2008). Such studies provide important

insight, in that they demonstrate that actively transcribed regions of the genome, as well as functional elements in general, bear distinct histone PTM signatures (ZHOU *et al.* 2011). Additional studies will surely expand upon whether the histone code is characterized by a fixed set of combinatorial patterns that establish defined chromatin states (also referred to frequently as chromatin 'signatures') or rather, if certain combinations tend to tip the balance in favor of a certain state. For example, recent work published by the modENCODE Consortium has provided great insight into the genome-wide chromatin organization in the model organisms *Caenorhabditis elegans* and *Drosophila melanogaster*, which together have vastly advanced our understanding how various histone PTMs are associated with genomic regulatory elements in defined developmental states (GERSTEIN *et al.* 2010; KHARCHENKO *et al.* 2010; ROY *et al.* 2010). Newcomers to this field should refer to these studies to become oriented not only to some of the principal PTMs that mark chromatin domains, but also to the staggering complexities underlying the combinatorial nature with which gene bodies and regulatory elements are specified and defined in a chromatin context. Indeed the language is colorful and must be interpreted in context, especially in a developmental setting.

Novel methods are also being developed to characterize combinatorial patterns that facilitate binding of effector proteins as well as identify novel proteins that can bind to modified histone tails. Use of combinatorial peptide libraries based on the N-terminal histone tails has become a widely used practice to identify how the presence of additional marks enhances or weakens the affinity of an effector protein for its target binding module. Peptide libraries have been synthesized as various types of platforms, including resin-bound PTM-containing histone tail libraries and custom peptide microarrays (BOCK *et al.* 2011; BUA *et al.* 2009; FUCHS *et al.* 2011; GARSKE *et al.* 2010). Such platforms have recently begun to be used to identify synergistic and antagonistic combinations of histone modifications that ultimately affect the binding of

effectors. For example, the H3K9me2 demethylase PHF8 binds to H3K4me3/2, and hybridization of a recombinant GST-PHD(PHF8) fusion protein to a synthetic peptide array containing combinatorial modifications patterns revealed that binding to H3K4me3/2 was also achieved when peptides were acetylated at the H3K9/K14 positions (KLEINE-KOHLBRECHER *et al.* 2010). While peptide libraries are advantageous at looking at how effector proteins respond to various combinatorial patterns of modifications, alternative functional technologies are being employed to screen for proteins that bind to a particular modification in an unbiased manner. Recently, a histone peptide pulldown approach paired with SILAC proteomics technology was used to define a large-scale methyl lysine interactome (VERMEULEN *et al.* 2010). Extending this concept further, designer synthetic nucleosomes in which nucleosomes are reconstituted using recombinant histones harboring specific modifications states have allowed for unbiased identification of cellular proteins that bind to a specific state on a nucleosomal substrate in a technique called SNAP (SILAC nucleosome affinity purification) (BARTKE *et al.* 2010). Because the DNA sequence and modifications of interest are user-defined, one could theoretically begin to make oligonucleosomes in which crosstalk both within and across nucleosomes can be addressed. This latter technology holds great potential for future studies in which peptides harboring several modifications are fused to multiple histone tails via native chemical ligation to reconstitute multiply-modified nucleosomes to give a more complete picture of how combinatorial patterns affect binding by chromatin readers in the more-physiologically relevant nucleosomal context.

Strict code versus rich language: exciting either way. At the time of inception, it is always difficult to discern how influential a hypothesis will truly be. We have been privileged to witness that François Jacob and Jacques Monod's report on the *lac* operon in the *Journal of Molecular*

Biology in 1961 has revolutionized our understanding of the basic mechanisms underlying gene regulation. We are also beginning to understand the richness of the histone code hypothesis. When we posited this hypothesis, now ten years ago, we had what in retrospect would be described as a quite limited scope of histone post-translational modifications. One decade later, we stand in awe at how the chromatin field, and scientific research community at large, has come together to expand this code to a scope beyond what was imaginable at the time of its conception. For example, never in our wildest dreams had we envisioned a Keystone meeting being dedicated to the singular topic of the 'Histone Code': Fact or Fiction (January 10-15, 2011 in Midway, Utah). However, it is with a sense of realism that we recognize that many obstacles remain to be overcome before we can officially declare that this code has been deciphered to its fullest potential. For example, it will be difficult to discern when saturation has been reached and all modifications have been identified, a reality complicated by the fact that organismal differences exist within the chromatin landscape. The staggering complexity of this proposed 'epigenetic code' promises to keep many talented scientists busy for the next decade with many more welcomed surprises along the way. Moreover, we are coming to realize that such a code may not pertain specifically to histones, but could potentially be extended to proteins in general. That proteins are modified post-translationally is by no means a novel concept, but the idea that modifications working in concert are predictive of defined downstream biological events has received more thought recently. The tumor suppressor p53 is highly regarded as the model for the existence of a more general protein code, as this protein is subject to a number of PTMs, including methylation, acetylation, phosphorylation, and ubiquitylation (SIMS and REINBERG 2008). The observation that modifications, such as acetylation, correlate with stabilization and activation of p53 (BODE and DONG 2004) in concert with the idea that one modification can enhance or preclude the placement of another supports a more general

mechanism in which modifications are tightly linked to p53 function in an analogous fashion as to how histone PTMs work together to form a functional code. Also worthy of noting is that many of the enzymes responsible for writing, erasing, and reading histone methylation and acetylation on histone proteins are also responsible for modifying the C-terminus of p53 and certainly other non-histone proteins (GLOZAK *et al.* 2005; HUANG and BERGER 2008), echoing Jacob's visionary sentiment that the same elements are often used to create new products of increasing complexity.

Our piece in 2000 was framed as a hypothesis with the hope that it would stimulate discussion and lead to subsequent tests of its central tenets. Much of this has happened, and we look forward to much more along these lines. While contention over use of the word "code" may eventually lead to an alternative designation in future years, we are confident that debates over diction will not hinder the elegant work that the chromatin community has collectively produced at a remarkable pace. We close with a prediction -- we will indeed witness a period of further enlightenment with regard to how cellular enzymes tinker with both histone and non-histone proteins alike to create increasingly complex patterns of regulatory mechanisms in the years to come. Coloring the chromatin code with even more shades will be part of the fun (FILION *et al.* 2010; SCHUBELER 2010).

Acknowledgements

We thank the many researchers whose studies have help to expand our understanding of both the *lac* operon and the histone code, and apologize to those whose work could not be cited here due to space constraints. We also thank Nara Lee, Scott Rothbart, and the members of the Allis lab for insightful conversations and comments on this review, and Nara Lee and Stephen Fuchs for assistance with the illustrations contained in this piece.

TABLE 4.1 | Histone modification types and the interacting domains that "read" them

Modification types	Residue(s) modified	Reader domain(s)
Unmodified lysine	Lysine	PHD
Acetylation	Lysine	Bromo
Methylation	Lysine/Arginine	Ankyrin, Chromo, HEAT, MBT, PHD, Tudor, PWWP, WD40
Phosphorylation	Serine/Threonine	14-3-3, BIR, BRCT
Ubiquitylation	Lysine	?
Sumoylation	Lysine	?
ADP-ribosylation	Lysine	?
Citrullination	Arginine	?
Butyrylation	Lysine	?
Propionylation	Lysine	?
Glycosylation	Serine/Threonine	?

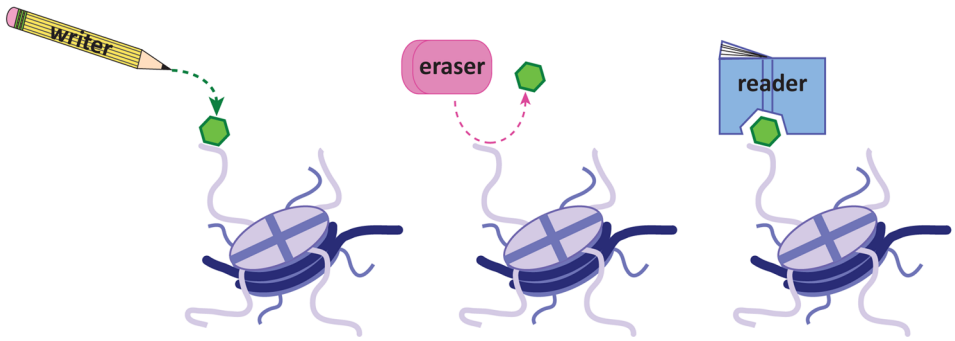

FIGURE 4.1 | Toolkit for modifying the chromatin template. Schematic illustrating the concept that writers place post-translational modifications on histone proteins (left), erasers remove such modifications from histone proteins (middle), and readers function to interpret these covalent modifications (right) to mediate diverse downstream processes.

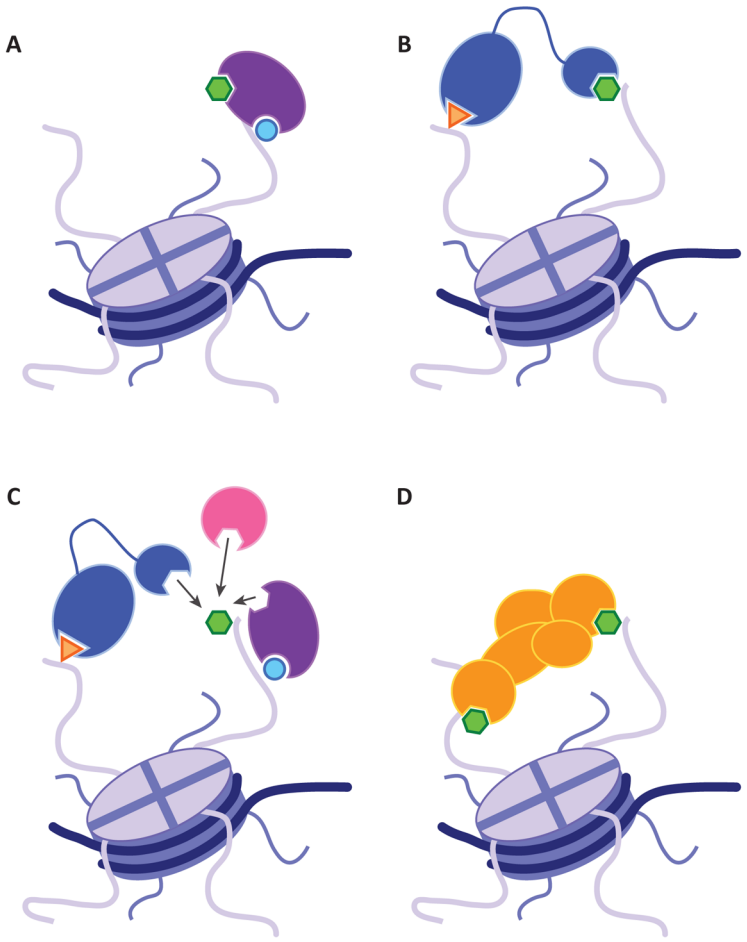

FIGURE 4.2 | Mechanisms of histone-recognition modules binding their target modification. Binding of specialized domains to histone post-translational modifications can occur in *cis*, where contact is made to a series of modifications on the same histone tail (**A**), or in *trans*, where contacts are made to distinct modifications across histone tails (**B**). Often, a single modification can serve as a docking site for more than one protein, in which secondary signals (e.g. other PTMs) may serve to dictate which protein is recruited to the specific mark (**C**). Proteins acting alone (**A-B**), or in the context of a macromolecular complex (**D**) can harbor multiple domains capable of facilitating chromatin recognition and binding. For clarity, no attempts have been made to depict histone recogntion between nucleosomes in either the same or distinct polynucleosome fibers, but these modes of binding recognition are also likely (reviewed in (RUTHENBURG *et al.* 2007b)).

Chapter Five

Perspectives

The unifying theme of the work contained within this dissertation is that of dynamic regulation of histone lysine methylation in the budding yeast *Saccharomyces cerevisiae*. Prior to the commencement of these studies, it had been well established that lysines residues 4, 36, and 79 of histone H3 were methylated in budding yeast by the enzymes Set1, Set2, and Dot1, respectively (MILLAR and GRUNSTEIN 2006). However, two prominent questions remained to be answered. The first was whether histone lysine methylation could be actively removed in *S. cerevisiae*. LSD1 had been shown to be an active histone demethylase in higher eukaryotes (SHI *et al.* 2004), but budding yeast lack a LSD1 homologue (KLOSE and ZHANG 2007). Thus, it was of general interest to determine whether methylation is indeed a dynamic mark in this model organism and if another class of enzymes existed that is capable of removing methyl marks. The second question was whether lysine residues other than 4, 36, and 79 on histone H3 could be methylated. While these three residues are by far the best-characterized sites of lysine methylation in budding yeast, it remained possible that other yet-to-be-identified sites could additionally be modified post-translationally by the addition methyl groups.

In what was an exciting time in the field of chromatin biology, JmjC-domain-containing proteins were identified as histone lysine demethylases, thus supporting the active removal of methyl marks from modified residues (KLOSE *et al.* 2006a; TSUKADA *et al.* 2006). The budding yeast protein Jhd1 was the first JmjC-domain-containing protein demonstrated to possess histone demethylase activity and shows specificity for mono- and dimethylated H3K36 (TSUKADA *et al.* 2006). Among the works described within these chapters is evidence that the yeast JmjC-domain-containing proteins Rph1 and Jhd2 also function as histone lysine demethylases with specificity for di- and trimethylated H3K36 and H3K4, respectively (KLOSE *et al.* 2007a; LIANG *et al.* 2007) (FIGURE 5.1).

In support of the notion that lysine resides other than 4, 36, and 79 on histone H3 can be methylated, work contained herein identifies lysine 37 of histone H2B as a novel site of histone methylation in budding yeast, and that this modification exists in the dimethyl state (GARDNER *et al.* 2011b) (FIGURE 5.1). Concurrent with the studies presented here, lysine 111 of histone H2B was also shown to be a site of histone methylation in *S. cerevisiae* (KYRISS *et al.* 2010). Comprehensive in-depth histone modification MS analysis has suggested that methylation also occurs on: H2BK22, H3K18, H3K23, H3K37, and H4K31 (GARCIA *et al.* 2007a; UNNIKRISHNAN *et al.* 2010; ZHANG *et al.* 2009) (FIGURE 5.1). Together these data corroborate that lysine methylation is not limited to the three previously characterized sites, but rather that additional sites of methylation likely await discovery.

Many questions regarding active demethylation of histones and identification and characterization of novel sites of histone lysine methylation remain. Topics to be explored in future studies regarding each area are discussed below.

Identification and Characterization of Histone Lysine Demethylases in *Saccharomyces cerevisiae* | The recent discovery of a number of histone lysine demethylases has demonstrated the dynamic nature of histone methylation. Three members from the JmjC-domain-containing family of proteins that have been demonstrated to function as active histone lysine demethylases in the budding yeast *Saccharomyces cerevisiae*: Jhd1, Rph1, and Jhd2 (KLOSE *et al.* 2007a; LIANG *et al.* 2007; TSUKADA *et al.* 2006). Beyond identification of enzymes capable of reversing histone methylation, in broad terms, the primary questions remaining point toward elucidating the mechanism by which the demethylases are regulated and targeted, as well as the functional consequence of the removal of histone methyl marks. Jhd1 is a mono- and dimethyl H3K36 demethylase, whose characterization to date has largely been discussed

elsewhere (FANG *et al.* 2007; KIM and BURATOWSKI 2007; TSUKADA *et al.* 2006). In addition to the work contained within this dissertation identifying Rph1 as a demethylase with specificity for di- and trimethyl lysine 36 of histone H3 (KLOSE *et al.* 2007a), preliminary studies were completed to explore the biological consequence of H3K36 demethylation during transcriptional elongation. Given the established role of H3K36 methylation in transcription elongation in promoting recruitment of the Rpd3S histone deacetylase complex (CARROZZA *et al.* 2005; JOSHI and STRUHL 2005; KEOGH *et al.* 2005), I sought to identify a role for removal of methyl marks from this residue during this process. Deletion of the H3K36 demethylases alone leads to no obvious cellular phenotypes or impaired cellular growth (FIGURE 5.2, panel A and see (FANG *et al.* 2007; KLOSE *et al.* 2007a)). However, recent studies have shows that the overexpression of Jhd1 or Rph1 can remove H3K36 methylation and promote transcription elongation (KIM and BURATOWSKI 2007). The lack of an overt cellular phenotype in the individual deletion strains alone thus prompted more specific analysis of the role of these enzymes in the context of H3K36 function during transcription elongation.

Previous studies revealed that the loss of H3K36me by either deletion of *SET2* or mutation of lysine 36 to a non-modifiable amino acid residue can bypass anomalous growth phenotypes of strains harboring mutations in factors necessary for proper transcription elongation, such as members of the FACT chaperone and the Bur1/2 kinase complexes. For example, the slow-growth phenotype of strains harboring a temperature sensitive mutant allele of *SPT16* (*spt16-11*), a member of the FACT histone chaperone complex that promotes transcription elongation (BELOTSERKOVSKAYA *et al.* 2003; BISWAS *et al.* 2006; FORMOSA 2003; FORMOSA *et al.* 2001; MASON and STRUHL 2003; SAUNDERS *et al.* 2003; SCHWABISH and STRUHL 2004), grown at the non-permissive temperature is suppressed by a loss of *SET2* or introduction of a histone H3 mutant containing an lysine-to-arginine substitution mutation at amino acid residue

36 (K36R) (FIGURE 5.2, panel A, and (BISWAS *et al.* 2006)). Likewise, deletion of *SET2* can improve the weak growth phenotype of strains deleted of *BUR2*, which encodes for a kinase linked to transcriptional regulation through its activity for phosphorylation of the CTD of the largest subunit of RNAPII (FIGURE 5.2, panel B, and (KEOGH *et al.* 2005; YAO *et al.* 2000)). Preliminary deletion analyses have revealed that loss the H3K36me3/2 demethylase Rph1 shows phenotypes supportive of a role in promoting transcription elongation. Genes encoding the knows H3K36 demethylases Jhd1 and Rph1 (as well as the putative H3K36 demethylase Gis1) were individually deleted in wild-type or *spt16-11* strains, and strains were assayed for growth at either the permissive (30°C) or semi-permissive (34°C) temperature. As shown in FIGURE 5.2 (panel A), deletion of the individual H3K36 demethylase genes in either the wild-type or *spt16-11* strains does not affect cellular growth at the permissive temperature. However, deletion of *RPH1* in the *spt16-11* strain shows in a synthetic effect at the semi-permissive temperature (FIGURE 5.2, panel A). Similarly, where a *bur2Δ* strain is viable, but slow growing, introduction of a secondary mutation in which *RPH1* is deleted results in a loss of cell viability (FIGURE 5.2, panel B). Collectively, these results suggest that Rph1 could function to promote transcription elongation by operating in pathways that overlap with the specific functions of the FACT and BUR complexes. It is possible that precise modulation of the levels of H3K36 methylation by Set2 and the H3K36 histone demethylase(s), such as Rph1, is necessary for proper transcription elongation.

That Rph1 definitely functions in the process of transcription elongation as well as the exact mechanism of action by which Rph1-mediated demethylation of H3K36 could promote faithful elongation remain to be conclusively demonstrated. The putative genetic interaction between *RPH1* and *SPT16* or *BUR2* was identified using genetic analysis. As alteration of chromatin-modifying machinery could lead to the introduction of unwanted mutations that are

capable of suppressing and/or enhancing cellular phenotypes, it is crucial to complete sequential rounds of backcrossing to an isogenic wild-type strain to ensure that the genotypes of the mutant strains are not compromised in an undesirable manner. Should the synthetic growth defect of a *spt16-11 rph1Δ* double mutant strain hold true, it would be necessary to demonstrate that such a synthetic phenotype is due to loss of Rph1 demethylase activity. To that end, a rescue experiment in which growth is assessed following introduction of a plasmid expressing either wild-type or catalytically inactive Rph1 into a *spt16-11 rph1Δ* strain should be completed (and likewise for the *bur2Δ rph1Δ* double mutant strain). If Rph1 demethylase activity does in fact promote proper FACT function in yeast, expression of wild-type Rph1, but not catalytically inactive Rph1, should restore growth to a level comparable to that of the *spt16-11* allele alone, in turn supporting the notion that Rph1 demethylase activity likely contributes to proper transcription elongation.

One possible means by which Rph1 could function during transcription elongation is through the proper recruitment and/or function of the Rpd3S histone deacetylase complex to chromatin. H3K36me is important for the stable association and function of the Rpd3S histone deacetylase complex on nucleosomes (CARROZZA *et al.* 2005; GOVIND *et al.* 2010; JOSHI and STRUHL 2005; KEOGH *et al.* 2005; LI *et al.* 2007b; LI *et al.* 2009a; YOUDELL *et al.* 2008). Similar to deletion of *SET2*, loss of the Rpd3S complex members Rco1 or Eaf3 in the *spt16-11* mutant background results in suppression of the synthetic growth defect observed at the semi-permissive temperature (BISWAS *et al.* 2006; BISWAS *et al.* 2008). It could be that the observed synthetic growth defect of the *spt16-11 rph1Δ* double mutant exhibited in FIGURE 5.2 (panel A) is dependent on the Rpd3S complex, as it is possible that increased levels of H3K36me on genes following deletion of Rph1 could lead to enhanced recruitment of the Rpd3S HDAC complex and subsequent deacetylation of nucleosomes in transcribed regions. If this model is correct,

deletion of Rpd3S complex members would suppress the observed slow growth defect of the *spt16-11 rph1Δ* double mutant strain. Thus, future experiment should include phenotypic growth assays testing how introduction of individual deletion of members of the Rpd3S complex, including Rco1 and Eaf3, affects the growth of a *spt16-11 rph1Δ* double mutant strain.

Unlike deletion of *SET2*, which leads to both global and gene-specific increases in acetylation levels due to the inability to recruit the Rpd3S complex, preliminary western blot and ChIP analyses indicate that loss of *RPH1* does not appear to result in altered acetylation levels either globally or on a subset of genes tested (*data not shown*). This suggests that Rph1 likely acts on a distinct group of genes whose identity remains to be determined. Thus, further work that remains to be completed also includes positive identification of Rph1 target genes, as such analyses could provide significant insight into the biological function of Rph1-mediated demethylation of H3K36. Microarray analyses of wild-type versus *RPH1* deletion strains would provide insight as to the genes whose expression changes upon loss of Rph1. Such analysis should be completed bearing in mind that Rph1 and its homolog Gis1 have been shown to be functionally redundant In the context of their previously identified roles as DNA damage-responsive repressors (JANG *et al.* 1999). Although it has been suggested that Gis1 is a histone lysine demethylase (TU *et al.* 2007), substitutions in necessary amino acids in its catalytic JmjC domain likely abrogate its enzymatic activity (KLOSE *et al.* 2007a; KLOSE and ZHANG 2007). Nonetheless, consideration for its putatively redundant function as a transcriptional repressor must be taken in such studies. As microarray analysis would merely provide information regarding categories of genes whose expression is changed upon loss of a demethylase, a more ideal approach to identify direct targets would be to perform chromatin immunoprecipitation coupled with deep sequencing (ChIP-seq) using an antibody capable of immunoprecipitating Rph1. However, we and others have experienced difficulty in performing ChIP analysis of JmjC-

domain-containing demethylases in yeast (INGVARSDOTTIR *et al.* 2007; KIM and BURATOWSKI 2007), which could perhaps be due to transient interactions between the demethylases and chromatin. Such limitations must be overcome if genome-wide localization of the demethylases by ChIP-seq analysis is to be completed. Should it be possible to identify target genes, subsequent studies must then be completed to determine the mechanism by which Rph1 is recruited to such genes, as well as how the different states of H3K36 methylation regulated by Set2 and Rph1 at this subset of genes ultimately control the recruitment and function of the Rpd3S complex during transcription elongation.

Originally identified as a transcriptional repressor of *PHR1*, a DNA repair gene that encodes a photolyase that repairs pyrimidine dimers, Rph1 does have one known target gene (JANG *et al.* 1999). EMSA and DNase I footprinting have suggested that *in vitro* Rph1 associates through its C-terminal zinc fingers with a single AG_4 sequence in the upstream repressive sequence of *PHR1* (URS_{PHR1}) (JANG *et al.* 1999; SANCAR *et al.* 1995). Upon DNA damage, Rph1 is released from the URS_{PHR1}, thereby allowing for transcription of *PHR1* and enhanced cellular DNA repair (JANG *et al.* 1999). Of interest would be to demonstrate a direct association of Rph1 with the URS_{PHR1} by ChIP analysis. Given the difficulties in immunoprecipitating JmjC-domain-containing demethylases (as described above) for ChIP analysis, it would at the very least be interesting to see evidence of demethylase activity at the URS_{PHR1} by assessing changes in H3K36 methylation states in the presence and absence of DNA damage in wild-type and Rph1 deletion strains using H3K36me modification-specific antibodies for ChIP analysis. Should changes in H3K36 methylation be detectable upon DNA damage, it would suggest action by Rph1. Subsequent analysis could be performed to see if other genomic regions that contain a AG_4 consensus sequence similar to that found in the URS_{PHR1} (see (JANG *et al.* 1999) for examples)

could putatively provide insight into additional targets of Rph1 by assessing H3K36 methylation levels at those genomic loci in the presence and absence of Rph1.

In this work, Jhd2 was identified as a H3K4 demethylase specific for the di- and trimethyl state (LIANG *et al.* 2007). At present, the regulation and functional consequence of Jhd2-mediated demethylation is better characterized than that of Jhd1 or Rph1. Where phenotypic analysis of a *JHD2* deletion strain revealed no obvious function, overexpression of Jhd2 disrupts silencing of telomeric regions (LIANG *et al.* 2007). This finding correlates well with the previously established role of H3K4 methylation in regulation of telomeric silencing (KROGAN *et al.* 2002a; NISLOW *et al.* 1997). Given the role of Set1-mediated H3K4 methylation in regulation of other biological processes in budding yeast including cellular growth, transcriptional regulation, rDNA silencing, meiotic differentiation, DNA repair, and chromosome segregation (DEHE and GELI 2006), and that Jhd2 is the only known H3K4 demethylase, it is somewhat surprising that phenotypic analysis of a *JHD2* deletion strain did not reveal more obvious cellular phenotypes, indicating that more specific assays are likely needed to provide greater insight into the role of H3K4 demethylation. For example, cell synchronization and release studies have revealed that Jhd2 contributes to cell cycle dynamics of H3K4 methylation (RADMAN-LIVAJA *et al.* 2010). Additionally, Jhd2 has been shown to be necessary to both establish proper levels of H3K4 methylation during activation of the *GAL1* gene and remove H3K4 trimethylation during the attenuation phase of transcription (INGVARSDOTTIR *et al.* 2007).

Studies are beginning to shed light onto the regulation and targeting of the histone demethylases. For example, it has been shown that levels of the H3K4 demethylase Jhd2 are controlled via polyubiquitylation by the E3 ubiquitin ligase Not4 and subsequent proteasome-mediated degradation (MERSMAN *et al.* 2009). Proteasomal degradation provides one potential means by which the precise amount of Jhd2 necessary to maintain the proper balance of H3K4

methylation for normal cellular activities can be controlled. It is possible, however, that other means such as post-translational modification of the demethylases themselves control enzymatic activity. Purification of native demethylases followed by mass spectrometric analysis could provide insight into differentially modified forms of the demethylases that exist which could potentially function in regulation of enzymatic activity. Investigations revealing how the demethylases are potentially targeted to their respective substrates have also recently been reported. One means by which targeting and regulation of chromatin modifying enzymes is often mediated is through association with other proteins in the form of high molecular weight complexes (CAIRNS 2005; CAO *et al.* 2002; KEOGH *et al.* 2005; KROGAN *et al.* 2002a; MILLER *et al.* 2001). However, all studies completed thus far have failed to reveal stable associations between the histone demethylases and auxiliary proteins that could putatively function in their targeting and/or regulation (FANG *et al.* 2007; KLOSE *et al.* 2007a; LIANG *et al.* 2007). Alternatively, domains within the proteins could likely facilitate binding to chromatin. For example, it has already been established that Rph1 can bind to DNA through its C-terminal zinc fingers (JANG *et al.* 1999). The ability to directly interact with DNA may circumvent the need for auxiliary proteins in recognizing target sites in chromatin. Both Jhd1 and Jhd2 have PHD fingers, a domain that has been shown to be able to bind to methylated lysine residues (TAVERNA *et al.* 2007a). Previous studies have indicated that Jhd1 can bind to methylated H3K4 *in vitro* (with a preference for the trimethyl state) through its PHD finger (SHI *et al.* 2007), and that the PHD finger of Jhd2 is necessary for chromatin association *in vivo* (HUANG *et al.* 2010). Such interactions likely provide one mechanism by which the demethylases are targeted to chromatin, thus necessitating further studies to decipher the connection between chromatin association and subsequent histone demethylation.

Although JmjC-domain-containing histone demethylases with specificity for H3K4 and H3K36 have been identified, an active demethylase with specificity for H3K79 remains to be discovered. Thus, a lingering question in the field of histone demethylation is whether H3K79 methylation is reversible. Where methylation of H3K4 and H3K36 is catalyzed by SET-domain-containing methyltransferases, methylation of H3K79 is catalyzed by Dot1, which lacks a SET domain altogether. The inability of JmjC-domain-containing proteins to catalyze removal of H3K79 methylation parallels the fact that a unique type of methyltransferase is responsible for its placement. It could be that a yet-to-be-identified class of novel histone demethylase enzymes with unique enzymatic properties facilitates the removal of H3K79 methylation, thereby necessitating further biochemical and genetic analyses to demonstrate that this mark is dynamically regulated. Experiments must be done thoughtfully, as H3K79 is highly methylated (estimated at 90%, (VAN LEEUWEN *et al.* 2002)), and high levels of methylation could mask slight changes arising from deletion of the responsible enzyme. Alternatively, it is possible that this modification may be enzymatically irreversible, and thus relies on passive mechanisms such as histone turnover for removal. Clearly, further biochemical investigations are necessary to delineate if either of these possibilities is correct.

The thermodynamic stability of the amino-methyl group within methylated histone residues led many to believe that histone lysine methylation was irreversible, thereby supporting a function in long-term epigenetic memory (KUBICEK and JENUWEIN 2004). While the existence of histone lysine demethylases has now been known for several years, studies on these enzymes are still in their infancy. The studies contained in this work greatly expanded our understanding of the dynamic nature of histone methylation in their identification of histone lysine demethylases in budding yeast. However, many questions remain thus prompting future investigations related to the enzymology and regulation of the histone demethylases that will

ultimately increase our genuine appreciation for the complexity associated with this highly stable, yet dynamic, modification.

Identification and Characterization of Novel Sites of Histone Lysine Methylation | At the time of inception of the histone code hypothesis, which posits that combinatorial and/or sequential patterns of post-translational modifications (PTMs) of histone tails dictate downstream biological phenomena (STRAHL and ALLIS 2000), we had quite a limited understanding of the breadth of the number and type of PTMs that existed on histone residues both on the unstructured N-terminal tails protruding from the nucleosomal surface and on the globular domain of the nucleosome core particle. Acetylation and phosphorylation were the best characterized at that time, as multiple sites and the enzymes responsible for their dynamic placement had been identified. However, investigations of modification of histones by methylation of lysine residues were just beginning to take off. While just a few sites of lysine methylation were known at the time, the past ten years has witnessed a dramatic expansion in our understanding of the dynamic nature of this modification. Numerous enzymes responsible for both the placement and removal of methylation events have been identified, and the biological consequences of histone lysine methylation are becoming apparent ((CLOOS *et al.* 2008; SHILATIFARD 2006) and FIGURE 5.1). In spite of this new knowledge, however, several sites of histone lysine methylation in budding yeast have been identified that have no ascribed methyltransferase, demethylase, and/or biological function, including H2BK22, H2BK37, H2BK111, H3K18, H3K23, H3K37, and H4K31 (GARCIA *et al.* 2007a; KYRISS *et al.* 2010; UNNIKRISHNAN *et al.* 2010; ZHANG *et al.* 2009), thereby necessitating continued biochemical and genetic analyses to delineate the enzymology, regulation, and functional consequence(s) of placement and removal of methyl marks at these sites.

A clear challenge that remains for future studies is to identify novel sites of histone lysine methylation (and PTMs in general) and decipher how such modifications function in the context of the same histone, within the same nucleosome, and within contiguous nucleosomes. Technological advancements have significantly advanced our efforts to both identify novel sites of histone modifications and the combinations in which they exist and to understand the biological significance of such combinatorial patterns. Histone modifications have typically been identified using mass spectrometry (MS) analysis. Recent advancements in MS technology include the development of top-down MS analysis, in which intact proteins samples are analyzed, as opposed to the more canonical bottom-up MS approach where proteins are fragmented prior to analysis. Because proteins are analyzed at the whole-molecule level, top-down MS allows for identification of combinatorial patterns of modifications that exist within one histone protein. Within this work, we used top-down MS analysis to analyze novel sites and combinations of modification on histones to begin to understand the totality of existent modifications and identified lysine 37 of histone H2B as a novel site of histone methylation in budding yeast (GARDNER *et al.* 2011b). This is likely to be one of numerous sites of histone methylation present on chromatin, thus necessitating continued MS studies to more fully catalogue modifications that potentially function within the context of the histone code. Although only recently developed, top-down MS analysis has already proven itself to be a powerful tool that will likely continue to push forward our understanding both the dynamics and combinatorial states of histone PTMs in future studies.

MS approaches employed to examine combinatorial modifications on histones must be complemented by comprehensive genomic location analysis, as identification of the histone PTMs themselves is not enough to understand functional consequences of their placement. The availability of modification specific antibodies has allowed for immunoprecipitation of DNA

fragments associated with a particular mark by chromatin immunoprecipitation (ChIP). The relatively small size of the genome has made hybridization of immunoprecipitated DNA to microarrays (ChIP-chip) a successful means by which genome-wide profiles of histone methylation in budding yeast have be assessed (POKHOLOK *et al.* 2005; RAO *et al.* 2005; SCHULZE *et al.* 2009). Coupled with ChIP, next-generation sequencing technology (ChIP-seq) has considerably advanced comprehensive mapping of genome-wide modification patterns in higher eukaryotes (BARSKI *et al.* 2007; MIKKELSEN *et al.* 2007). Given the great insight that ChIP-chip and ChIP-seq studies have provided with respect to the coordinated placement of histone marks, these techniques are likely to be readily called upon in future studies to map genome-wide patterns of novel marks as well as patterns of transcription factors and chromatin modifying machinery that place, remove, and interpret modifications.

The rapid pace of research in chromatin biology means that we are continually refining how we define the histone code. Recently, it has come to be seen less as a set of definite combinations that lead to an absolute outcome (as would be dictated, for example, by the genetic code), and more as patterns of modifications that when in combination tend to tip the balance toward a specific outcome. Perhaps, then, the ramifications of the histone code are correlative rather than causal in that combinatorial patterns provide a bias for a specific outcome rather than serve as an absolute mark of one. Regardless of how the code is defined, it is certain that continued efforts are needed to both identify and characterize novel histone modifications and the combinatorial patterns in which they exist and map the genome-wide localization patterns of such modifications so that we can continue to refine instead our understanding of how histone modifications work together in concert to shape defined biological outcomes.

Closing Thoughts | From the onset of the studies included within this body of work, the overarching objective was to understand how dynamic changes in histone lysine methylation regulate chromatin structure and function. Although the budding yeast *Saccharomyces cerevisiae* has a relatively simplistic histone lysine methylation system compared to higher eukaryotes, elegant studies can be conducted in this model organism that are simply not possible in higher organisms. For example, where the budding yeast protein Set2 is the sole methyltransferase responsible for methylation of H3K36 (STRAHL *et al.* 2002), mammals have at least three proteins that can methylate H3K36 (LI *et al.* 2009b; RAYASAM *et al.* 2003; SUN *et al.* 2005). Where depletion of the murine Setd2 in cultured cells results in a loss of trimethylated H3K36, mono- and dimethyl H3K36 remain largely unaffected, supporting the view that Setd2 is a trimethyl specific H3K36 methyltransferase (EDMUNDS *et al.* 2008). Such an example of the complexity associated with the histone lysine methylation system in higher eukaryotes makes the difficulties of conducting functional studies in which a methylation event is completely abrogated immediately apparent. For studies related to identification of novel enzymes and sites of modification by dynamic methylation as were conducted within this piece, budding yeast therefore offers the advantage of a system in which biochemical and genetic studies that reveal insight into the enzymology and regulation of such events can be completed with relative ease and can then be translated to higher eukaryotes. Moving forward, cross-disciplinary investigations involving exploratory studies in model organisms followed by translation to higher organisms seem most advantageous in creating a comprehensive picture of the dynamics of histone methylation, and future studies in budding yeast will thus continue to contribute significantly to our understanding of the intricacies of this modification.

As our understanding of the sites on which histone lysine methylation occur as well as the proteins that place, interpret, and remove this modification increases, the importance of a

histone methylation system in the regulation of chromatin-based processes becomes more apparent. A major challenge in chromatin biology is to integrate how the addition and removal of specific modifications such as lysine methylation on histones fits into the fluid picture of the continually changing chromatin template. Fortunately, technological advancements continue to further our research efforts, arming us with a continually polished understanding of methylation patterns. Although the scientific investigations within this dissertation individually advance our basic knowledge of the histone methylation system in yeast, it is with great optimism that they will contribute to even more sophisticated analysis of defining how histone lysine methylation dictate epigenetic states involved in more complex biological processes such as human disease in future studies.

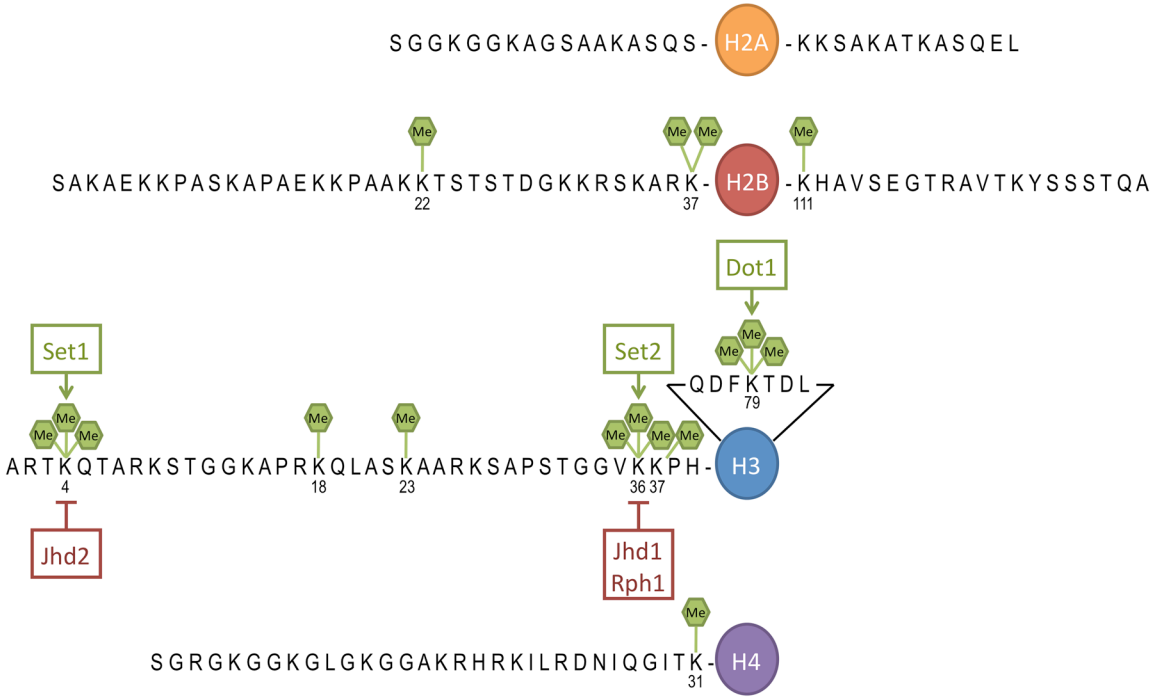

FIGURE 5.1 | Current atlas of histone lysine methylation in *Saccharomyces cerevisiae*. Illustrated are the presently known sites of histone lysine methylation in the budding yeast *Saccharomyces cerevisiae* along with the known histone methyltransferases and histone demethylases that catalyze placement and removal of methyl groups (Me; depicted by green hexagons), respectively. The number of methyl groups shown represents the maximum state to which the modified residue has been shown to methylated. Work contained within this doctoral dissertation contributed to this atlas by demonstrating that the budding yeast proteins Rph1 and Jhd2 function as histone lysine demethylases with specificity for di- and trimethylated histone H3 lysine residues 36 and 4, respectively, and by also showing that lysine 37 of histone H2B is a site of histone methylation.

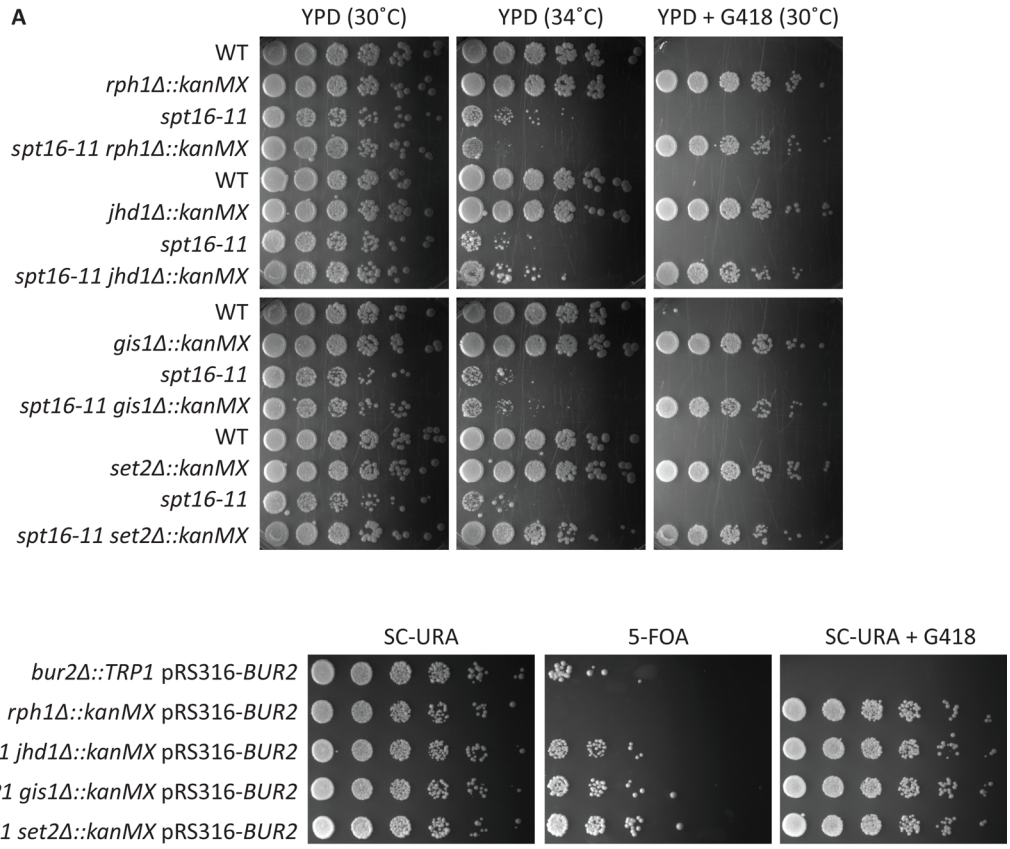

FIGURE 5.2 | Deletion of *RPH1* results in phenotypes supportive of a role in transcription elongation. (**A**) *JHD1*, *RPH1*, *GIS1*, and *SET2* were individually deleted in isogenic wild-type (W303) and *spt16-11* haploid strains, which were subsequently backcrossed to wild-type (W303) for three generations. Segregants from individual tetrads from the third backcross were assayed for growth on rich YPD media (spotted in 5-fold serial dilutions) at either the permissive (30°C) or semi-permissive (34°C) temperature. Cells were grown on selective rich media (YPD + G418) as a control to demonstrate appropriate *kanMX* deletions. *rph1Δ::kanMX*, *jhd1Δ::kanMX*, *gis1Δ::kanMX*, and *set2Δ::kanMX* single mutant strains grow equivalently to wild-type strains. A *spt16-11 rph1Δ::kanMX* double mutant strain exhibits a synthetic growth defect, as compared to growth of a *spt16-11* strain alone. This is in direct contrast to the enhanced cellular growth observed for cells upon loss of *SET2* in combination with the *spt16-11* allele grown at the semi-permissive temperature (as previously established (BISWAS *et al.* 2006)). (**B**) *RPH1*, *JHD1*, *GIS1*, and *SET2* were individually deleted in a *bur2Δ::TRP1* strain containing a pRS316-*BUR2* plasmid. Shown is the growth of the *bur2Δ::TRP1*, *bur2Δ::TRP1 rph1Δ::kanMX*, *bur2Δ::TRP1 jhd1Δ::kanMX*, *bur2Δ::TRP1 gis1Δ::kanMX*, and *bur2Δ::TRP1 set2Δ::kanMX* strains spotted in 5-fold serial dilutions on SC-URA and SC-URA containing 5-fluoroorotic acid (5-FOA) plates. Cells were grown on selective rich media (SC-URA + G418) as a control to demonstrate appropriate *kanMX* deletions. Deletion of *RPH1* is lethal when combined with loss of *BUR2*, as opposed to the combined loss of *SET2* and *BUR2*, which results in enhanced cellular growth as compared to the *bur2Δ::TRP1* strain alone (in agreement with previously published data (KEOGH *et al.* 2005; YAO *et al.* 2000)).

Appendices

Appendix One

Yeast Jhd2p is a Histone H3 Lys4 Trimethyl Demethylase

*G.L. and R.J.K. contributed equally to this study

Histone methylation is important in regulating chromatin structure and function. In budding yeast, methylation of histone H3 at Lys4 (H3-K4) is associated with active transcription and is enriched at the 5' regions of transcribed genes. Here we identify a novel budding yeast JmjC-domain-containing H3-K4 demethylase, Jhd2p, that antagonizes the trimethyl modification state and contributes to regulation of telomeric silencing.

Histone methylation on lysine residues contributes to transcriptional regulation, maintenance of genome integrity, and epigenetic inheritance (MARTIN and ZHANG 2005). Characterization of individual histone lysine methylation marks has revealed that specific modifications can have very defined functional effects on surrounding chromatin. In addition, each modified lysine can exist in the mono- (me1), di- (me2), or trimethyl (me3) state, increasing the potential complexity of the histone lysine modification system. The effects of histone methylation are mediated largely through recruitment of effector proteins that can recognize regions of differentially modified chromatin (BANNISTER *et al.* 2001; LACHNER *et al.* 2001; MARTIN *et al.* 2006a; WYSOCKA *et al.* 2006). In budding yeast (*Saccharomyces cerevisiae*), histone lysine methylation occurs exclusively on histone H3 at Lys4, Lys36, and Lys79 (MILLAR and GRUNSTEIN 2006). H3-K4 is methylated by the Set1p methyltransferase during transcriptional initiation, through association with the Ser5-phosphorylated CTD of RNA polymerase II (NG *et al.* 2003). Although the dynamics of H3-K4 methylation in budding yeast are poorly characterized, some rapid changes in H3-K4 methylation have been reported, suggesting an active mechanism exists to counteract this modification (KATAN-KHAYKOVICH and STRUHL 2005; ZHANG *et al.* 2005).

Recently, JmjC-domain-containing enzymes have been shown to directly remove histone lysine methylation via a hydroxylation reaction that requires iron and α-ketoglutarate as co-factors

(KLOSE *et al.* 2006a; TSUKADA *et al.* 2006). In budding yeast, there are five JmjC-domain-containing proteins: Jhd1p, Rph1p, Gis1p, Yjr119Cp, and Ecm5p (FIGURE A1.1, panel a). Jhd1p has previously been shown to catalyze demethylation of H3-K36me2 and H3-K36me1 (TSUKADA *et al.* 2006), but the potential enzymatic activity of other JmjC-domain-containing proteins remains uncharacterized. Bioinformatic analysis has indicated that substitution mutations in important catalytic residues of the JmjC domains of Gis1p and Ecm5p render the JmjC domain enzymatically inactive (KLOSE *et al.* 2006a). We have recently demonstrated that Rph1p catalyzes demethylation of H3-K36me3 and H3-K36me2 (KLOSE *et al.* 2007a). To determine whether the remaining JmjC-domain-containing protein, Yjr119Cp, is a histone demethylase, we expressed recombinant Yjr119Cp in insect cells using a baculovirus expression system and purified the recombinant protein by affinity chromatography (FIGURE A1.1, panel b). Recombinant Yjr119Cp protein was incubated with radioactively labeled histone substrates corresponding to all three histone lysine methylation sites in yeast, and demethylase activity was analyzed by release of the labeled reaction product formaldehyde (FIGURE A1.1, panel c). Yjr119Cp catalyzed demethylation of substrates labeled by Set7p Y245A, an enzyme capable of producing H3-K4me3 and H3-K4me2 modification states (XIAO *et al.* 2003a), but not substrates labeled in the H3-K36 or H3-K79 positions (FIGURE A1.1, panel c). Notably, Yjr119Cp was catalytically inactive towards substrates produced by wild-type Set7p, which generates the H3-K4me1 modification state (*data not shown*), suggesting that Yjr119Cp targets H3-K4me2/3 but not H3-K4me1. To reflect the protein's newly identified enzymatic activity, we have renamed the YJR119C gene as JmjC-domain-containing histone demethylase 2 (*JHD2*).

Many chromatin-modifying proteins in yeast are found in large multiprotein complexes with auxiliary proteins that function to target, and in some cases, regulate enzymatic activity (MILLAR

and GRUNSTEIN 2006; MILLER *et al.* 2001). To gain insight into potential functional partners of Jhd2p, we sought to analyze whether Jhd2p forms a high-molecular weight multiprotein complex. To this end, extract from a strain containing Flag-tagged Jhd2p was separated by size-exclusion chromatography and Jhd2p-containing fractions were identified by western blot analysis. The molecular weight of Jhd2p, as determined by size exclusion chromatography, is between 80 and 100 kDa, which corresponds to the predicted molecular weight of monomeric Jhd2p (85 kDa) (FIGURE A1.1, panel d, top). The elution profile of Jhd2p from yeast extracts mirrors the profile of recombinant protein fractionated on the same column, verifying the monomeric nature of this enzyme (FIGURE A1.1, panel d, bottom). Therefore, Jhd2p seems to function as an H3-K4 demethylase in the absence of stably associated protein factors.

To examine whether Jhd2 can target demethylation of H3-K4 *in vivo*, wild-type (WT) Jhd2p and Jhd2p with a mutation in a proposed iron-binding site (H427A) were overexpressed in budding yeast, and the resulting H3-K4 methylation states were analyzed with modification-specific antibodies (FIGURE A1.2, panel a). Overexpression of WT Jhd2p resulted in a reduction of H3-K4me3 and H3-K4me2 and an increase in the levels of H3-K4me1 (FIGURE A1.2, panel a, middle lanes). Jhd2p demethylase activity was specific for H3-K4, as the levels of H3-K36me3 and H3-K79me3 remained unchanged (FIGURE A1.2, panel a). The effect of Jhd2p on H3-K4 methylation was completely dependent on an intact JmjC domain, as mutation of a predicted iron-binding residue abrogated demethylase activity (FIGURE A1.2, panel a, right lane). Alteration of H3-K4 methylation levels by deletion of Set1p or the Cps30p component of the Set1 complex causes defects in telomeric silencing and sensitivity to agents that inhibit DNA replication (KROGAN *et al.* 2002; MUELLER *et al.* 2006; NAGY *et al.* 2002; NISLOW *et al.* 1997; SCHLICHTER and CAIRNS 2005). To examine whether elevated levels of Jhd2p result in similar cellular defects, Jhd2p was

overexpressed in a strain containing the *URA3* telomeric reporter (SMITH *et al.* 2000) or treated with the DNA replication-inhibiting agent hydroxyurea (FIGURE A1.2, panels b and c). Notably, elevated levels of Jhd2 and demethylation of H3-K4 caused partial reactivation of the telomeric *URA3* reporter gene, as evidenced by reduced growth on 5-fluoroorotic acid (FOA)-containing media, and also reduced growth after treatment with hydroxyurea (FIGURE A1.2, panels b and c). These effects were completely dependent on the enzymatic activity of Jhd2p, they did not occur upon overexpression of catalytically inactive Jhd2p H427A (FIGURE A1.2, panels b and c). These data indicate that Jhd2p can demethylate H3-K4 *in vivo* to counteract cellular functions mediated by Set1 methylation. Furthermore, overexpression of Jhd2p resulted in DNA replication defects and loss of telomeric silencing similar to strains with perturbed Set1 function (KROGAN *et al.* 2002a; MUELLER *et al.* 2006; NAGY *et al.* 2002; NISLOW *et al.* 1997; SCHLICHTER and CAIRNS 2005), indicating that these effects are dependent on H3-K4 methylation and not other regulatory properties of the Set1 complex.

To determine whether endogenous Jhd2p contributes to regulation of normal H3-K4 methylation levels, we deleted the *JHD2* gene and analyzed the H3-K4 methylation levels using modification-specific antibodies (FIGURE A1.2, panel d). In strains lacking Jhd2p, there was an increase in the levels of H3-K4me3 (FIGURE A1.2, panels d and e), whereas H3-K4me2 were reduced (FIGURE A1.2, panels d and e), indicating a global shift from H3-K4me2 to the H3-K4me3 modification state, while H3-K4me1 levels remained constant (FIGURE A1.2, panel d). Together these data indicate that H3-K4me3 is preferentially demethylated by Jhd2p *in vivo* and that Jhd2p contributes to global regulation of H3-K4me3 levels. Despite global changes in H3-K4 methylation, Jhd2p deletion strains are viable and healthy. To try to uncover functional defects in the Jhd2p deletion strain, we examined the strain's phenotypes under a number of conditions

(TABLE A1.1). This analysis revealed no phenotypic defects in the Jhd2p deletion strain, with the exception of a subtle enhancement of telomeric silencing (FIGURE A1.2, panel f). Therefore, disruption of Jhd2p and overexpression of Jhd2p have the opposite effects on telomeric silencing. Although the possibility cannot be ruled out that the observed phenotypes are due to demethylation of nonhistone substrates, these observations presumably demonstrate a role for Jhd2p and H3-K4 methylation in regulation of telomeric chromatin function.

The identification of histone demethylases has revealed that histone methylation can be dynamically regulated, like histone acetylation or phosphorylation (KLOSE *et al.* 2006a; SHI *et al.* 2004; TSUKADA *et al.* 2006). Here we demonstrate that Jhd2p dynamically regulations H3-K4 methylation in budding yeast. Jhd2p preferentially demethylates the H3-K4me3 modification state *in vivo* and contributes to regulation of telomeric silencing. Notably, none of the JmjC-domain-containing proteins in budding yeast can remove H3-K79 methylation, suggesting that this modification may be enzymatically irreversible or that an uncharacterized class of demethylase enzyme with unique enzymatic properties remains to be identified. Further analysis of the dynamics of H3-K79 methylation in budding yeast will be important in determining whether this modification can be dynamically regulated and will help to identify enzymes that could remove this histone modification.

Materials and Methods

Yeast strains. All strains, except those in telomeric silencing assay, were derived from BY4741. Strains used in telomeric silencing assay were of the YCB647 background (SMITH *et al.* 2000). The *jhd2Δ* strains were generated by homologous recombination of PCR-amplified *hphMX* (GOLDSTEIN and MCCUSKER 1999) or *kanMX* (BRACHMANN *et al.* 1998) knock-out cassettes.

Endogenous Jhd2 was C-terminal 3xFlag-tagged by amplifying the p3Flag-KanMX cassette (GELBART *et al.* 2001) using primers A and B (sequences listed below) and was introduced into the BY4741 strain by homologous recombination.

A: GAAGGATATTGACTCTTTAATAAAGCAAGTTGGTGTTAAGTTAGATAGAAGGGAACAAAAGCTGGAG

B: GTATTATTCTAAAAAATCATTACGCCATACACAAATATTGAAGACTACTATAGGGCGAATTGGGT

Recombinant protein, histone demethylase assay, and plasmid constructs. For recombinant protein expression, *JHD2* was PCR amplified from yeast genomic DNA and cloned into a modified pFastbacHT vector (Invitrogen) containing an N-terminal Flag-tag. Recombinant Yjr119c/Jhd2 baculovirus was generated to purify recombinant protein as described previously (CAO and ZHANG 2004). The histone demethylase assay was carried out as described previously (TSUKADA *et al.* 2006). The H427A substitution mutation in the predicted iron-binding site was generated by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene). Full-length Jhd2 or Jhd2-H427A was cloned into a 2 µm *LEU2* plasmid under *ADH1* promoter containing an N-terminal Flag-tag for Jhd2 over-expression in yeast. In all cases the sequences of PCR amplified clones were confirmed by sequencing.

***Antibodies*.** For western blot analysis of yeast histones the following antibodies were used at dilutions ranging from 1:200 to 1:1000: α-H3K4me3 (Abcam, ab8580), α-H3K4me2 (Abcam, ab7766), α-H3K4me1 (Abcam, abAb8895), α-H3K36me3 (Abcam, ab9050), and α-H3K79me3 (Abcam, ab2621). Anti-Flag M2 (Sigma, F3165) was used to western blot for Flag-tagged proteins.

Size exclusion chromatography and sucrose gradient analysis. Whole cell yeast extract or recombinant Jhd2 were fractionated over a 24 mL Superose 6 size exclusion column (Amersham Biosciences) equilibrated with BC400 [40 mM HEPES (pH 7.9), 400 mM KCl, 0.5 mM DTT, 10% glycerol, 0.2mM PMSF] with the aid of an ÄKTA purifier (Amersham Biosciences) at a flow rate of 0.2 ml/min and 250 µl fractions were collected. Every other fraction was analyzed for Jhd2 by western blot or Coomassie staining.

Acknowledgments

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TABLE A1.1| Phenotype analysis of the *jhd2Δ* strain

Phenotype	Functional implication (HAMPSEY 1997)	Control (Reference)
Slow growth	General protein defects indicating important genes	
Heat sensitivity	General protein defects indicating important genes	*spt4Δ* (BASRAI *et al.* 1996)
Mycophenolic acid (MPA) sensitivity	Transcriptional elongation	*rtf1Δ* (DESMOUCELLES *et al.* 2002)
Galactose fermentation	Transcriptional activation	*snf2Δ* (NEIGEBORN and CARLSON 1984)
Raffinose fermentation	Transcriptional derepression	*snf2Δ* (NEIGEBORN and CARLSON 1984)
Inositol auxotrophy	Inositol biosynthesis; transcriptional activation	*spt7Δ* (PATTON-VOGT and HENRY 1998)
Hydroxyurea sensitivity	DNA replication	*htz1Δ* (MIZUGUCHI *et al.* 2004)
Caffeine sensitivity	Mitogen-activated protein (MAP) kinase pathway; chromatin remodeling	*htz1Δ* (MIZUGUCHI *et al.* 2004)
Telomeric silencing defect	Heterochromatin silencing	*sir2Δ* (SMITH *et al.* 2000)

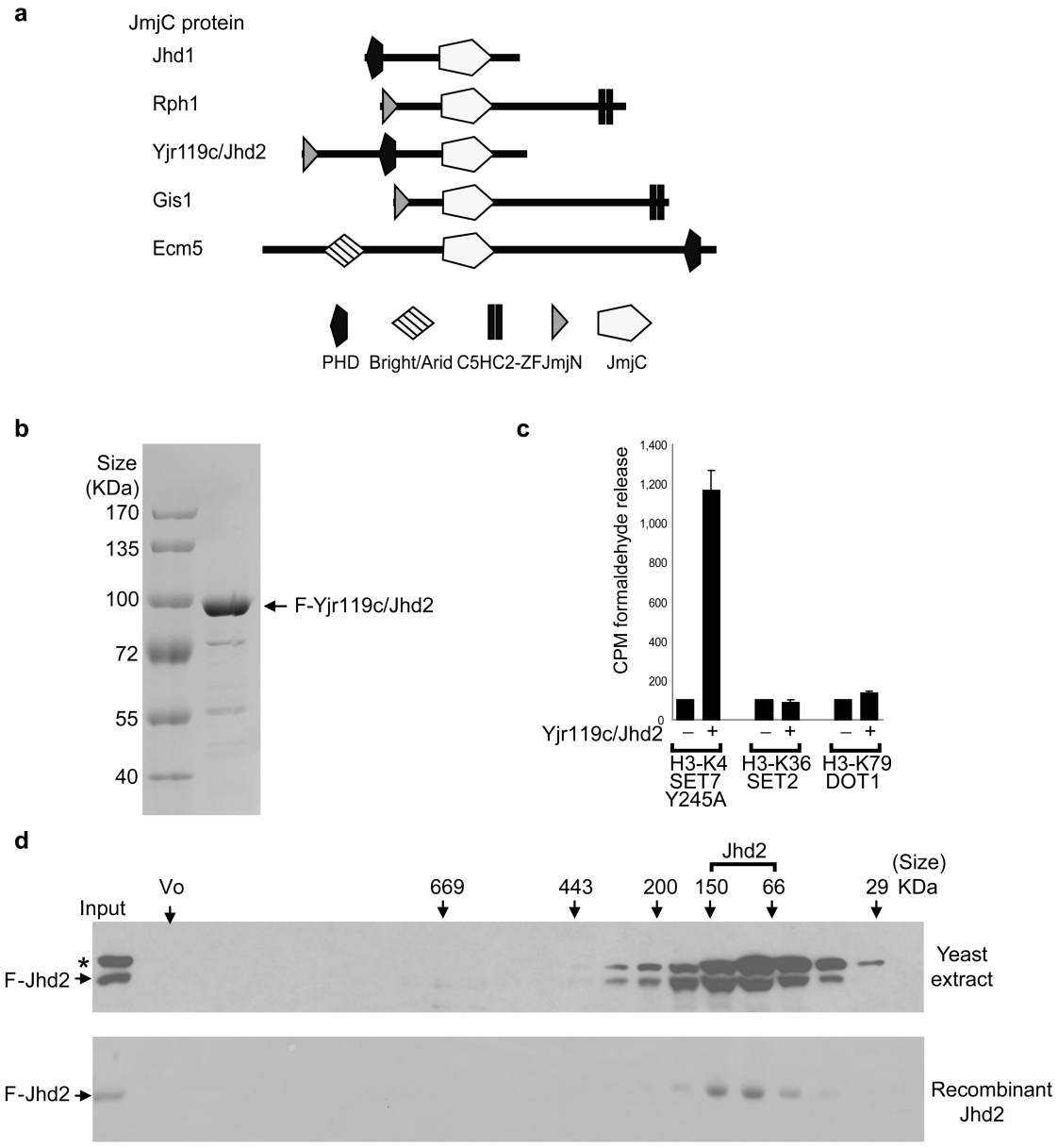

FIGURE A1.1 | Budding yeast Yjr119Cp (renamed Jhd2p) is an H3-K4 demethylase. (**a**) Schematic illustration of domain architecture of the five JmjC-domain-containing proteins in budding yeast. (**b**) Coomassie-stained gel showing affinity-purified recombinant Yjr119Cp/Jhd2p produced in insect cells. (**c**) Histone substrates were labeled using methyltransferase enzymes that modify known histone methylation sites in budding yeast and incubated with recombinant Yjr119Cp/Jhd2p. Histone demethylase activity was monitored as the release of labeled formaldehyde, plotted as a bar graph (error bars show s.d.). Yjr119Cp/Jhd2p specifically demethylates H3-K4me3 and H3-K4me2. (**d**) Top, Flag-Jhd2 (F-Jhd2p) in yeast extract was fractionated by size-exclusion chromatography and identified by Flag-specific western blotting (top gel). Asterisk (*) indicates a cross-reacting band found in yeast extracts. Size-exclusion chromatography molecular weight markers are indicated above the panel. Jhd2p eluted with an apparent molecular weight of 80-100 kDa. Bottom, recombinant Jhd2p was fractionated by

size-exclusion chromatography and identified by Coomassie staining. Recombinant Jhd2 eluted with the same apparent molecular weight as endogenous Jhd2p, suggesting Jhd2p is a monomeric protein that does not form a stable high-molecular molecular weight protein complex.

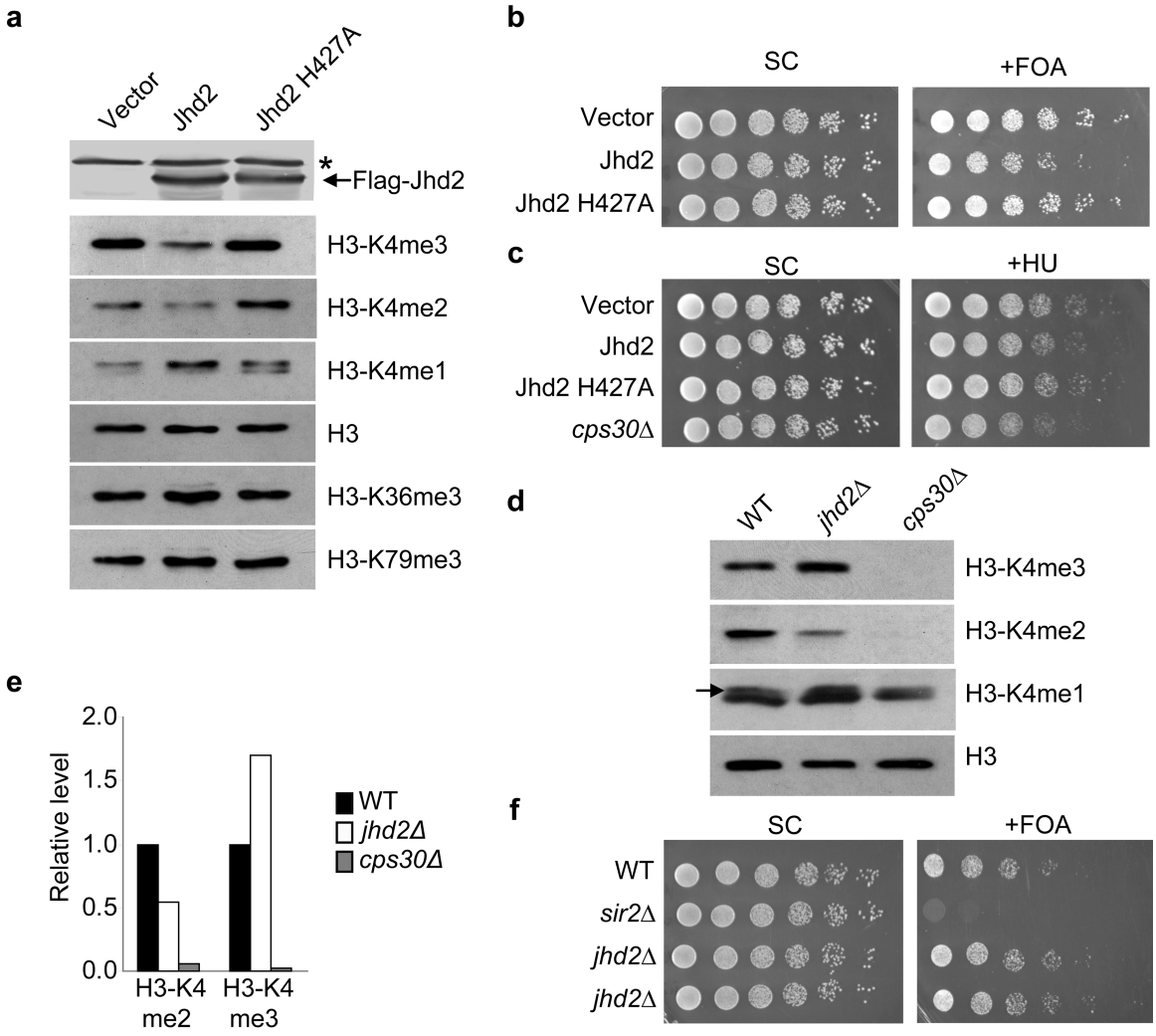

FIGURE A1.2 | Jhd2p antagonizes H3-K4me3 methylation and regulates telomeric silencing. (**a**) Whole-cell extracts were prepared from strains containing either empty vector or constructs expressing Flag-tagged wild-type Jhd2p or the H427A mutant. Histone methylation levels were analyzed by western blotting using modification-specific antibodies. (**b**) WT Jhd2p or Jhd2p H427A was overexpressed in a strain carrying a silenced telomeric *URA3* reporter gene. After normalization for cell number, each strain was serially diluted in five-fold increments and spotted on SC medium or SC containing 100 µg/µL 5-fluoroorotic acid (FOA). Strains overexpressing Jhd2p show reactivation of the *URA3* gene, evident from growth sensitivity on plates containing FOA. (**c**) WT Jhd2p or Jhd2p H427A was overexpressed in wild-type yeast. Each strain was serially diluted as in **b** and spotted on SC medium or SC containing 100 mM hydroxyurea (HU). Strains overexpressing Jhd2p are sensitive to hydroxyurea, as is evident from reduced growth. A *cps30Δ* strain, which has compromised Set1 enzymatic function, was used as a control for sensitivity to hydroxyurea. (**d**) Whole-cell extracts were prepared from wild-type, *jhd2Δ*, and *cps30Δ* strains. H3-K4 methylation levels were analyzed by western blotting using modification-specific antibodies. The *jhd2Δ* strain has increased levels of H3-K4me3 and reduced levels of H3-K4me2. (**e**) Plot of quantified relative changes in **d**. (**f**) Cells from wild-type (WT), *sir2Δ*, and *jhd2Δ* strains were normalized for cell density and spotted in five-fold dilutions

onto SC medium or SC medium containing 100 μg/μL FOA. *sir2Δ* strain is a control for loss of telomeric silencing. Two independent *jhd2Δ* strains show enhanced telomeric silencing, evident from reduced sensitivity to FOA in the *URA3* reporter strain.

Appendix Two

Histone H2BK123 Monoubiquitination is the Critical Determinant for H3K4 and H3K79 Trimethylation by COMPASS and Dot1

Histone H2B monoubiquitination by Rad6/Bre1 is required for the trimethylation of both histone H3K4 and H3K79 by COMPASS and Dot1 methyltransferases, respectively. The dependency of methylation at H3K4 and H3K79 on the monoubiquitination of H2BK123 was recently challenged, and extragenic mutations in the strain background used for previous studies or epitope-tagged proteins were suggested to be the sources of this discrepancy. In this study, we show that H3K4 and H3K79 methylation is solely dependent on H2B monoubiquitination regardless of any additional alteration to the H2B sequence or genome. Furthermore, we report that Y131, one of the yeast histone H2A/H2B shuffle strains widely used for the last decade in the field of chromatin and transcription biology, carries a wild-type copy of *HTA2* and *HTB2* genes under the *GAL1/10* promoter on chromosome II. Therefore, we generated the entire histone H2A and H2B alanine-scanning mutant strains in another background, which does not express wild-type histones.

Introduction

A nucleosome contains 146 bp of DNA wrapped twice around an octamer composed of two copies of each histone protein: H2A, H2B, H3, and H4 (KORNBERG 1974; KORNBERG and LORCH 1999). Nucleosomes are observed as a series of "beads on a string" via electron microscopy with the "beads" being the individual nucleosomes connected by the linker DNA, the "string". Structural studies have demonstrated that histone N-terminal tails protrude outward from the nucleosome and can be posttranslationally modified by different enzymes (LUGER *et al.* 1997; SHILATIFARD 2006). Such posttranslational modifications of histone tails include phosphorylation, acetylation, sumoylation, ADP ribosylation, ubiquitination, and methylation.

The first H3K4 (histone H3 lysine 4) methylase, Set1/COMPASS, was isolated from *Saccharomyces cerevisiae* and was demonstrated to be capable of mono-, di-, and trimethylating

H3K4 (KROGAN *et al.* 2002a; MILLER *et al.* 2001; ROGUEV *et al.* 2001). This posttranslational modification of H3K4 by COMPASS requires prior H2BK123 (histone H2B lysine 123) monoubiquitination in yeast and H2BK120 in vertebrates, which is a process known as histone cross talk (DOVER *et al.* 2002; SHILATIFARD 2006; SUN and ALLIS 2002; WOOD *et al.* 2005). It has also been demonstrated that histone H3K79 methylation by Dot1 also requires H2BK123 monoubiquitination (BRIGGS *et al.* 2002; WOOD *et al.* 2003b). Monoubiquitination of histone H2BK123 is mediated by the macromolecular complex containing the E2-conjugating enzyme Rad6 and the E3 ligase Bre1 in *S. cerevisiae* (HWANG *et al.* 2003; ROBZYK *et al.* 2000; WOOD *et al.* 2003a). This modification has been linked to transcriptional activation and elongation (HENRY *et al.* 2003; KAO *et al.* 2004; KIM and BURATOWSKI 2009; PAVRI *et al.* 2006; SHILATIFARD 2006; TANNY *et al.* 2007; XIAO *et al.* 2005). Studies in other eukaryotic organisms also have confirmed that this mode of regulation is well conserved from yeast to human (PAVRI *et al.* 2006; SMITH and SHILATIFARD 2009). However, a recent study performed by Foster and Downs (2009) argued that monoubiquitination of H2BK123 in yeast is not the sole determinant for the methylation of H3K4 and H3K79 (FOSTER and DOWNS 2009). In their study, Foster and Downs (2009) observed the presence of both H3K4 and H3K79 trimethylation in the H2BK123R mutant strain derived from an FY406 parental strain, which is contradictory to the previous findings, whereas both modifications were lost in an H2BK123R mutant derived in the Y131 background. Surprisingly, a triple KSS mutant (H2BK123R-S125A-S126A) in the FY406 background resulted in the loss of trimethylation of both H3K4 and H3K79. Additionally, Foster and Downs (2009) demonstrated that Flag-tagged H2B containing the K123R mutation (Flag-H2BK123R) abolished the trimethylation of H3K4 and H3K79, whereas untagged H2BK123R still possessed normal levels of trimethylation of both of the lysine residues in strain FY406. These observations led them to conclude that monoubiquitination of histone H2B alone is not required for the trimethylation of

either H3K4 or H3K79, but that an additional "unknown" alteration to H2B or the mutation in the genome in combination with the K123 mutation caused the loss of histone H3K4 and H3K79 trimethylation. In this study, we have addressed a possible role for the Flag tag on histone H2B in the regulation of H3K4 and H3K79 methylation. Our collective experiments have demonstrated that histone H3K4 and H3K79 methylation is solely dependent on H2B monoubiquitination and is independent of any other unknown factors or genetic backgrounds.

Materials and Methods

Generation of histone mutants. Strains used in this study are listed in TABLE A2.1. We used a plasmid containing *HTA1* and *HTB1* genes and a plasmid containing all four histones, *HTA1*, *HTB1*, *HHT2*, and *HHF2* genes, as shown in FIGURE A2.1, panel B. Plasmids bearing an alanine or arginine mutation in the Flag-tagged *HTB1* gene and Flag-less *HTB1* gene were generated by site-directed mutagenesis (QuickChange II kit; Agilent Technologies). Products were transformed into *E. cloni* 10G ELITE electrocompetent cells (Lucigen). Mutated targets were confirmed by sequencing using the primers (HTBseqF) 5'-GGCAAATACTACCTTGGTTGG-3' and (HTBseqR) 5'-TTTCGAGAACACAATTTTACAACCGA-3'. Each plasmid was transformed manually into yeast shuffle strains Y131, FY406, DY20D, and JHY205 using a standard yeast transformation protocol, and strains were grown on a synthetic dropout (SD) medium lacking histidine, SD-His (for Y131 and FY406 strains), or a medium lacking leucine, SD-Leu (for DY20D and JHY205 mutants). After 2 days of incubation, transformants were replica plated onto plates containing either SD-His plus 5-FOA or SD-Leu plus 5-FOA to select single-colony cells that had lost the plasmid containing the wild-type histones. Each colony was inoculated into YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) plus 5-FOA.

Western blot analyses. Cells are grown in YPD or YPGal [1% yeast extract, 2% peptone, and 2% galactose] to mid-log phase. Whole cell extracts were prepared from the wild-type and histone mutant strains as previously described (WOOD *et al.* 2003b) with some minor modifications. In brief, cell pellets were washed and resuspended in 400 µL NIB [0.25 M sucrose, 60 mM KCl, 14 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.8% Triton X-100]. After the addition of 250 µL of 0.5-mm glass beads to the tubes containing the suspension, the tubes were vortexed for 20 min at 4°C. Cell lysates were recovered by puncturing the bottom of the tube and centrifuging the contents at 3,000 rpm. After removal of the supernatant, the pellet was suspended in 150 µL of sterile water and 75 µL of 4× loading buffer and heated at 95°C for 5 min. Extracts were subjected to 18% SDS-PAGE electrophoresis, transferred to nitrocellulose membrane, and probed with either anti-Flag or H2B ubiquitin-specific antibodies, as well as antibodies specific for H3K4 di- and trimethylation and H3K79 trimethylation, followed by detection of the bound antibody with horseradish peroxidase-conjugated secondary antibodies. An antibody to histone H3 was used as a loading control.

Generation of H2A/H2B histone mutant library. The *h2a/h2b* histone mutant library was generated in a FY406 background as described previously (NAKANISHI *et al.* 2008). In brief, plasmids bearing alanine mutations in the *HTA1* and *HTB1* genes were systematically generated by site-directed mutagenesis in 96-well format. After transformation into *E. cloni* 10G ELITE electrocompetent cells using a 96-well electroporator, plasmids were prepared with a BioMekFX (Beckman Coulter) using the CosMCPrep kit (Agencourt). Each mutation was confirmed by sequencing using the aforementioned primers, and each plasmid was transformed into FY406 using a standard yeast transformation protocol. Transformants were selected on an SD-His medium followed by the second selection on a plate containing SD-His plus 5-FOA. To ensure

the complete removal of the plasmid containing the wild-type histones, each single colony was inoculated into YPD medium plus 5-FOA in 96-well plates. Finally, histone mutant strains were confirmed by sequencing, and the glycerol stocks of the library were generated.

Results and Discussion

To further investigate the role of H2BK123 monoubiquitination in H3K4 and H3K79 trimethylation and a possible role for the Flag epitope of H2B in this process, we set out to analyze the role of Flag-less H2B mutated in K123 for H3K4 and H3K79 methylation in several different strain backgrounds. We first wanted to be able to detect the presence of monoubiquitinated H2B in a Flag-less H2B background because the observation made by Foster and Downs (2009) that H3K4 trimethylation could be detected in a Flag-less H2BK123R strain could be because of the inadvertent presence of a wild-type copy of H2B. To address this, we generated polyclonal antibodies specific to monoubiquitinated H2B (FIGURE A2.1, panel A) and then shuffled a plasmid expressing H2B lacking an N-terminal Flag tag (FIGURE A2.1, panel B) into several different backgrounds, including Y131, FY406, DY20D, and JHY205. We analyzed the trimethylation levels of both H3K4 and H3K79 as well as the monoubiquitination of H2B in these backgrounds (FIGURE A2.2, panels A and B). Our antibody generated in this study is specific for monoubiquitinated H2B in yeast and is capable of detecting this modification in the presence or absence of a Flag tag (FIGURE A2.1, panel A; FIGURE A2.2, panel A; FIGURE A2.3, panel B; FIGURE A2.5, panel B). When this antibody was used, Flag-less wild-type H2B showed a band migrating slightly faster than that of Flag-tagged wild-type H2B as a result of the deletion of the Flag tag (FIGURE A2.2, panel A). However, no monoubiquitinated form of H2B was detected from K123A mutants in both Y131 and FY406 backgrounds and K123R mutants in DY20D and JHY205, regardless of the attachment of a Flag tag to H2B (FIGURE A2.2, panels A and B). Furthermore,

we tested these strains for the methylation of histone H3K4 and H3K79 (FIGURE A2.2). Regardless of the presence or absence of a Flag-tag on H2B, H3K4 methylation was detected in strains bearing wild-type H2B but not in strains in which H2B carries a point mutation at the K123 residue. Similar strains were generated independently in all of our laboratories, and we have each confirmed that there is no difference between Flag-tagged H2B and untagged H2B and that all of the histone modifications are consistent among the different strain backgrounds, including the FY406 background used in the study performed by Foster and Downs ((FOSTER and DOWNS 2009), FIGURE A2.2). Therefore, we conclude that H3K4 and H3K79 trimethylation is indeed solely dependent on H2B monoubiquitination regardless of an additional alteration to the H2B sequence or strain backgrounds.

The Y131 strain was originally generated for plasmid shuffling of H2A-H2B genes, as a simultaneous deletion of the *HTA1-HTB1* and *HTA2-HTB2* loci is lethal (ROBZYK *et al.* 2000). The genotype of Y131 describes that *hta1-htb1* was replaced with *LEU2*, whereas *hta2-htb2* was replaced with *URA3* in the presence of a *HIS3 HTA2-HTB2* plasmid. After selection for tight 5-FOA resistance, a *URA3* plasmid carrying *HTA1-HTB1* was substituted for the *HIS3-HTA2-HTB2* plasmid, thus creating the shuffle strain that was used to introduce a *HIS3*-marked plasmid containing *HTA1* and *Flag-HTB1*. The Y131 strain expressing Flag-tagged wild-type H2B under either glucose or galactose media has normal levels of H2B monoubiquitination and methylation of H3K4 and K79 (FIGURE A2.3, panel A, lanes 3–6). To our surprise, when Flag-H2BK123R in the Y131 background was grown continuously in a galactose-containing media, the methylation of both of the H3 residues was present at levels close to those in the wild-type cells (FIGURE A2.3, panel A, lanes 7–10). Because the N-terminus of H2BK123R was tagged with Flag, we tested for the presence of monoubiquitination using a Flag antibody. There was no slower-migrating band in the histone H2BK123R bearing strain, indicating that there is no monoubiquitinated form of

Flag-H2B (FIGURE A2.3, panel A, lanes 3–10). The H3K4 and H3K79 methylation patterns appear to be normal.

Because H2B monoubiquitination is required for the methylation of these lysine residues, we suspected that one of the two genomic *HTB* genes was not deleted in Y131 and that the genomic wild-type H2B was expressed in the K123R mutant strain in a galactose-dependent manner. We tested this possibility by using our antibody specific to monoubiquitinated histone H2B (FIGURE A2.3, panel B). In wild-type Y131 under glucose media, the Flag-tagged wild-type H2B produced only an upper band (Flag-tagged, monoubiquitinated H2B; FIGURE A2.3, panel B, lane 5, blue arrow). However, in wild-type Y131 under galactose media there are two bands (FIGURE A2.3, panel B, lane 6). The upper band is Flag-tagged, monoubiquitinated histone H2B, and the lower band is Flag-less, monoubiquitinated histone H2B (FIGURE A2.3, panel B, lane 6). Similarly, the Flag-H2BK123R strain shows no H2B monoubiquitination under the glucose media (FIGURE A2.3, panel B, lane 7). However, the Flag-tagged H2BK123R strain grown under galactose media shows the band corresponding to Flag-less, monoubiquitinated histone H2B (FIGURE A2.3, panel B, lane 8). We have also detected the expression of wild-type H2B under the galactose condition when using H2B-specific antibodies (*unpublished data*). This suggests that Y131 contains a galactose-inducible version of wild-type H2B somewhere in the genome.

To understand the basis for these results between the two growth conditions, we sequenced the genomic regions around the deleted *hta1-htb1* (chromosome IV) and *hta2-htb2* (chromosome II) genes in strain Y131. We found that intact *HTA2* and *HTB2* genes were still present on chromosome II in the Y131 strains, and surprisingly, the bidirectional *GAL1/10* promoter was inserted between the two genes to exactly replace the bidirectional *HTA2-HTB2* promoter (FIGURE A2.4). The *GAL10* promoter drives the expression of *HTA2*, and *GAL1* drives

the expression of *HTB2*. The presence of the *GAL1/10* promoter explains why wild-type H2B was expressed only under the galactose media and not under the glucose media. It remains somewhat of a mystery how *GAL1/10* was inserted exactly between the two genes in the first place, although the *HIS3* plasmid in the original Y131 strain carried a *GAL1/10*-regulated *HTA2-HTB2* locus that may have been incorporated into the genome by a rare recombination event. Because Y131 is a widely used strain, mutants that affect *GAL1/10* transcription may not appropriately repress wild-type *HTA2-HTB2* in glucose in this strain, and data obtained from its usage under prolonged growth in galactose conditions must be interpreted carefully. Studies using this strain to analyze effects of the H2B-K123R mutation on *GAL1* transcription were performed for short periods of galactose induction (2 hr) when the expression of the genomic *HTA2-HTB2* genes was not detected, and the transcription results have been recapitulated in other strain backgrounds ((XIAO *et al.* 2005); *unpublished data*).

Finally, we have now generated a histone H2A and H2B alanine mutant library in the FY406 background (FIGURE A2.5, panel A) in addition to the library we reported earlier in Y131 (NAKANISHI *et al.* 2008), as one may wish to use this collection under galactose conditions for different genetic and biochemical screens. From our new library in FY406, we found three H2A (Y58, E62, and D91) residues and one H2B (L109) residue that are essential for viability. These data are in agreement with our previous published results in Y131 (NAKANISHI *et al.* 2008). Furthermore, we tested our new library for H3K4 methylation and H2B monoubiquitination and identified the same residues in Y131, specifically those that regulate normal levels of H3K4 methylation in FY406, as well (FIGURE A2.5, panel B; (NAKANISHI *et al.* 2008)). These residues include four residues within H2A (Glu65, Leu66, Asn69, and Asp73) and three residues within H2B (His 112, Arg119, and Lys123). This observation is consistent with our previous findings. Thus, strain differences did not cause any discordant results when grown under glucose media.

In summary, our data clearly demonstrate that monoubiquitination of histone H2B on lysine 123 and the machinery required for its implementation are the sole requirements for the regulation of the trimethylation of H3K4 and H3K79 in yeast *S. cerevisiae*. Our collective experiments indicate that there are no "unknown" mutations as proposed by Foster and Downs (2009) that function with H2BK123R in the regulation of H3K4 and H3K79 trimethylation. Furthermore, we have discovered that the widely used Y131 strain background expresses a previously undetected copy of the *HTA2-HTB2* genes when this strain is grown for many generations in the presence of galactose. Therefore, we generated the entire *h2a*/*h2b* mutant collection in a background that can be readily used under galactose conditions and have now made this collection available to the entire scientific community.

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TABLE A2.1 | Strains used in this study

Strain	Genotype	Source/Reference
Y131	*MAT***a** *(hta1-htb1)Δ::LEU2, (hta2-htb2)Δ::URA3* and selected for strong 5-FOA-r, *leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can 1-100* [pRS426-*HTA1-HTB1-URA3*]	(ROBZYK *ET AL.* 2000)
YSN430	*MAT***a** *(hta1-htb1)Δ::LEU2, (hta2-htb2)Δ::URA3* and selected for strong 5-FOA-r, *leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can 1-100* [pZS145-*HTA1-Flag-HTB1-HIS3*]	(NAKANISHI *et al.* 2008)
YSN536	*MAT***a** *(hta1-htb1)Δ::LEU2, (hta2-htb2)Δ::URA3* and selected for strong 5-FOA-r, *leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can 1-100* [pZS145-*HTA1-Flag-htb1K123A-HIS3*]	(NAKANISHI *et al.* 2008)
YSN66	*MAT***a** *(hta1-htb1)Δ::LEU2, (hta2-htb2)Δ::URA3* and selected for strong 5-FOA-r, *leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can 1-100* [pSN888-*HTA1-HTB1-HIS3*]	This study
YSN68	*MAT***a** *(hta1-htb1)Δ::LEU2, (hta2-htb2)Δ::URA3* and selected for strong 5-FOA-r, *leu2-3,-112 his3-11,-15 trp1-1 ura3-1 ade2-1 can 1-100* [pSN890-*HTA1-htb1K123A-HIS3*]	This study
FY406	*MAT***a** *(hta1-htb1)Δ::LEU2, (hta2-htb2)Δ::TRP1, his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128δ* [pSAB6 *(HTA1-HTB1-URA3)*]	(HIRSCHHORN *et al.* 1995)
YSN545	*MAT***a** *(hta1-htb1)Δ::LEU2, (hta2-htb2)Δ::TRP1, his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128δ* [pZS145-*HTA1-Flag-HTB1-HIS3*]	This study
YSN763	*MAT***a** *(hta1-htb1)Δ::LEU2, (hta2-htb2)Δ::TRP1, his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128δ* [pZS145-*HTA1-Flag-htb1K123A-HIS3*]	This study
YSN61	*MAT***a** *(hta1-htb1)Δ::LEU2, (hta2-htb2)Δ::TRP1, his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128δ* [pSN888-*HTA1-HTB1-HIS3*]	This study
YSN63	*MAT***a** *(hta1-htb1)Δ::LEU2, (hta2-htb2)Δ::TRP1, his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128δ* [pSN890-*HTA1-htb1K123A-HIS3*]	This study
BY4742	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*	Open Biosystems
JL026	*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 rad6Δ::kanMX*	Open Biosystems
DY20D	*MAT***a** *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 hhf2-hht2::NAT hta1-htb1::HPH hhf2-hht2::KAN hta2-htb2::NAT* [pJH33-*HHT2-HHF2-HTA1-HTB1-URA3*]	M. Smith[a] & C.D. Allis[b]
YAF120	*MAT***a** *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 hhf2-hht2::NAT hta1-htb1::HPH hht1-hht2::KAN hta2-htb2::NAT* [pRS315-*HHT2-HHF2-HTA1-Flag-HTB1*]	(FLEMING *et al.* 2008)
YAF121	*MAT***a** *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 hhf2-hht2::NAT hta1-htb1::HPH hht1-hht2::KAN hta2-htb2::NAT* [pRS315-	(FLEMING *et al.* 2008)

	HHT2-HHF2-HTA1-Flag-htb1K123R]	
YJL031	*MAT***a** *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 hhf2-hht2::NAT hta1-htb1::HPH hht1-hht2::KAN hta2-htb2::NAT* [pRS315-*HHT2-HHF2-HTA1-HTB1*]	This study
YJL032	*MAT***a** *leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 hhf2-hht2::NAT hta1-htb1::HPH hht1-hht2::KAN hta2-htb2::NAT* [pRS315-*HHT2-HHF2-HTA1-htb1K123R*]	This study
JHY205	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3 hhf2-hht2::NAT hta1-htb1::HPH hhf2-hht2::KAN hta2-htb2::NAT* [pJH33-*HHT2-HHF2-HTA1-HTB1-URA3*]	(AHN *et al.* 2005)
YSF200	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3 hhf2-hht2::NAT hta1-htb1::HPH hht1-hht2::KAN hta2-htb2::NAT* [pRS315-*HHT2-HHF2-HTA1-Flag-HTB1*]	This study
YSF201	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3 hhf2-hht2::NAT hta1-htb1::HPH hht1-hht2::KAN hta2-htb2::NAT* [pRS315-*HHT2-HHF2-HTA1-Flag-htb1K123R*]	This study
YJL033	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3 hhf2-hht2::NAT hta1-htb1::HPH hht1-hht2::KAN hta2-htb2::NAT* [pRS315-*HHT2-HHF2-HTA1-HTB1*]	This study
YJL034	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3 hhf2-hht2::NAT hta1-htb1::HPH hht1-hht2::KAN hta2-htb2::NAT* [pRS315-*HHT2-HHF2-HTA1-htb1K123R*]	This study
YKG001	*MAT***a** *(hta1-htb1)Δ::LEU2, (hta2-htb2)Δ::TRP1, his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128δ* [pZS145 *HTA1-FLAG-HTB1-HIS3*]	This study
YKG002	*MAT***a** *(hta1-htb1)Δ::LEU2, (hta2-htb2)Δ::TRP1, his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128δ* [pZS146 *HTA1-FLAG-htb1K123R-HIS3*]	This study
YKG003	*MAT***a** *(hta1-htb1)Δ::LEU2, (hta2-htb2)Δ::TRP1, his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128δ* [pKG3 *HTA1 -HTB1-HIS3*]	This study
YKG004	*MAT***a** *(hta1-htb1)Δ::LEU2, (hta2-htb2)Δ::TRP1, his3Δ200 leu2Δ1 ura3-52 trp1Δ63 lys2-128δ* [pKG4 *HTA1 -htb1K123R-HIS3*]	This study

[a] The University of Virginia Health System, Charlottesville, VA
[b] The Rockefeller University, New York, NY

TABLE A2.2 | Key for the histone H2A (*HTA1*) library in FY406 background

	1	2	3	4	5	6	7	8	9	10	11	12
Plate 1												
A	S1A	S10A	G23A	V31A	N39A	P49A	Y58A	N69A	I79A	I88A	K96A	Q105A
B	G2A	K13A	L24A	H32A	Y40A	V50A	L59A	R72A	I80A	R89A	L97A	G106A
C	G3A	S15A	T25A	R33A	Q42A	Y51A	E62A	D73A	P81A	N90A	L98A	G107A
D	K4A	Q16A	F26A	L34A	R43A	L52A	I63A	N74A	R82A	D91A	G99A	V108A
E	G5A	S17A	P27A	L35A	I44A	T53A	L64A	K75A	H83A	D92A	N100A	L109A
F	G6A	R18A	V28A	R36A	G45A	V55A	E65A	K76A	L84A	E93A	V101A	P110A
G	K7A	S19A	G29A	R37A	S46A	L56A	L66A	T77A	Q85A	L94A	T102A	N111A
H	G9A	K21A	R30A	G38A	G47A	E57A	G68A	R78A	L86A	N95A	I103A	I112A
Plate 2												
A	H113A	S121A	WT									
B	Q114A	K123A										
C	N115A	T125A										
D	L116A	K126A										
E	L117A	S128A										
F	P118A	Q129A										
G	K119A	E130A										
H	K120A	L131A										

TABLE A2.3 | Key for the histone H2B (*HTB1*) library in FY406 background

	1	2	3	4	5	6	7	8	9	10	11	12
Plate 1												
A	S1A	P13A	S24A	R32A	S41A	K49A	I57A	L65A	F73A	L83A	S93A	L103A
B	K3A	E15A	T25A	S33A	S42A	Q50A	S58A	N66A	E74A	Y86A	R95A	I104A
C	E5A	K16A	S26A	K34A	Y43A	T51A	Q59A	S67A	R75A	N87A	E96A	L105A
D	K6A	K17A	T27A	R36A	I44A	H52A	K60A	F68A	I76A	K88A	I97A	P106A
E	K7A	P18A	D28A	K37A	Y45A	P53A	S61A	V69A	T78A	K89A	Q98A	G107A
F	P8A	K21A	G29A	E38A	K46A	D54A	M62A	N70A	E79A	S90A	T99A	E108A
G	S10A	K22A	K30A	T39A	V47A	T55A	S63A	D71A	S81A	T91A	V101A	L109A
H	K11A	T23A	K31A	Y40A	L48A	G56A	I64A	I72A	K82A	I92A	R102A	K111A
Plate 2												
A	H112A	T122A	WT									
B	V114A	K123A										
C	S115A	Y124A										
D	E116A	S125A										
E	G117A	S126A										
F	T118A	S127A										
G	R119A	T128A										
H	V121A	Q129A										

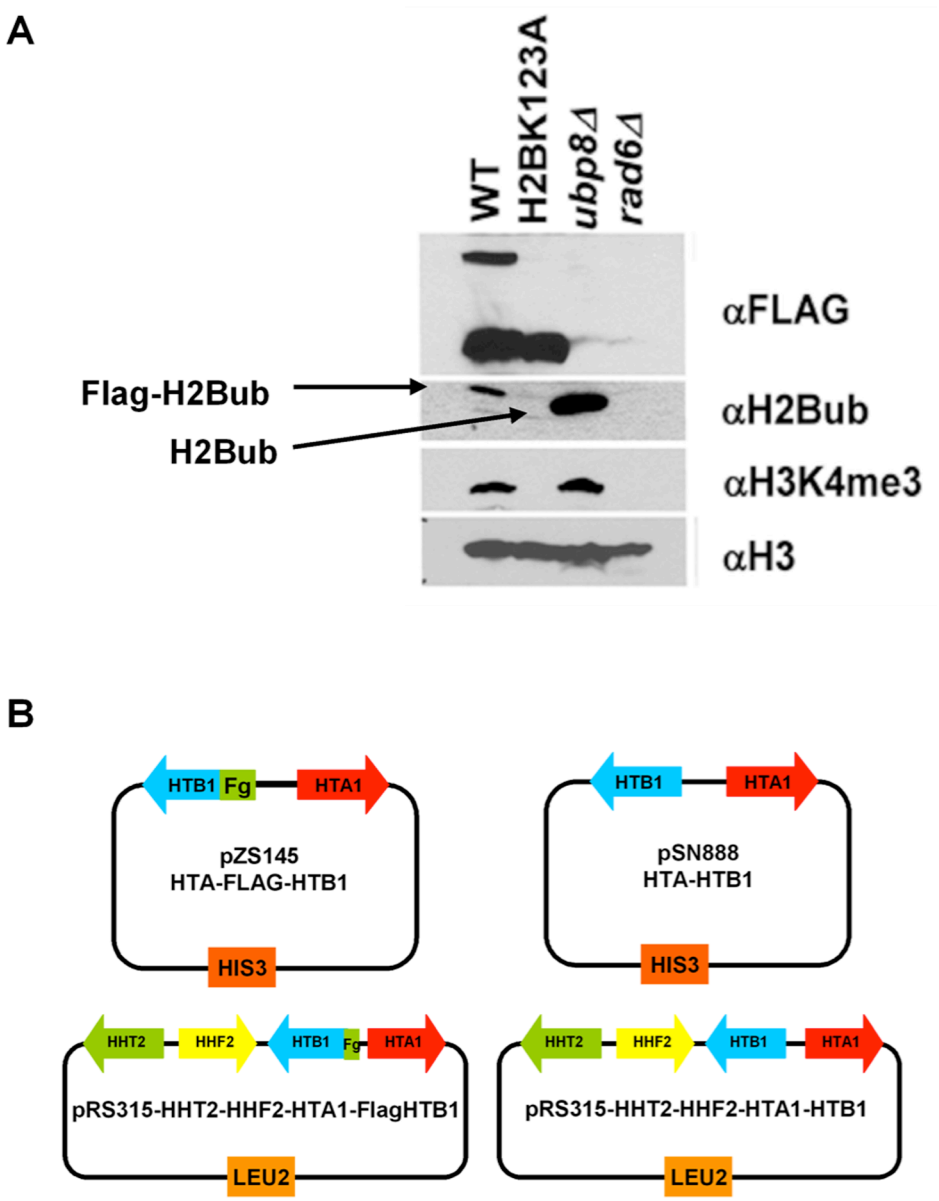

FIGURE A2.1 | Generation of antibodies specific to K123-monoubiquitinated H2B. (**A**) Development of polyclonal antibodies specific to monoubiquitinated H2B. Ubiquitinated H2B–specific antibodies generated in rabbit were affinity purified and characterized by Western blot analysis of whole cell extracts prepared from wild type (WT), H2BK123A, *ubp8Δ*, and *rad6Δ*. Both wild-type and *ubp8Δ* strains show the presence of monoubiquitinated H2B (24 kD and 23 kD, respectively); however, an increased amount of the ubiquitinated form of H2B was observed in the *ubp8Δ* strain as expected. No bands were detected in either H2BK123A or the *rad6Δ* strains, showing that our H2Bub antibody is capable of specifically recognizing ubiquitinated H2B in yeast. (**B**) Schematics of the plasmids used in this study. pSN888 was generated from pZS145 by deletion of the N-terminal Flag tag (Fg) from the *HTB1* gene. Similarly, the Flag on H2B was removed in pRS315-*HHT2-HHF2-HTA1-FlagHTB1* to generate H2B Flagless strains.

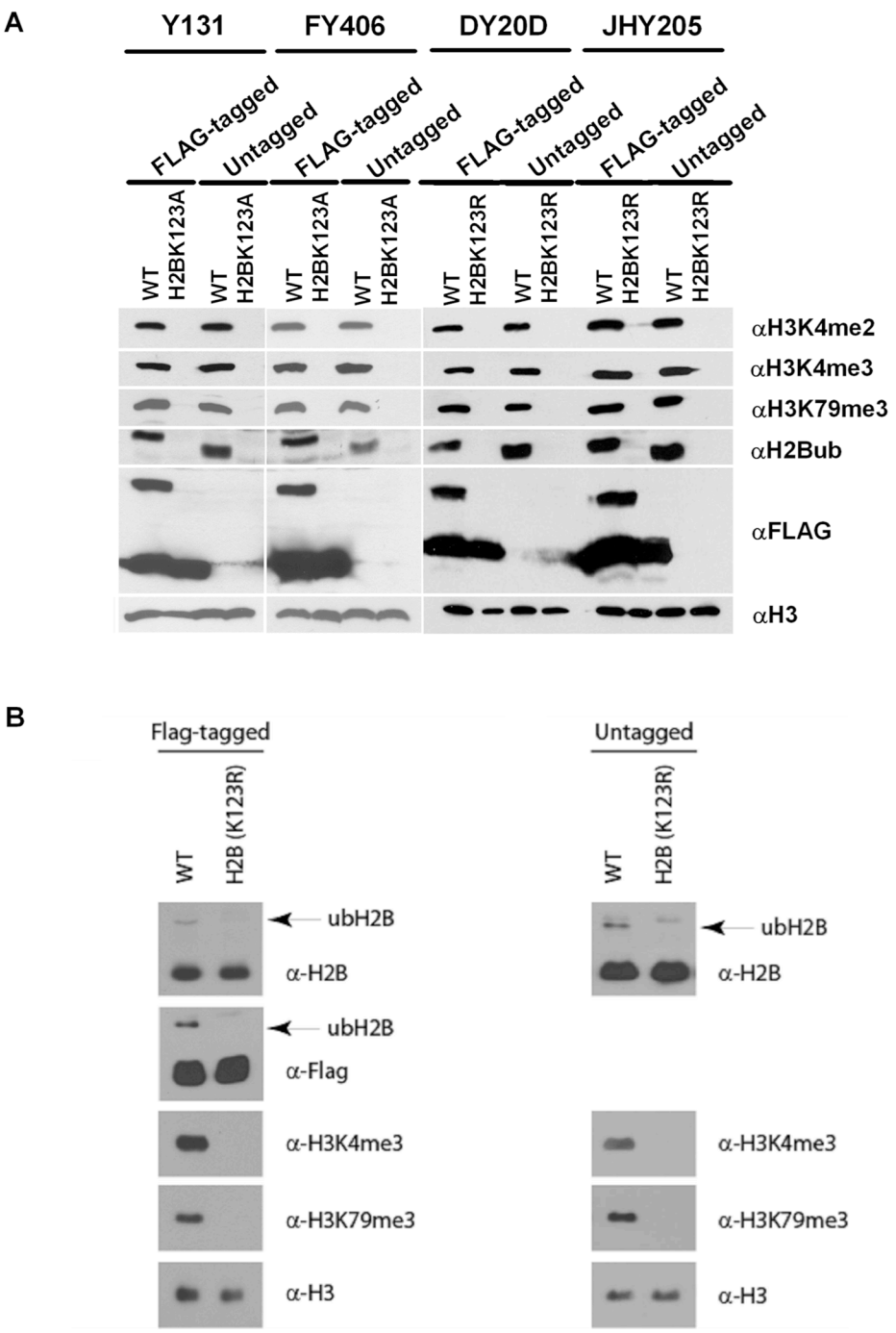

FIGURE A2.2 | Di- and trimethylation of histone H3K4 and trimethylation of H3K79 are dependent solely on monoubiquitination of H2BK123. (**A**) Western blotting of whole cell extracts from strains transformed with a plasmid carrying wild-type (WT) H2B or mutant H2BK123 (K123A; K123R) either with or without an N-terminal Flag tag. Cell extracts were prepared from wild-type H2B or H2BK123 mutants in three different strain backgrounds and were subjected to SDS-PAGE and analyzed by Western blot analysis with antibodies to dimethyl H3K4, trimethyl H3K4, trimethyl H3K79 (α-H3K4me2, α-H3K4me3, and α-H3K79me3),

monoubiquitinated H2BK123 (α-H2Bub), or Flag (α-FLAG). An antibody against H3 (α-H3) was used as a loading control. The calculated molecular masses of H2B, ubiquitinated H2B, and H3 are 14 kD, 23 kD, and 15 kD, respectively. The calculated molecular masses of Flag-tagged H2B and ubiquitinated, Flag-tagged H2B are 15 kD and 24 kD, respectively. White lines indicate that intervening lanes have been spliced out. (**B**) Western blotting of whole cell extracts from strains transformed with plasmid carrying wild-type or mutant H2BK123R in FY406 background.

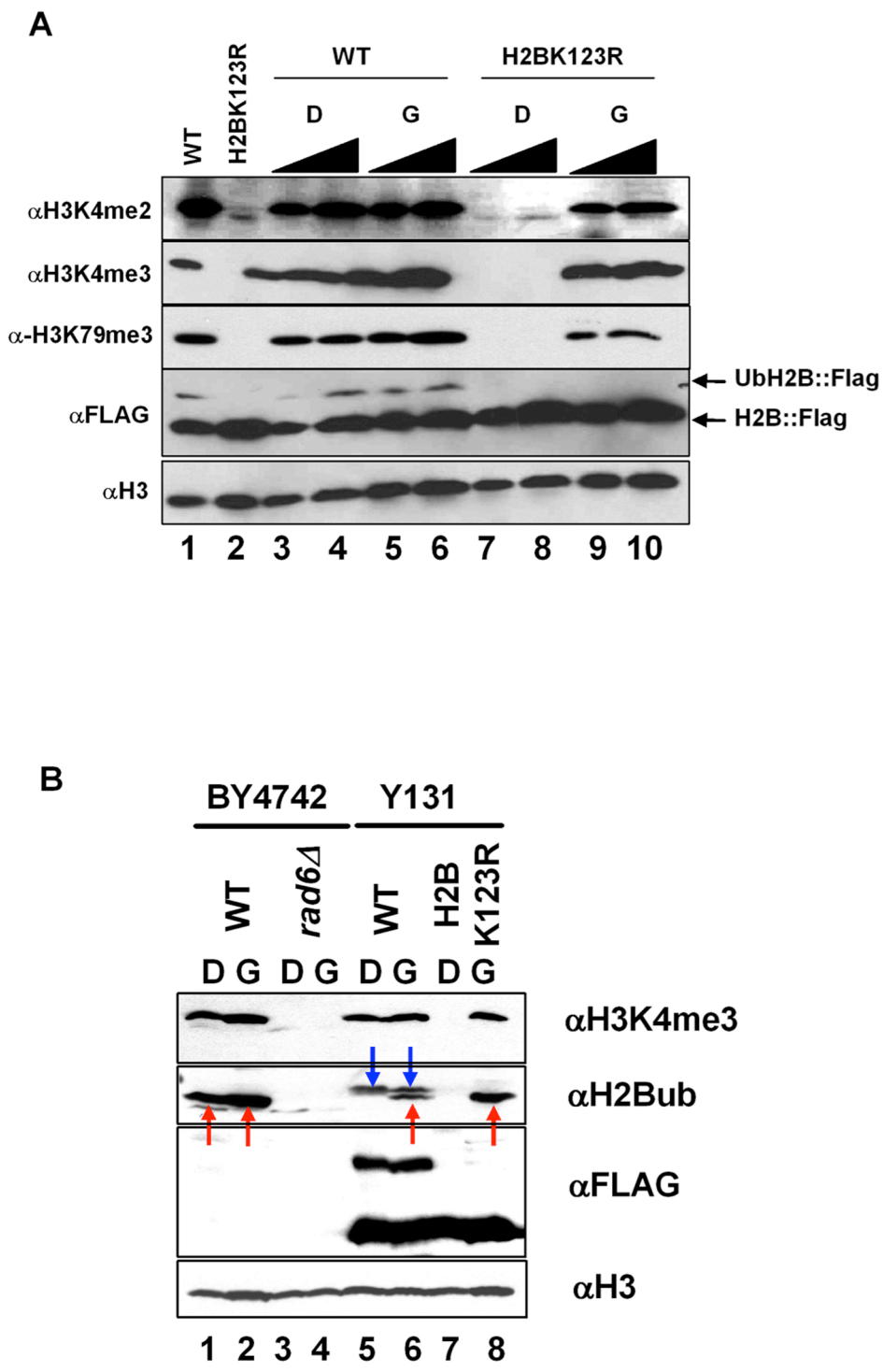

FIGURE A2.3 | The H2A/H2B shuffle strain Y131 contains a galactose-regulated copy of *HTA2-HTB2* genes on chromosome II. (**A**) Western blot analysis of whole cell extracts prepared from Flag-tagged wild-type H2B and Flag-H2BK123R grown under either glucose or galactose media. Cell extracts were subjected to SDS-PAGE and Western analysis with antibodies specific to H3K4me2, H3K4me3, H3K79me3, and Flag. An antibody against H3 (α-H3) was used as a loading

control. Triangles describe the increasing amounts of proteins loaded onto the gel. Letters D and G denote glucose and galactose, respectively. (**B**) Western analysis of whole cell extracts prepared from wild-type (WT) BY4742 (FM392) and its derivative *rad6Δ*, Flag-H2B (Y131 background), and Flag-H2BK123R (Y131 background). Cell extracts were subjected to SDS-PAGE and Western analysis with antibodies to trimethyl H3K4 (α-H3K4me3), monoubiquitinated H2BK123 (α-H2Bub), or Flag (α-FLAG). An antibody against H3 (α-H3) was used as a loading control. The H2B monoubiquitination-specific antibody detected a faster migrating band, which is indicated by red arrows. This band represents an untagged version of H2B, which is only seen in Y131 when cells are grown in galactose-containing media. Blue arrows indicate the slower migrating Flag-tagged, monoubiquitinated H2B seen under both dextrose and galactose growth conditions only in wild-type cells and not H2BK123R. Black lines indicate that intervening lanes have been spliced out.

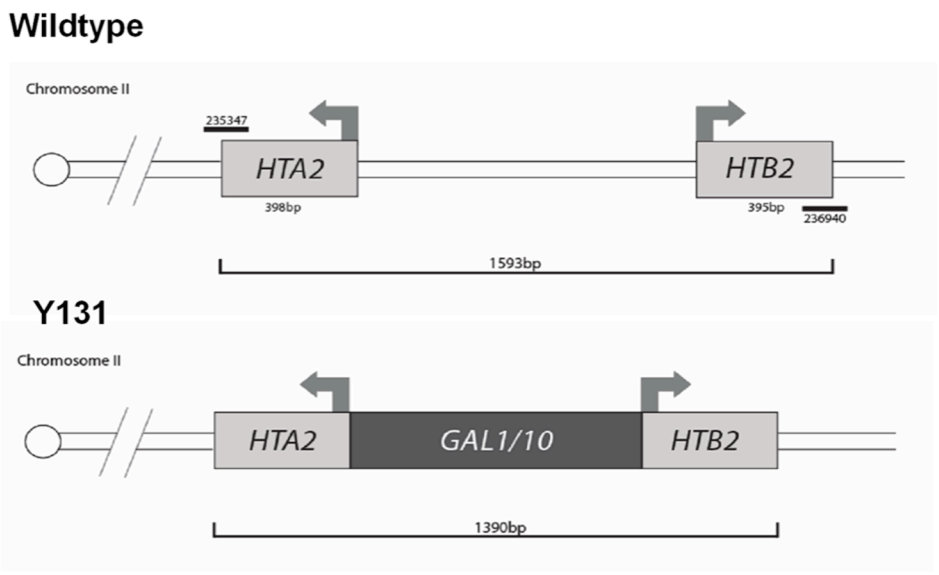

FIGURE A2.4 | In the Y131 strain, the *GAL1/10* promoter is inserted between *HTA2* and *HTB2* on chromosome II. Schematic of the regions containing *HTA2-HTB2* genes on chromosome II. Although this region was deleted and replaced with *URA3* in the Y131 strain, a wild-type copy of *HTA2-HTB2* controlled by the *GAL1/10* promoter is present in this region.

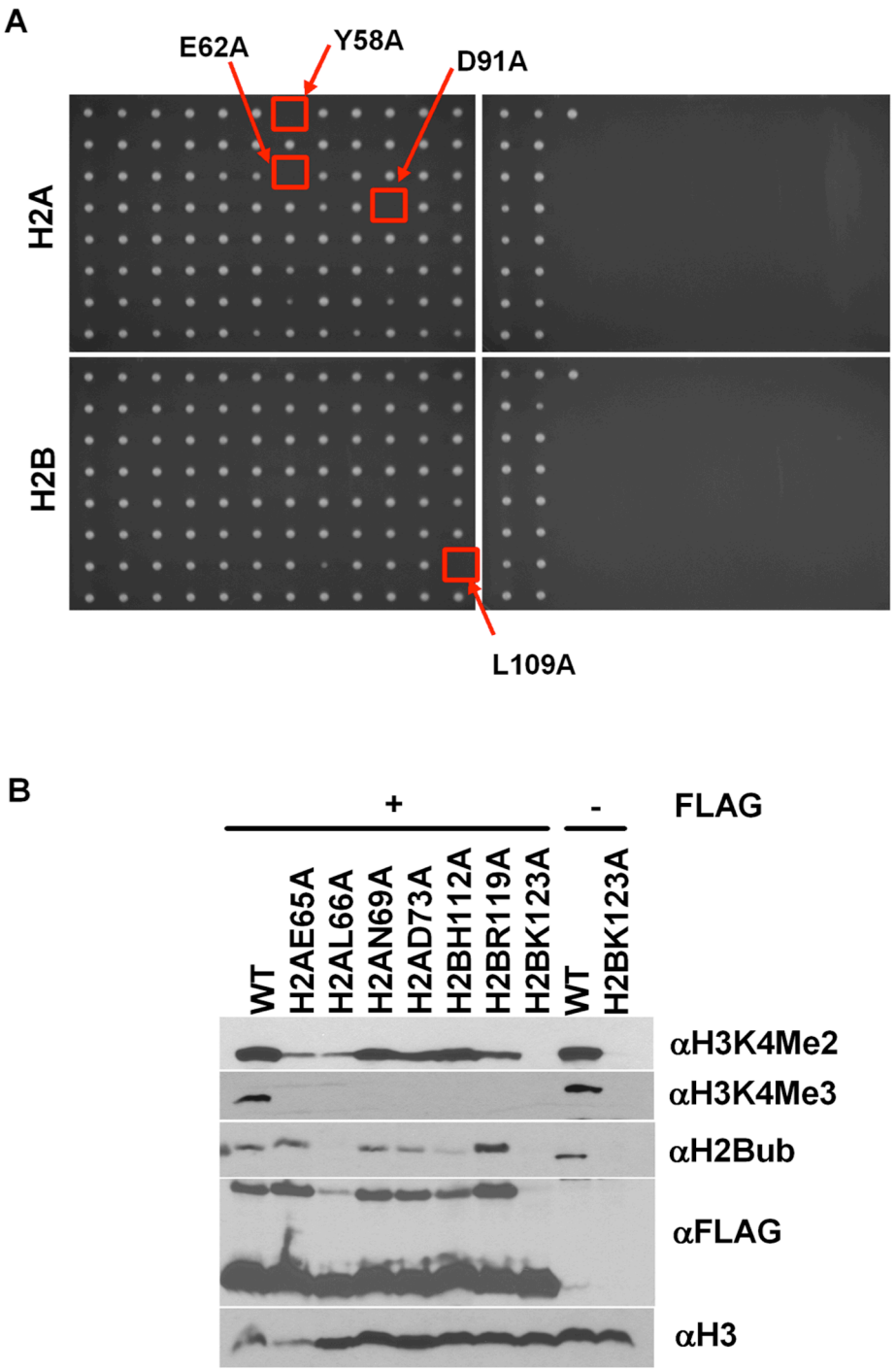

FIGURE A2.5 | Generation of the entire H2A/H2B alanine-scanning collection in an FY406 background. (**A**) The H2A/H2B alanine-scanning library was generated as described in our previous study (NAKANISHI *et al.* 2008). The complete removal of wild-type (WT) H2B was ensured by multiple rounds of 5-FOA selection and verified by sequencing. Each colony represents a strain expressing histones containing a single alanine substitution mutation of each of the residues of H2A and H2B. Red squares indicate the location of strains that are inviable in

SD media containing 5-FOA (lethal mutants). For the key to the corresponding mutant strains within each plate, see TABLES A2.2 and A2.3. (**B**) Western blot analysis of mutant strains in the FY406 background identified as defective for proper methylation of H3K4. Cell extracts prepared from each mutant strain were subjected to SDS-PAGE and analyzed by Western analysis with antibodies to dimethyl H3K4, trimethyl H3K4 (α-H3K4me2 and α-H3K4me3, respectively), monoubiquitinated H2BK123 (α-H2Bub), or Flag (α-FLAG). An antibody against H3 (α-H3) was used as a loading control. White lines indicate that intervening lanes have been spliced out.

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