INVESTIGATING HISTONE METHYLATION IN YEAST; REGULATION OF H3K4me3, AND THE ROLE OF THE METHYL-HISTONE BINDING DOMAINS OF ISW1B

by

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B.Sc., The University of Calgary, 2005

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

(Biochemistry and Molecular Biology)

THE UNIVERSITY OF BRITISH COLUMBIA
(VANCOUVER)

July 2010

Abstract

Chromatin structure is regulated in part by the post-translational modification of histones. Histone methylation is highly conserved amongst eukaryotes, and is arguably one of the best characterized indicators of whether a gene is repressed or active. There are several unique states of histone methylation, each capable of specific downstream effects through the recruitment of highly specific methyl-histone binding domains and their associated chromatin-altering protein complexes. Histone H3 lysine 4 tri-methylation (H3K4me3) is a well known mark of actively transcribed genes, and co-localizes with histone H3 lysine 14 acetylation (H3K14ac), another mark of actively transcribed genes. The discovery that H3K4me3 is lost when H3K14 is substituted with another residue, led to the possibility of cross-talk between H3K4me3 and H3K14ac. The first part of this thesis demonstrates that H3K4me3 is indeed dependent on H3K14ac. Furthermore, we go on to show for the first time, that H3K14ac protects H3K4me3 from demethylation by the histone demethylase Jhd2.

Though the mechanisms by which methyl-histone binding domains recognize methylated chromatin have been well studied, the specific physiological roles of the numerous methyl-histone binding domains have yet to be investigated. Isw1 is a highly conserved catalytic subunit of several ATP-dependent chromatin-modifying complexes. One of these complexes, Isw1b, has two putative methyl-histone binding domains, the PHD finger of Ioc2, and the PWWP domain of Ioc4. The second part of this thesis investigates the role that these domains play in the localization of the Isw1b complex to a specific region of the genome. Though we were unable to demonstrate a role for the PHD finger of Ioc2, we did demonstrate that the PWWP domain of Ioc4 is involved in

chromatin localization. Additionally we found that Ioc2's ability to bind chromatin is negatively affected by association with Ioc4 in the Isw1b complex, though the significance of this finding has yet to be determined.

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List of Abbreviations

5-FOA	5-FluoroOrotic Acid
ac	acetyl
ADA	transcriptional ADApter
ATP	Adenosine Tri-phosphate
BHC	BRAF-HDAC Complex
bp	Base Pairs
BRE	BREfeldin A sensitivity
BSA	Bovine Serum Albumin
CHD	Chromatin organization modifier, Helicase, and DNA-binding domains
ChIP	Chromatin Imuno-precipitation
COMPASS	COMplex Proteins ASsociated with Set1
CTD	C-terminal Domain of RNA Pol II
CTK	Carboxy-Terminal domain Kinase
DMSO	Di-Methyl-SulfOxide
DNA	DeoxyriboNucleic Acid
DOT	Disruptor Of Teolomeric silencing
ECM	ExtraCellular Mutant
GCN	General Control Non-derepressible
GIS	GIg 1-2 Suppressor
H1	Histone 1
H2A	Histone 2A
H2B	Histone 2B
H3	Histone 3
H3K#	Histone 3 Lysine #
H4	Histone 4
HA	influenza HemAgglutinin epitope
HAT	Histone Acetyl Transferase
HDAC	Histone DeACetylase
HHF	Histone H Four
HHT	Histone H Three
HIS	HIStadine
HMGN	High Mobility Group N
HMT	Histone Methyl-Transferase
HP1	Heterochromatin Protein 1
IgG	Imunoglobulin G
INO	INOsitol
IOC	Isw One Complex

ISW	Imitation SWitch
JHD	Jumonji c domain Histone Demethylase
JHDM	Jumonji c domain Histone DeMethylase
JMJC	JuMonJi C
KAN	KANomycine
LEU	LEUcine
LSD	Lysine-Specific Demethylase
MBD	Methyl-lysine Binding Domain
MBT	Malignant Brain Tumor domain
me	methyl
MET	METhionine
MOT	MOdifier of Transcription
NuA	Nucleosome Acetyl transferase
OD	Optical Density
PAF	RNA Polymerase-Associated Factor
PBS-T	Phosphate Buffered Saline – Tris
PCR	Polymerase Chain Reaction
PHD	Plant Homeo Domain
PWWP	Proline Tryptophan Tryptophan Proline domain
RNA	RiboNucleic Acid
RNAP	RiboNucleic Acid Polymerase
RPD	Reduces Potassium Dependency
RPH	Regulator of PHr1
RSC	Remodle the Structure of Chromatin
SANT	Swi-snf, Ada, N-cor, TfIIb
SAS	Something About Silencing
SDC	Set1c, homologue of Dpy30 from C.elegans
SDS	Sodium Dodecyl Sulphate
SET	Su(var)3-9, Enhancer-of-zeste, Trithorax
SIR	Silent Information Regulator
SLIDE	Sant Like Domain
SWD	Set1c, WD40 repeat protein
SWI	SWItch
SWR	SWi2/snf2 Related
TAP	Tandem Affinity Purification
TBP	Tata Binding Protein
TrxG	Trithorax Group
URA	URAcile
WD40	Tryptophan Aspartic acid containing region repeated 40 times domain
WT	Wilde Type

YPD	Yeast extract, Peptone, Dextrose

Nomenclature

The text follows the conventional method for designating genetic symbols and protein products. Alleles of wild type genetic loci are designated by italicized upper case letters (e.g. *IOC2*) while mutant genes are designated with italicized lower case letters (e.g. *ioc2*). Gene deletions are written as a mutation with a Greek ‘delta’ following the designation (e.g. *ioc2*□ indicates deletion of the *IOC2* locus). Insertion mutations are indicated with the symbol ::. For example *ioc2::HISMX6* indicates that the *HISMX6* gene is inserted within the *IOC2* locus. Gene products are not italicized and only the first letter is capitalized (e.g. Ioc2 is the gene product of the *IOC2* locus).

Acknowledgments and Dedication

First and foremost I offer my gratitude to Dr. LeAnn Howe, my supervisor, whose guidance, wisdom, and support made this thesis possible. It is difficult to express just how grateful I am to LeAnn for all she has done to help bring this thesis to conclusion. Next I thank my fellow lab members, especially Vicki MacDonald, for making the lab an enjoyable place to work with her positive attitude, and for keeping the lab running far more efficiently then it would without her. Additionally I offer thanks to my fellow lab mates Jenifer Choi, Adam Chruscicki, and Dr. Lorena Rosaleny for their friendship, and being fun to work with, as well as for their help making strains and plasmids. I must also offer thanks to our undergrads Keegan Rowe and Eric Chi, for their help on this project. Last, but not least, I thank Erynne Green for an endless supply of clean glass wear and fresh media.

My gratitude also goes out to my committee members, Ivan Sadowski and Vivien Measday, especially for ideas during the difficult "find a phenotype for *isw1Δ*" stage of this thesis. As well as friends and family for endless support and encouragement, especially my Mom, who has always been understanding, no matter what.

Lastly I would like to dedicate this thesis to science, for science is the light that fends of the darkness.

Chapter 1 - Introduction

1.1 Chromatin

All the genetic information required to build and maintain an organism is contained within a set of double helical DNA molecules contained in every cell of that organism, with very few exceptions. In humans this information is encoded onto approximately 3 billon base pairs (bp) of DNA. If both copies of the 23 chromosomes were aligned end to end, they would be over 2m in length. Every living cell, with few exceptions, contains the entire genome of its species, which must not only be compacted into the confines of a single cell, or nucleus, but must also be organized and regulated. This insures that genetic information is available when it is needed. In eukaryotes the protein and nucleic acid structure responsible for this packaging is chromatin.

Chromatin structure was first postulated to regulate access to DNA after the discovery of heterochromatin, which is seen as densely staining regions on chromosomes that contain, for the most part, transcriptionally inactive DNA. The light staining, actively transcribed regions of chromatin are called euchromatin. Additionally there are regions of facultative heterochromatin that switch back and forth between heterochromatin and euchromatin as needed (Trojer and Reinberg., 2007). The mechanisms involved in determining if DNA is packaged in euchromatin or heterochromatin primarily revolve around the alterations to the basic structural unit of chromatin, the nucleosome.

Nucleosomes form the basic structure of chromatin. Each one consists of 147 base pairs of DNA wrapped around a core of two copies of each of the four histones H2A, H2B, H3, and H4 (Luger *et. al.,* 1997). Two H3-H4 dimers are bridged forming a

tetramer, which is flanked by two H2A-H2B dimers. Protruding out of the core nucleosomes, past the enwrapped DNA, are the unstructured N-terminal tails of the histones (Luger *et. al.,* 1997). This structure repeats approximately every 200bp along the DNA molecule of most eukaryotes (Kornberg, 1974) resulting in a 30-40 fold linear compaction of the DNA molecule (Luger *et. al.,* 1997). The nucleosome-bound DNA is further compacted into the 30nm fiber by a combination of inter-nucleosome contacts between the N-terminal tail of H4 with neighboring nucleosomes (Wang and Hayes, 2008), and histone H1 which binds and structures the inter-nucleosomal linker DNA (Thoma *et. al.,* 1979). Additionally, higher order folding structures are formed by a variety of inter-strand interactions (Kan, *et. al.,* 2009) (Horn and Pererson, 2002). By controlling which regions of DNA are exposed and which are not, these ordered chromatin structures represent one of the mechanisms involved in regulating access to the genome.

Even actively transcribed euchromatic chromatin is structured by nucleosomes, which would hinder transcription if there were not mechanisms in place to move them (Lorch *et. al.,* 1987). Initially histone octamers must be displaced from the DNA in promoter regions in order for the subunits of the pre-initation complex to access the promoter elemetns (Workman and Kingston, 1998). Then, during elongation, histone chaperones must disassemble and remove nucleosomes ahead of the transcriptional machinery and reassemble them behind RNA polymerase (Koning *et. al.,* 2007). Similarly, chromatin structure plays a role in DNA damage repair, replication, and recombination, as all manipulations of DNA in eukaryotes take place within the context

of chromatin. Thus understanding chromatin structure and function is an essential part of our understanding of eukaryotic genetics.

Investigation of the nucleosome's role in the regulation of transcription has revealed a vast array of functionally different chromatin altering mechanisms. For instance, AT rich regions of DNA seem to be unfavorable for wrapping around nucleosomes thus excluding them from these regions (Anderson, and Windom, 2001). This is especially evident in the AT rich promoters of *Saccharomyces cerevisiae,* which are predominantly nucleosome free. Furthermore the core histones of a nucleosome can be replaced with several variant forms resulting in altered chromatin properties (Sarma and Reinberg, 2005). Non-histone proteins can interact with chromatin to either stabilize structures, as is the case with the heterochromatin protein one (HP1) (Eissenberg *et. al.,* 1990), or destabilize structures such as the high mobility group N (HMGN) proteins (Bustin, 2001). Also, there are ATP-dependent chromatin-remodeling proteins that utilize the energy of ATP to actively move histones around on the DNA, thus exposing or covering different DNA segments (Flaus and Owen-Hughes, 2003). Further complicating these interactions are the post-translational histone modifications, consisting of small molecular additions placed on the core histones. All of these distinct mechanisms have been observed to functionally interact with one another resulting in a vast regulatory network constantly altering chromatin structure for various purposes and functions.

1.2 Post-translational modifications

Histones can be phosphorylated, methylated, ubiquitinated, and acetylated by a myriad of different enzymes. Usually, but not exclusively, occurring on the N-terminal histone tails (Fig. 1.1). These modifications correlate with various chromatin states and interact with chromatin altering mechanisms. The first observed correlation was between histone acetylation and active transcription (Hebbes *et. al.,* 1998). Transcriptionally active genes are packaged with acetylated histones and the level of histone acetylation correlates with the rate of transcription genome-wide. Research into the connection between histone acetylation and transcription exploded with the discovery that the yeast transcriptional co-activator Gcn5 was a histone acetyltransferase (Brownell *et. al.,* 1996). Acetylation of the basic lysines on the tail of histone H4 disrupts its inter-nucleosomal contacts inhibiting the formation of the 30nm fiber (Wang and Hayes, 2008), thus making the chromatin more accessible to transcriptional machinery. Additionally some chromatin remodeling enzymes that aid active transcription recognize acetylated histones and remain localized to these regions (Hassan *et. al.,* 2002). Similar correlations with transcription are observed with other histone modifications, including histone H3 lysine 4 and lysine 36 methylation.

Closer investigation of the correlation between histone modifications and actively transcribed DNA revealed that these modifications are targeted, not only to actively transcribed regions, but to specific parts of actively transcribed genes. This was determined by mapping the precise localization of acetylated and methylated histones across the entire *Saccharomyces cerevisiae* genome and demonstrated that certain modifications such as acetylation and some forms of methylation occur at the promoter

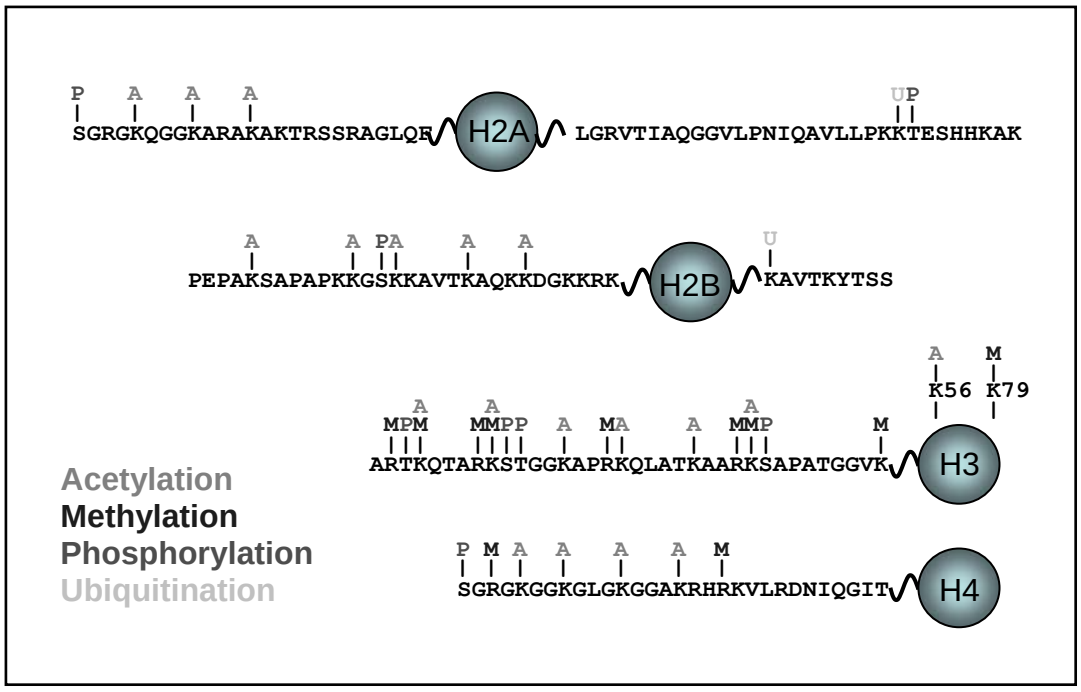


Figure 1.1 Post-translational modifications of human histones

Schematic of the sites for acetylation, methylation, phosphorylation, and ubiquitination on Human histones. Adapted from Bhaumik et al. 2007. Nature Struct. Molec. Biol. 14 p.1008

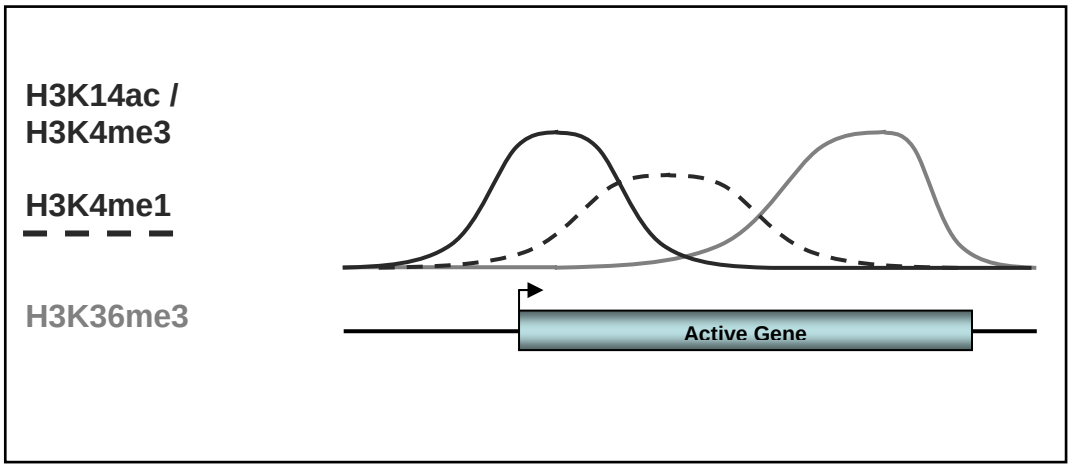


Figure 1.2 Localization of post-translational histone modifications of an actively transcribed gene

Schematic representation of the abundance of histone modifications on an average transcribed gene in yeast. The grey bar represents the entire transcribed unit and the arrow the transcriptional start site. Adapted from Pokholok *et al*., 2005. *Cell*, **122** p.517.

and 5' region of transcribed genes, while other forms of methylation occur at the 3' region, or equally spread across the entire coding region (Pokholok *et. al.,* 2005; Fig. 1.2). Similar mapping of mammalian post translational modifications (Wang *et. al.,* 2009), as well as other higher eukaryotes (Schübeler *et. al.,* 2004) (Schneider *et. al.,* 2003) and even *Arabidopsis thaliana* (Zhang *et. al.*, 2009), has demonstrated that these trends have been conserved through evolution.

A great deal of cross-talk has been observed between various histone modifications, which may help maintain correlative and anti-correlative associations of the modifications with each other. Cross-talk occurs on several levels, the simplest being that of H3K9, which is methylated in repressed chromatin and acetylated in active chromatin (Zhang *et.al.,* 2002) (Fischle *et. al.,* 2003). H3K9 cannot be both methylated and acetylated, and thus these marks are never found together. Another layer of cross-talk is the ability of one modification to inhibit or promote another. Again, using H3K9me as an example, once this repressive modification has been established, the non-histone protein HP1 is recruited to the site of methylation, which in turn recruits a histone deacetylase complex that removes acetylation from the neighbouring nucleosomes (Yamada *et. al.,* 2005). There are numerous examples of cross-talk between histone modifications. Of particular importance to this thesis is the relationship between H3K4 methylation and H3K14 acetylation, which will be discussed in subsequent sections.

1.3 Histone methylation

Histone methylation does not alter the charge of the methylated residue in the manner that acetylation and phosphorylation do, and unlike ubiquitination (8564 Da per ubiquitin), methylation results in a very minor change in mass of the residue (14 Da per methyl group). Yet, despite the seemingly benign nature of methylation, it is the most complex modification currently observed. Both lysines and arginines can be methylated, each with three different methylation states. Lysines can be mono-, di-, or tri- methylated, while arginines can be mono-, symmetrically di-, or asymmetrically di- methylated (Turner, 2005). Furthermore, methylation can correlate with a variety of chromatin structures and functions, depending on the methylation pattern of specific histone residues. For instance H3K4me3 peaks at the promoter of actively transcribed genes (Barski *et. al.,* 2007), while H3K36me3 localization tends to peak near the 3' end of actively transcribed genes (Pokholok *et. al.*, 2005). The complexity and specificity of the various methylation marks can be attributed to the highly specific, and tightly regulated, methytransferase enzymes that place them.

Transcription-associated histone methylation is best characterized in *S. cerevisiae*. There are three yeast histone residues that undergo methylation; H3K4, H3K36, and H3K79. Each is methylated by its own individual methyltransferase; Set1, Set2, and Dot1 respectively. Dot1 and H3K79 methylation function primarily to protect actively transcribed genes from the telomeric-silencing Sir proteins (Ng *et. al.,* 2002). H3K4me3 is catalyzed by Set1, a component of the COMPASS complex, which is localized to the promoters of actively transcribed genes due to its interaction with serine 5 phosphorylated C-terminal domain (CTD) of RNA polymerase II (RNAP II) (Krogan *et.*

al., 2003). Serine 5 is phosphorylated by Kin28, a TFIIH-associated kinase, during transcription initiation. When RNAP II transitions to an elongating form, the kinase Ctk1 phosphorylates serine 2 of the CTD and serine 5 phosphorylation is lost. As this happens COMPASS disassociates form RNAP II and Set1 can only di- or mono-methylate H3K4, resulting in a gradient of H3K4 tri-, to di-, to mono-methylation across the 5' end of the transcribed gene (Wood *et al.,* 2006). Furthermore, when Ctk1 phosphorylates serine 2 of the RNAP II CTD, Set2 becomes associated with the elongating polymerase, and begins methylating H3K36 (Krogan *et. al.,* 2003). This results in a counter gradient of H3K36 methylation that starts as light mono-methylation near the 5'end of the gene, and peaks as tri-methylation at the 3'end of the actively transcribed gene (Barski *et. al.,* 2007; Pokholok *et. al.*, 2005).

H3K4 methylation is a dynamic mark that is actively removed, and this removal has been associated with transcriptional repression, though the involved mechanisms are still under investigation. The recently discovered histone demethylases may be instrumental for the dynamic nature of histone methylation. Characterized by Shi *et. al.* in 2004, LSD1 was the first enzyme shown to be able to remove methylation from histones. LSD1 is a conserved protein capable of removing methyl groups from mono- and di-methylated lysines, specifically H3K4 (Shi *et. al.,* 2004). Later the Jumonji C (JmjC) domain family of proteins were found to demethylate the histones of *S. cerevisiae*, which lack an LSD1 homologue (Tsukada *et. al.,* 2006). These JmjC domain histone demethylases (JHDM's) can specifically demethylate histone lysine mono-, di-, and tri-methylation. One JHDM, Jhd2, has been shown to be capable of demethylating histone H3K4 *in vitro*. Deletion of the gene encoding Jhd2 however results in only subtle

increases in global H3K4me3 levels and thus the contribution of this JHDM in maintaining H3K4me3 levels is unclear.

1.4 Methyl-lysine binding domains

The specificity of the downstream effects resulting from various methylation marks is accomplished by proteins that contain methyl-lysine binding domains (MBD). These domains are targeted to specific methylation marks and carry with them associated chromatin-modifying enzymes. The MBD families that have been characterized so far include; chromodomains, double chromodomains, tudour domains, double tudour domains, tandem tudour domains, malignant brain tumour domains (MBT), WD40 repeats, PWWP domains, and PHD fingers. All but the PHD fingers belong to the Royal super family of MBDs (Taverna *et. al.,* 2007; Wang *et. al.,* 2009). They bind methyl-lysines in a binding pocket known as an aromatic cage, held together by a β-sheet barrel like structure (Jacobs and Khorasanizadeh, 2002), whereas the PHD finger relies on a zinc finger to hold together its aromatic cage.

The aromatic cage of the royal family usually consists of 3 to 4 aromatic residues, and can be supplemented with an acidic residue for the purpose of hydrogen bonding. Highly methylated lysines, tri- and di-, are recognized by cages located in surface grooves, allowing more room for the large methyl-lysine. These cages mainly rely on the cation-π interaction between the positively charged tri- or di- methylated lysine and the aromatic residues. Lower methylation states, mono- and di-, are recognized by deep pocket insertion aromatic cages. This allows for steric exclusion of the larger tri-methyl lysines. Additionally these cages often employ the supplemental acidic residue to form hydrogen bonds with the methyl-lysine (Taverna *et. al.*, 2007). The royal family of

MBDs also bind the neighboring residues of the unstructured N-terminal histone tail by an induced fit mechanism, thus imparting lysine specificity on the MBD (Taverna *et. al.*, 2007).These combined interactions result in binding affinities in the micro molar range (Kd ~ 2-30 μM) (Taverna *et. al.*, 2007). However, due to the fact that chromatin modifying protein complexes often contain multiple domains capable of binding modified histones, it is possible that these domains work together in a multivalent fashion in order to increase the binding affinity of the overall complex.

PHD fingers bind methyl-lysines through an aromatic cage, in much the same way that the royal family of MBDs do, only the aromatic cage is held together with a very different structure. The PHD finger (Plant Homeodomain) is a zinc finger held together by the inter-chelation of two zinc ions between 7 cysteines and a histidine. Most of the PHD fingers characterized so far bind very specifically to H3K4me3 (Mellor, 2006). However, there are a few PHD fingers with other specificity (Shi *et. al.,* 2006), including one, BHC80, that is specific for unmethylated H3K4 (Lan *et. al.,* 2007). Similar to the royal family, the PHD fingers have micro-molar dissociation constants but are often found in complex with other domains that bind histone modifications, and may work in a multivalent fashion (Mellor, 2006; Shi *et. al.,* 2006).

1.5 The ISW1 chromatin remodeling complexes

Isw1 (Imitation SWitch) is an ATP-dependent chromatin-remodeling protein that actively slides nucleosomes along DNA to expose or conceal different regions of the DNA. Study of Isw1 has focused on its role in gene activation and repression, although deletion of *ISW1* has relatively little effect on genome-wide gene expression (Lindstrom *et. al.* 2006). The impact of deleting *ISW1* becomes more evident when combined with

the deletion of one of the other chromatin-remodeling protein-encoding genes such as *ISW2, SWI2, RSC, CHD1, INO80,* or *SWR1*. This is due to gene-specific functional interplay between the various chromatin-remodelers (Erkina *et. al.* 2010).

In addition to genetically interacting with a variety of partners, Isw1 is important for both gene activation and repression by sliding nucleosomes either away from or over top of promoter sequences respectively (Erkina *et. al.* 2010). In fact, Isw1 is associated with both active and repressed *MET16* (Morillon *et. al.* 2003). All of these diverse roles for Isw1 are regulated by incorporation of Isw1 into distinct complexes that associate differently with other chromatin-related proteins, and chromatin itself.

There are three forms of Isw1 that perform different roles in the cell. Independent Isw1 has no strong binding partners and is required for silencing of RNA polymerase II transcribed genes in the ribosomal DNA locus (Mueller and Bryk. 2007). How Isw1 is targeted to ribosomal DNA is unknown. It may be through the SANT and SLIDE domains on Isw1, which interact with chromatin (Pinskaya *et. al.* 2009), or it may be through some unidentified binding partner. Isw1a is a complex of two proteins; Isw1 and Ioc3 (Isw One Complex 3) and was identified by TAP purification of Isw1 followed by mass spectrometry (Vary *et. al.* 2003). This complex is associated with repression of inducible genes (Pinskaya *et. al.* 2009). Additionally it has appeared in TAP purifications of Mot1, a protein that dissociates TBP from DNA, as well as Rpd3, a HDAC that represses cryptic transcription initiation (Mellor and Morillon. 2004). Ioc3 has no known chromatin binding domains, but may be responsible for interactions with other chromatin-binding complexes, thus differentiating Isw1a activity from other forms of Isw1.

The third form of Isw1 is the Isw1b complex, identified along with the Isw1a complex (Vary *et. al.* 2003), consisting of Isw1, Ioc2, and Ioc4. This complex is associated with activation of inducible genes, and is believed to facilitate transcription elongation and termination (Morillon *et. al.* 2003). Ioc2 and Ioc4 both contain a single putative MBD, a PHD finger and PWWP domain respectively. Like many of the MBDs of yeast, the PHD finger of Ioc2 and the PWWP domain of Ioc4 have not actually been demonstrated to bind methylated histones. However, it has been shown that Isw1 localization to chromatin is strongly influenced by histone methylation (Santos-Rosa *et. al.* 2003; Morillon *et al*. 2005), and thus we propose that the MBDs of Isw1b may play a role in the localization of Isw1b.

1.6 Summary of research hypotheses

H3K4me3 and H3K14ac co-localize through-out the yeast genome, which may suggest that one modification mediates the establishment of the other. However, loss of *SET1*, the sole H3K4 HMT, does not result in a significant loss of acetylation. In section 3.1.2 of this thesis we show that methylation of H3K4 is dependent on H3K14ac. Additionally, time course studies of histone post-translational modifications have shown that H3K4me3 is lost after loss of histone acetylation when histone deacetylase inhibitors are removed from a cell culture (Nightingale *et. al.* 2007). This led us to hypothesize that histone acetylation present at the promoters of actively transcribed genes protects the H3K4me3 mark from demethylation. We tested this hypothesis in section 3.1.3 of this thesis, and demonstrated that histone acetylation protects chromatin from demethylation by the histone demethylase Jhd2.

Isw1 is a chromatin-remodelling protein that performs a variety of tasks in association with different complexes. Furthermore, Isw1 preferentially localizes to specific actively transcribed genes that bear H3K4 and K36 methylation. This led us to hypothesize that Isw1b, which contains two MBDs, depends on these domains for localization. We tested the dependence of Isw1b on the PHD finger of Ioc2 in section 3.2.2.2 of this thesis, and on the PWWP domain of Ioc4 in section 3.2.2.3 of this thesis. In doing so we demonstrated the methyl-binding capability of Ioc4's PWWP domain, as well as identified a negative effect that association with Ioc4 has on Ioc2's ability to bind chromatin.

Chapter 2 - Materials and Methods

Table 2.1 Yeast Strains Used in This Study

Strain	Mating type	Genotype
BY4741ΔΔΔ	Mat a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SAS4TAP::HISMX6*
BY4741 (186)	Mat a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SAS4TAP::HISMX6 jhd1::KANMX*
BY4741 (4031)	Mat a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SAS4TAP::HISMX6 gis1::KANMX*
BY4741 (6165)	Mat a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SAS4TAP::HISMX6 rph1::KANMX*
BY4741 (6922)	Mat a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SAS4TAP::HISMX6 jhd2::KANMX*
BY4741 (7408)	Mat a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SAS4TAP::HISMX6 set1::KANMX*
BY4741 (761)	Mat a	*his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 SAS4TAP::HISMX6 ecm5::KANMX*
FY602	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63*
yIJ27	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc2::HISMX6*
yIJ28	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6*
yIJ29	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6*
yIJ31	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 isw1::HISMX6*
yIJ37	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 jhd2::KANMX6*
yIJ51	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ada2::HIS3 sas3::HISMX6 jhd2::NATMX4*
yIJ57	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ura3::pIOC2-6HA.406*
yIJ63	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pIOC2-6HA.406 leu2::pRS405*
yIJ64	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pIOC2-6HA.406 leu2::pIOC4.405*
yIJ65	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pIOC2-6HA.406 leu2::p.ioc4P21A.405*
yIJ66	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pRS406 leu2::pIOC4-6HA.405*
yIJ67	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pIOC2.406 leu2::pIOC4-6HA.405*
yIJ68	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pIOC2D574A.406 leu2::pIOC4-6HA.405*
yIJ69	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pIOC2D574A.406 leu2::p.ioc4P21A-6HA.405*
yIJ70	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pRS406 leu2::p.ioc4P21A-6HA.405*
yIJ71	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pIOC2.406 leu2::p.ioc4P21A-6HA.405*
yIJ73	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pIOC2.406 leu2::pIOC4.405 ISW1TAP::TRP*
yIJ74	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pIOC2.406 leu2::pIOC4-6HA.405 ISW1TAP::TRP*
yIJ75	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pRS406 leu2::pIOC4-6HA.405 ISW1TAP::TRP*
yIJ76	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pIOC2D574A.406 leu2::pIOC4-6HA.405 ISW1TAP::TRP*
yIJ77	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pIOC2.406 leu2::p.ioc4P21A-6HA.405 ISW1TAP::TRP*
yIJ78	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pRS406 leu2::p.ioc4P21A-6HA.405 ISW1TAP::TRP*
yIJ79	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pIOC2D574A.406 leu2::p.ioc4P21A-6HA.405 ISW1TAP::TRP*
yIJ80	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pIOC2-6HA.406 leu2::pIOC4.405 ISW1TAP::TRP*
yIJ81	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pIOC2-6HA.406 leu2::pRS405 ISW1TAP::TRP*
yIJ82	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ioc4::KANMX6 ioc2::HISMX6 ura3::pIOC2-6HA.406 leu2::p.ioc4P21A.405 ISW1TAP::TRP*
yIJ83	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 set1::HISMX6 ura3::pIOC2-6HA.406*

Table 2.1 Yeast Strains Used in This Study

Strain	Mating type	Genotype
yIJ84	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 set2::TRP ura3::pIOC2-6HA.406*
yIJ85	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 set2::TRP set1::KAN ura3::pIOC2-6HA.406*
yIJ86	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 leu2::pIOC4-6HA.405*
yIJ87	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 set1::HISMX6 leu2::pIOC4-6HA.405*
yIJ88	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 set2::TRP leu2::pIOC4-6HA.405*
yIJ89	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 set2::TRP set1::KAN leu2::pIOC4-6HA.405*
yIJ90	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63(hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912δ 35::his4 pHHF2.hht2K14Q.314*
yIJ91	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63(hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912δ 35::his4 pHHF2.hht2K14R.314*
yIJ92	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63(hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912δ 35::his4 pHHF2.hht2K14A.314*
yLH104	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 gcn5::HIS3*
yLH106	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 sas3::HISMX6*
yLH146	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 ada2::HIS3 sas3::HISMX6*
yLH220	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 set1::HISMX6*
yLH224	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63(hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912δ 35::his4 pHHF2.HHT2.314*
yLH353	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 set2::TRP*
yLH354	Mat a	*his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 set2::TRP set1::KAN*

Table 2.2 Plasmids Used in This Study

Plasmid	Description	Reference
p*HHT2*.314	*HHT2-HHF2* locus on pRS414 vector	Martin *et al.* 2006
p*hht2K14A*.314	substitution mutation *K14A* of H3 on p*HHT2*	Choi, J.
p*hht2K14R*.314	substitution mutation *K14R* of H3 on p*HHT2*	Choi, J.
p*hht2K14Q*.314	substitution mutation *K14Q* of H3 on p*HHT2*	Choi, J.
pFA6a-HisMX6	HisMX6 cassette on pFA6a vector	Longtine *et. al.* 1998
pFA6a-KanMX6	KanMX6 cassette on pFA6a vector	Longtine *et. al.* 1998
pAG25-NatMX4	NatMx4 cassette on pAG vector	Goldstein and McCusker 1999
pRS405	pRS. Vector that integrates into *leu2Δ1*	Sikorski and Hieter 1989
pRS406	pRS. Vector that integrates into *ura3-52*	Sikorski and Hieter 1989
pBS1479	Vector containing TAP purification cassette with *TRP* marker	Puig *et. al.* 2001
p*IOC2*.406	*IOC2* locus on pRS406 vector	This study
p*IOC2-6HA*.406	6HA tag cassette cloned onto 3' end of *IOC2* in p*IOC2.406*	This study
p*ioc2D574A*.406	substitution mutation *D574A* of *IOC2* on p*IOC2*.406	This study
p*IOC4*.405	*IOC4* locus on pRS405 vector	This study
p*IOC4-6HA*.405	6HA tag cassette cloned onto 3' end of *IOC4* in p*IOC4*.405	This study
p*ioc4P21A*.405	substitution mutation *P21A* of *IOC4* on p*IOC4*.405	This study
p*ioc4P21A-6HA*.405	6HA tag cassette cloned onto 3' end of *ioc4P21A* in *pioc4P21A*.405	This study

2.1 Preparation of yeast whole cell extracts

Yeast were inoculated from plates into 10ml YPD cultures and grown to an OD_{600} of 0.8 to 1.0. For confirmation of expression in ChIP conditions, 10ml -met cultures (Hartwell's complete medium –methionine, 0.5% glucose) were used instead. Cells were pelleted by centrifugation (2844 X g, 3min), washed 1X with 5ml deionized water, resuspended in 1ml dH_2O and transferred to pre-weighed 1.6ml microfuge tubes. Cells were then pelleted again and supernatant removed by pipette. The pellets were then weighed and resuspended in dH_2O to 200µg/µl. Sixty microlitres of suspension was transferred to a new 1.6ml microfuge tube where NaOH was added to a final concentration of 182mM. The suspension was then incubated at room temperature for 15min, followed by the addition of 70µl of 3X SDS solvent (360mM Tris-HCl pH 6.8, 18% SDS, 54% Glycerol, 20% β-mercaptoethanol, 0.06% Bromophenol Blue) and 40µl of 5X electrophoresis buffer (125mM Tris, 1.25M Glycine, 0.5% SDS). The mixture was boiled for 5min, and then centrifuged for 5min at 14550 X g. The soluble material represented the yeast whole cell extract.

2.2 Western blot of non-histone proteins

Five microlitres of whole cell extract was loaded onto a 12% poly-acrylamide gel and run at 120V until dye front was at the bottom of the gel. The protein was transferred to a nitrocellulose membrane using a BioRad Mini Trans-Blot Electrophoretic Transfer Cell at 30V overnight at 4^oC. The membrane was then blocked with 1% BSA in PBS-T (137mM NaCl, 2.68mM KCl, 10.1mM Na_2HPO_4, 1.76mM KH_2PO_4, 0.1% Tween-20, and adjust to pH 7.4) for 1h, shaking at room temperature. Afterwards the primary

antibody was added (Applied Biological Materials Inc. Anti-HA Mouse Monoclonal Antibody cat. # G036, Clone HA.C5, 1:1000 in PBS-T) and incubated for 1h at room temperature while shaking. The membrane was then washed 3X in PBS-T for 5min each, followed by incubation with secondary antibody [LI-COR IRDye 800CW Goat anti-Mouse IgG (cat. # 926-32210), 1:25000 in PBS-T] for 30min at room temperature while shaking. The membrane was then washed 4X 5min in PBS-T and imaged using the LI-COR Odyssey Infrared Imaging system.

To confirm expression of HA-tagged proteins in ChIP conditions (Fig. 3.7) the primary antibody used was a horse radish peroxidase conjugated anti-HA antibody [Roche Anti-HA-peroxidase, High Affinity (3F10) (cat. # 2013819), 1:1000 in PBS-T]. No secondary was needed, and the blot was imaged using Thermo Scientific Super Signal West Femto Maximum Sensitivity Substrate (cat. # PI34096).

2.3 Western blot of histone proteins

Ten microlitres of whole cell extract was loaded onto a 10% poly-acrylamide gel and run at 120V until the the dye front was at the bottom of the gel. The gel was equilibrated for 30min. in 62.5 mM Tris pH 6.8, 2.3% SDS and the protein was transfered to a nitrocellulose membrane in transfer buffer (25 mM ethanloamine/glycine pH 9.5 20% methanol) using Bio Rad Mini Trans-Blot® Electrophoretic Transfer Cell at 30V for 1h at room temperature. The membrane was then blocked with 5% milk in PBS-T for 1h, shaking at room temperature. Afterwards the primary antibody was added [our in house anti-H3 antibody, or Active Motif anti histone H3 trimethyl Lys4 Rabbit pAb (cat. # 39159), 1:1000 in PBS-T] and incubated for 1h at room temperature while shaking. The membrane was then washed 3X in PBS-T for 5min each, followed by incubation with

secondary antibody [LI-COR IRDye 680 conjugated Goat polyclonal anti-rabbit IgG (cat. # 926-32221), 1:50000 in PBS-T] for 30min at room temperature while shaking. The membrane was then washed 4X 5min in PBS-T and imaged using the LI-COR Odyssey Infrared Imaging system.

2.4 Plasmid shuffle for histones

To generate strains of yeast expressing mutated forms of histones we started with the parent strain (yLH224: *MATa his3Δ200 leu2Δ1 lys2-128Δ ura3-52 trp1Δ63 (hht1-hhf1)::LEU2 (hht2-hhf2)::HIS3 Ty912δ 35::his4*) that only has one copy of the genes encoding histones H3 and H4 (*HHT2* and *HHF2* respectively) on a pRS416 vector (Sikorski and Hieter 1989) which contains an *URA3* gene. Next we transformed in the *TRP1*-based plasmids *pHHF2.HHT2.314, pHHF2.hht2K14A.314, pHHF2.hht2K14Q.314,* and *pHHF2.hht2K14R.314,* which encode H3, H3K14A, H3K14Q, and H3K14R respectively along with H4. Selection was performed by plating on synthetic media lacking tryptophan. Successful transformants were replica-plated onto 5-FOA plates (0.1% of 5-fluoroorotic acid) that selects against yeast expressing the *URA3* gene. Surviving colonies were then replica-plated onto –Trp synthetic media as well as –Ura synthetic media and $Trp1^{+}$ $Ura3^{-}$ colonies were chosen for use in experiments.

2.5 Generation of parent strain for designer Isw1b complexes

The genes *IOC2* and *IOC4* were deleted from wild type S288C *Saccharomyces cerevisiae* using PCR-based gene deletion (Ausubel 1987). Knock out cassettes were created as follows. Forward primer TATTTTACTCATACACACTGGCTAATCAT-

ATATAGATACAAAGCGGTGAATCAGCGATCGCGGATCCCCGGGTTAATTAA, and reverse primer ATGAAGAACCAGTTGATATATATTTTACTATATACTTT-TGCTGTGTATTTCTATATGAGGGAATTCGAGCTCGTTTAAAC were used to PCR amplify the *HISMX6* region from pFA6a-HisMX6 (Longtine *et. al.* 1998). This cassette was used to knock out *IOC2*. The cassette used to knock out *IOC4* was created by PCR amplifying the KanMX6 region of pFA6a-KanMX6, using forward primer TAAGAATTGACGAAGAAATTGTTAACTACATTTTTCAGAACGGCGTGTCATT-CTCCGATACGGATCCCCGGGTTAATTAA and reverse primer TAGTTTTAGTCT-ATTTTATAGTACACAGGTTTCCCCTCTATTGTTCAAAAGCAGAGTACAGAAT-TCGAGCTCGTTTAAAC (Longtine *et. al.* 1998). His^+ Kan^+ colonies, confirmed as *ioc2*Δ *ioc4*Δ by PCR, were used for experimentation.

2.6 Plasmid construction for designer Isw1b complexes

IOC2 was cloned into the *EcoRI* site of the multiple cloning site of pRS406 (Sikorski and Hieter 1989). *IOC2* was PCR amplified from S288C genomic DNA (Ausubel 1987) using forward primer GCATTAAAGCTTGATTTGT-GTTCACGTCCAGC which anneals 523 bp downstream of the gene and adds an *EcoRI* restriction site to the end of the PCR product, and reverse primer TGGCAAAAGCTTACTTCTAGGTCCACTTGAGC which anneals 249 bp upstream of the gene and also adds an *EcoRI* restriction site to the end of the PCR product. The PCR was done in 50µl reactions using Pfu Ultra (Stratagene cat# 600382-51) (1X Pfu Ultra Buffer, 250µM dNTP's, 5nM forward primer, 5nM reverse primer, 2µg genomic DNA, 0.05 Units Pfu Ultra, diluted in miliQ water). The PCR program used was: 95^0C 1min, followed by 29 repeats of 95^oC for 30s, 55^oC for 30s, and 72^oC for 2min 20s. *IOC4* was

cloned the same way except utilizing the *HindIII* site in the multiple cloning site of pRS405, using forward primer TACCAAAAGCTTTTTCAAGAACCGCA-ACAAGG (682 bp upstream), and reverse primer ACAGACAAGCTTTACA-GCACAGTCTAGGTGAG (152 bp downstream). p*IOC2*.406 was then integrated into the *ioc2*Δ *ioc4*Δ strain as needed by digestion with *NcoI* and integration into the *ura3-52* gene. p*IOC4*.405 was integrated by digestion with *BsrGI* and integration into the *leu2Δ1* gene.

Site directed mutagenesis was used to generate mutant *ioc2D574A*. First PCR was used to generate a mega primer for the 5' end of the gene from the clone. The forward primer used was the same one used to clone the gene, and the reverse primer used was CACCTGTAACAATGAGCACAGCATATCAATG, which spans the region to be mutated and replaces the D574 GAT codon with the alanine codon GCT. This was done using the same 50µl Pfu Ultra PCR reaction as the initial cloning step. The 3' end megaprimer was created in the same manner using the forward primer CATTGATATGCTGTGCTCATTGTTACAGGTG, which spans the same region as the reverse primer of the 5' megaprimer and induces the same mutation, with the reverse primer used for cloning. These two PCR products were gel purified and then pooled together to be used as template in a third PCR reaction. A final 50µl Pfu Ultra PCR reaction was prepared using the initial cloning primers and 1µl of pooled megaprimer as template, and run using the PCR program 95^0C 1min, followed by 29 repeats of 95^oC for 30s, 55^oC for 30s, and 72^oC for 4min.

The same was done to generate *ioc4P21A* only using GTTCATTA-GGGATAATCATTGCAGCCCATGCTGAAAAGCCTTTAAC as the reverse primer of

the 5' end, and GTTAAAGGCTTTTCAGCATGGGCTGCAATGATTATCCC-TAATGAAC as the forward primer for the 3'end. The mutant genes were then cloned into pRS406 and pRS405 and then integrated into the parent strain as needed, exactly as was done for the vectors containing wild type genes.

To 6-HA tag the Ioc subunits, vectors were created that would add the tag to the 3' end of any gene subcloned in-frame with with a *SacII* site. To generate this vector, a cassette that had a *SacII* site joined in-frame to 6 repeats of the *HA* encoding gene followed by a stop codon, *CYC1* terminator was PCR amplified from our plasmid p410 using the forward primer TAGGACGATACCGCGGTCCGGTTCTGCTGCTAG containing a *SacII* site, and the reverse primer TACGAGTTCAGAGCTC-CAGCAGGCCTCTCAATAGAG that adds a *SacI* site to the end of the PCR product. The cassette was then cloned into the multiple cloning sites of both pRS406 and pRS405 using *SacI* and *SacII*. The *IOC2*, *IOC4*, and *ioc4P21A* were then PCR amplified from the untagged plasmids using a T3 forward primer and either AAGCCTCAGACCGCGGGTTGGTTGGTGGGAACTG for *IOC2* or, AAGCCTCA-GACCGCGGTGCTCCTACTTTGCTTTC for *IOC4* and *ioc4P21A*. These reverse primers replaced the stop codons on the 3' ends of the genes with a *SacII* cut site in frame with the gene. The PCR products were then cloned into the desired 6-HA vector using *SacII* and *SmaI*. The resulting 6-HA tagged gene expressing vectors (p*IOC2-6HA.406*, p*IOC4-6HA.405* and, p*ioc4P21A-6HA.405*) were then integrated into the parent strain as needed in the same manner as the vectors containing wild type genes.

2.7 Chromatin immuno-precipitation

For each strain used in an experiment three 50ml cultures were grown in –met synthetic media to an OD_{600} 0.8. Cells were then cross linked by shaking at room temperature after the addition of 1.35ml formaldehyde for 30min. Cross linking was stopped by the addition 1g glycine and a further 15min of shaking at room temperature. The cells were then washed 2X with cold PBS, pelleted, and stored at -80^{o}C.

For each cell pellet 110µl of Invitrogen Dynabeads Protein G (cat# 100.03D) suspended in PBS was prepared. Twenty-five microlitres of bead slurry was washed 3X with PBS. Two micrograms of Anti-HA High Affinity Rat monoclonal antibody [Roche (clone 3F10) cat# 11 867 423 001] was added along with 11µg of BSA and the final volume was adjusted to 110µl with PBS. Beads were prepared the day before needed and rotated overnight at 4^{o}C.

To extract chromatin form the frozen cell pellets, the pellets were thawed on ice for 20min then resuspended in 300ul of lysis buffer [(50mM HEPES pH 7.5, 140mM NaCl, 500µM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, and freshly added 1mM PMSF, 1mM Benzamidine, 0.02 tablet/ml Roche complete protease inhibitor cocktail tablet (cat#11 697 498 001)]. The suspension was then vortexed on the disrupter genie with 500µl of glass beads at 4^{o}C for 20min. The lysate was transferred to new tubes and sonicated on high in a Diogenode Biorupter water bath sonicator, 30s on 30s off for 10min at 4^{o}C. Four hundred microlitres of lysis buffer was added to each sample followed by centrifugation at 21885 X g for 10min at 4^{o}C. Six hundred microlitres of supernatant was transferred to a new tube and vortexed thoroughly. One hundred microlitres was transferred to a new tube and saved on ice as input DNA, the remaining

500µl was mixed with 100µl of magnetic beads and rotated at 4°C for 3hr. The beads were washed 2X with 1ml cold lysis buffer, followed by 2X with 1ml lysis buffer with 500mM NaCl, then 2X with freshly prepared lithium buffer (10mM Tris-HCl pH 8.0, 250mM LiCl, 0.6% NP-40, 0.5% Na-deoxycholate, 1mM EDTA, 1mM PMSF, 1mM Benzamidine, and 1X Roche cocktail), then finally once with 1ml 1M TE pH 8.0. One hundred microlitres of 10% (wt/vol) chelex 100 was then added directly to the beads. The slurry was then vortexed and boiled for 10min. The samples were cooled and 1µl of 20mg/ml proteinase K was added to each sample. The samples were incubated for 30min at 55^{0}C with brief vortexing every 10min. Samples were boiled and briefly centrifuged to pellet the beads and chelex resin. Eighty microlitres of supernatant was transferred to a new tube and the remaining beads were resuspended in 120µl of dH_2O. The suspensions were vortexed, briefly centrifuged, and the resulting supernatants pooled with the previously collected 80µl of supernatant and vortexed. This DNA was used for real time PCR.

The cross-links between the proteins and DNA from the input samples were reversed by overnight incubation at 65°C. Next 120µg of proteinase K was added to each sample which were then incubated at 37°C for1hr. Afterwards 96µl of 5M LiCl and 2µl of 20mg/ml glycogen were added to the samples and the samples were vortexed. The DNA was phenol chloroform extracted with 500µl of phenol/ chloroform/isoamyl alcohol (25:24:1). The supernatant-containing DNA was collected from vortexed extractions after 10min of centrifugation at 21885 X g. The DNA was ethanol precipitated from the supernatants using 1ml EtOH at -20°C for 1h followed by centrifugation at 16800 X g for 10min. The pellets were then washed once with 70% EtOH and dried with a Savant DNA

speed vac. Afterwards the pellets were incubated at 37°C for 30min in 60µl of 666 µg/ml RNase A, followed by the addition of 60µl of dH_2O, and then frozen at -20°C.

2.8 Real time PCR

A 25µl PCR reaction was set up for each DNA sample (20mM Tris-HCl pH 8.8, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgSO_4$, 0.1% Triton X-100, 0.2nM dNTPs, 0.89nM forward primer CGAATCGGCTGGCTTCAT, 0.89nM reverse primer CATTCCACACAACCCGTCAA,) in white 8 tube PCR strips. The reactions were then run on an Applied Biosystems Step One Plus Real-Time PCR system with the program 95°C for 3min followed by 40 repeats of 95°C 15s, 60°C 20s, and 72°C 1min. Ct values from the amplification curves were compared to a standard curve to determine concentration of the PCR templates. The concentrations of template in the imunoprecipitates were divided by concentrations of template in the input to generate a relative signal strength in arbitrary units.

2.9 Generation of TAP tagged Isw1

After generating strains expressing designer Isw1b complexes the *ISW1* gene was TAP tagged as described in Puig *et. al.* 2001. The TAP tag cassette was amplified from pBS1479 using primers Upstream A GTTGGTAGCAGAGAAAATTCCGG-AAAACGAAACCACTCATTCCATGGAAAAGAGAAG, and downstream B AGGATATATTAAAAAAAATCGAAATATAAAAAAAGAAGGTTACGACTCACT ATAGGG.

2.10 Purification of Isw1

Fifty millilitre YPD cultures of yeast were grown to an OD_{600} of 1 at 30°C. Cells were pelleted and washed once in sdH_2O, pelleted then transferred to a 1.6ml microfuge tube, pelleted and frozen at -80°C. Pellets were then thawed on ice in 350µl of EX350 buffer (20mM HEPES pH8.0, 350mM NaCl, 0.1% Tween-20, and freshly added 1mM Benzamidine, 1mM PMSF, 50X Roche Cocktail), and resuspended. Seven hundred and fifty microlitres of glass beads were added to each suspension, which were then vortexed for 3min in a vortex genie. The cell lysates were then transferred to a new tube and centrifuged at 21885 X g for 5min at 4°C. Five hundred microlitres of supernatant was collected from each lysate and used for purification after a 5µl "input" sample was collected and stored on ice.

To pull down the TAP tagged protein, 20µl of Dynabeads pan mouse IgG (Invitrogen cat# 110.41) was prepared for each sample by washing 3X with 400µl of EX350 buffer. Four hundred microlitres of each collected lysate was then transferred to separate tubes containing washed resin. The resin and lysate were incubated at 4°C for 2h while rotating. The resin was washed 4X with EX350 buffer then resuspended in 40µl of 3X SDS buffer (180mM Tris-HCl pH 6.8, 18% SDS, 54% Glycerol, 20% 2-mercaptoethanol, 0.06% bromophenol blue). At this time the input extract on ice was suspended in 45µl of 3X SDS buffer. All the samples were boiled for 5min and used for western blots.

Chapter 3 - Results

3.1 A novel H3K4me regulatory pathway

Tri-methylation of lysine 4 on histone H3 (H3K4me3) is a post-translational modification that correlates with the 5' end of actively transcribed genes (Pokholok, et al. 2005). The current model for H3K4me3 deposition readily explains this observed pattern. H3K4me3 is deposited by Set1 of the COMPASS protein complex when in association with Rpb1, the largest subunit of RNA Pol II, which has been phosphorylated on the 5th serine of its C-terminal heptapeptide repeat (S5). S5 of RNA Pol II is phosphorylated during initiation of transcription, which is subsequently lost, along with COMPASS association, during elongation. This results in a co-localization of RNA Pol II CTD S5P, COMPASS, Set1, and H3K4me3 with the 5' end of actively transcribed genes (Wood *et al.* 2007).

In addition to S5 phosphorylation of RNA Pol II localizing Set1, the Shilatifard lab found that several residues on the N-terminal tail of H3 were also required for H3K4me3 (Nakanishi *et al.* 2008). This was found using a library of mutant yeast strains, each containing a single alanine substitution for one of the residues of the four core histones. Western blots were prepared using whole cell extracts from each strain and probed for the presence of H3K4me3 using an H3K4me3 specific antibody. The five residues required for H3K4me3 were H3R2, T3, K4, Q5, and K14. K4 is required as it is the site of methylation. R2 has been shown to be required for regulation of COMPASS activity (Kirmizis *et al.* 2007, and Guccione *et al.* 2007). T3 and Q5 are immediately adjacent to K4 and mutations of these may mask the epitope recognized by the anti-H3K4me3 antibody or alter the substrate recognized by the COMPASS complex. K14 on

the other hand lies ten residues away from the site of methylation and, interestingly, is acetylated at the 5' end of actively transcribed genes.

Due to the spatial correlation of H3K4me3 and H3K14ac at the 5' end of active genes, and the observed dependence of H3K4me3 on the H3K14 residue, we endeavored to test the hypothesis that H3K4me3 is dependent on H3K14ac. Furthermore we intended to investigate how the loss of H3K14ac could possibly lead to a loss of H3K4me3.

3.1.1 K14 is required for H3K4me3

We first confirmed the result observed by the Shilatifard lab by creating our own mutant H3 strains and testing for H3K4me3. Owing to the redundancy of *HHT1* and *HHT2*, the two copies of the gene encoding histone H3, the plasmid shuffle technique was employed to create yeast strains that express various mutant forms of histone H3. This was done by deleting *HHT1* and *HHT2* in a strain containing *HHT2* on a plasmid along with the *URA3* gene. We then transformed in another plasmid containing a *hht2K14A* mutation along with a *TRP1* gene and plated the strains on 5-fluoroorotic Acid (5-FOA) plates to select against the *URA3 HHT2* plasmids (Boeke *et al.* 1984). This results in a strain of yeast expressing only plasmid born mutant *hht2K14A*.

Whole cell extracts were prepared from the *hht2K14A* strain as well as a strain expressing wild type *HHT2* off a similar plasmid, and normalized by cell pellet weight. A western blot of the extracts was probed with an H3K4me3-specific antibody, to detect global levels of H3K4me3. We observed a loss H3K4me3 in the *hht2K14A* strain confirming published results (Fig. 3.1, lanes 5 and 6 compared to lanes 7 and 8).

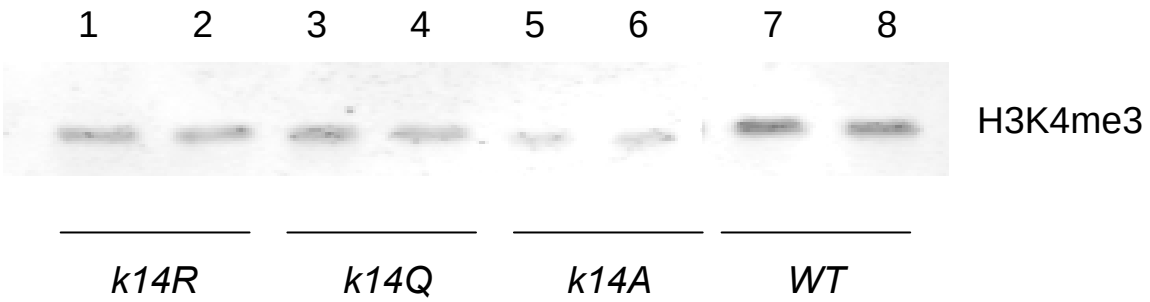


Figure 3.1 Mutation of lysine 14 on histone H3 reduces the levels of tri-methylation on lysine 4 of histone H3.

Whole cell extracts were prepared from yeast strains expressing their sole histone H3 from plasmid born *HHT2*, or a mutant version of the *HHT2* gene. Western blot analysis was performed using anti-H3K4me3 antibodies. Each whole cell extract was prepared from independent cultures normalized by cell weight.

Contrary to the results obtained by the Shilatifard lab, where a K14A mutation abolished all detectable H3K4me3, we observed a residual H3K4me3 signal in our *K14A* mutant. We later found that this was due to our antibody cross reacting with H3K4me2 which is still present in an *H3K14A* strain (Nakanishi *et al.* 2009).

We then performed the same experiment using *hht2K14R* and *hhtK14Q,* which mimic the charge of an un-acetylated and acetylated lysine respectively, in order to test if it is the positive charge of the lysine that is required (Fig. 3.1, lanes 1-4). The diminishment of H3K4me3 in the K14R and K14Q mutants further demonstrated the importance of K14 for H3K4me3, while the similar effects observed between the positively charged K14R and the uncharged K14Q indicates that the necessity of the lysine residue is not due to charge effects.

3.1.2 Deletion of K14 acetyltransferases results in loss of global H3K4me3

As mentioned previously H3K14 can be acetylated by histone acetyltransferases (HAT's). Interestingly H3K14ac placement strongly correlates with the 5' end of actively transcribed genes, much the same way as H3K4me3 (Pokholok *et. al.* 2005). Therefore we hypothesized that the importance of H3K14 for H3K4me3 was its ability to be acetylated. To test this we deleted *SAS3* and *GCN5,* the genes encoding the catalytic subunits of the H3-specific acetyltransferases (Table 1), thus inhibiting the yeasts ability to acetylate H3K14. Whole cell extracts were prepared from the two mutant strains as well as a wild type strain, normalized by weight of cells used to make the extract. A western blot using H3K4me3 specific antibody was then performed using the extracts in order to see the effect that deleting the HAT's had on total H3K4me3 levels. Both *sas3Δ*

and *gcn5Δ* strains have reduced global levels of H3K4me3 supporting our hypothesis (Fig. 3.2, compare lanes 3 and 4 with 5).

Our next step was to create a yeast strain that lacked H3K14ac completely. Unfortunately *sas3Δ* and *gcn5Δ* are synthetically lethal when knocked out together due to the role of Gcn5 in acetylating Rsc4, a subunit of the essential RSC nucleosome complex (Choi *et al.* 2008). However all the Gcn5 containing HATs posses an Ada2 subunit that is essential for their ability to acetylate histones, but not Rsc4 (Table 1). *ada2Δ* strains have the same levels of H3K14ac as *gcn5Δ* strains thus *ada2Δ* strains can be used in place of *gcn5Δ* strains when working with H3 acetylation (Howe *et.al.* 2001). Hence we were able to create a *sas3Δ ada2Δ* strain that was viable yet, lacked all H3K14ac. An H3K4me3 western blot performed with extract from this strain showed an even more profound loss of global H3K4me3 levels providing yet more support for a link between H3K14ac and H3K4me3 (Fig. 3.2, lane 2). Interestingly one of the proposed methods of action for acetylation of histone lysines is the neutralization of its positive charge. However this is unlikely the case here as *hht2K14Q* mutants, which mimic the neutral charge of an acetylated lysine, and an *hht2K14R* mutation, which mimics a positively charged unacetylated lysine, both result in a loss of H3K4me3 (Fig. 3.1 lanes 1 - 4). Thus H3K14ac is required for H3K4me, and the mode of action is not through charge neutralization.

Table 3.1 Histone Acetyltransferase complexes in Saccharomyces cerevisiae

HAT Complex	Catalytic Subunit	Substrate Specificity
ADA	Gcn5p	H3
SAGA	Gcn5p	H3
SLIK	Gcn5p	H3
HAT-A2	Gcn5p	H3
NuA3	Sas3p	H3
NuA4	Esa1p	H4
SAS	Sas2p	H4

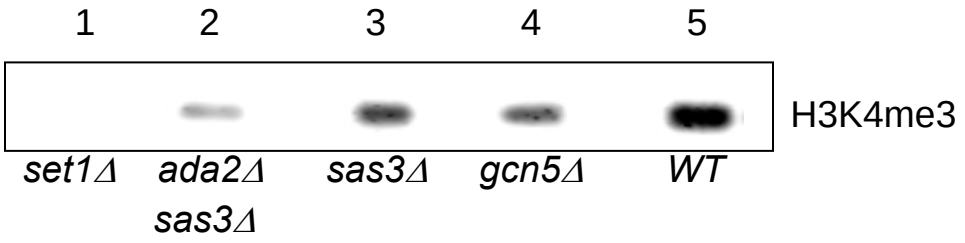


Figure 3.2 Deletion of the catalytic subunits of the histone acetyltransferases reduces the levels of tri-methylation on histone H3.

Whole cell extracts were prepared from yeast strains lacking the genes encoding the histone acetyltransferases Sas3 and Gcn5. In lane 2 *ada2Δ* was used in place of *gcn5Δ*. The H3K4 specific methyltransferase encoding gene *SET1* was deleted as a negative control. Western blot analysis was performed using anti-H3K4me3 antibodies.

3.1.3 H3K14ac protects H3K4me3 from demethylation

There are several possible explanations for the link between H3K14ac and H3K4me3. One is transcription. H3K4me3 is deposited by Set1 of the COMPASS complex in association with RNA Pol II (Ng *et al.* 2003), and it has been known for some time that acetylation of lysines on histones is strongly correlated with transcription (For Review see MacDonald, and Howe 2009). Perhaps H3K14ac is required for transcription, which is required for methylation of H3K4 by Set1. This is unlikely though as many histone lysines are acetylated in actively transcribed genes, and not a single one has been shown to be independently necessary for transcription, yet H3K4me3 is directly dependent on only H3K14 (Fig. 3.1, and Nakanishi *et al.* 2009).

Another possibility is that H3K14ac acts as a docking site that recruits some unidentified protein complex that is required for the proper tri-methylation of H3K4 by Set1. This would be difficult to test as one would need to identify the unknown interacting partner, most likely through mass screening. Another hypothesis is that H3K14ac protects H3K4me3 from demethylation. This could easily be confirmed by eliminating the demethylase activity of the yeast. Without demethylation, there would be nothing to protect H3K4me3 from, and removal of H3K14ac would have no effect on global H3K4me3 levels. We decided that investigation of this hypothesis would be the most feasible and proceeded to do so.

In order to abolish the demethylase activity of yeast, we decided to delete the known demethylase enzyme encoding genes. There are five demethylase enzymes in yeast identified by homology to the human JmjC domain containing demethylases.

These are encoded by the genes *ECM5, JHD1, JHD2, GIS1,* and *RPH1*. Deletion of any of these genes does not have an effect on global levels of histone methylation detectable by western blot, which we confirmed using strains from the *Saccharomyces* deletion project (Kelly *et al.* 2001, Fig. 3.3.).

Rather than creating a strain lacking all five demethylases to remove the demethylase activity, we deleted the gene encoding the strongest known H3K4-specific demethylase, *JHD2* (Tu *et. al.* 2007), and performed our experiments in a *jhd2Δ* background. As previously mentioned an *ada2Δ sas3Δ* strain, which lacks H3 acetylation, has less H3K4me3 than a wild type strain as detected by western blot analysis normalized by weight of cells used to prepare the whole cell extracts (Fig. 3.2, lanes 2 and 5). When we deleted *JHD2,* H3K4me3 levels were restored (Fig. 3.4, compare lanes 4 - 6 with 7 - 9) supporting the hypothesis that one of the roles of H3K14ac is to protect H3K4me3 from demethylation.

To rule out the possibility that the total amount of H3 present in either a cell, or extractable into an extract, is affected by mutation of *ADA2* and *SAS3,* we raised our own H3-specific antibody, and used it as a loading control for the western blot. The western blot for each strain was performed in triplicate, and when the average intensities of the bands are expressed as a bar graph, it is easier to see the rescuing effect that deleting the *JHD2* gene has (Fig. 3.4B. column 2 and 3). The bar graph also shows a detectable 51% global increase of H3K4me3 in the *jhd2Δ* (Fig. 3.4B. column 4) strain as compared to *WT* (Fig. 3.4B. column 1), yet this cannot account for the observed 221% increase of H3K4me3 signal in the *ada2 sas3 jhd2* strain as compared to the *ada2 sas3* strain (Fig. 3.4B. column 3 compared to 2). These results suggest that H3K4me3 is partially

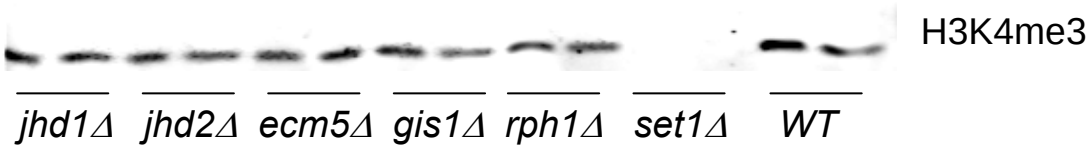


Figure 3.3 Deletion of histone demethylase encoding genes does not result in an increase of tri-methylation of histone H3.

Whole cell extracts were prepared from yeast strains lacking the genes encoding the histone demethylases Jhd1, Jhd2, Ecm5, Gis1, and Rph1. The H3K4 specific methyltransferase encoding gene *SET1* was deleted as a negative control. Western blot analysis was performed using anti-H3K4me3 antibodies. Each whole cell extract was prepared from independent cultures normalized by cell weight.

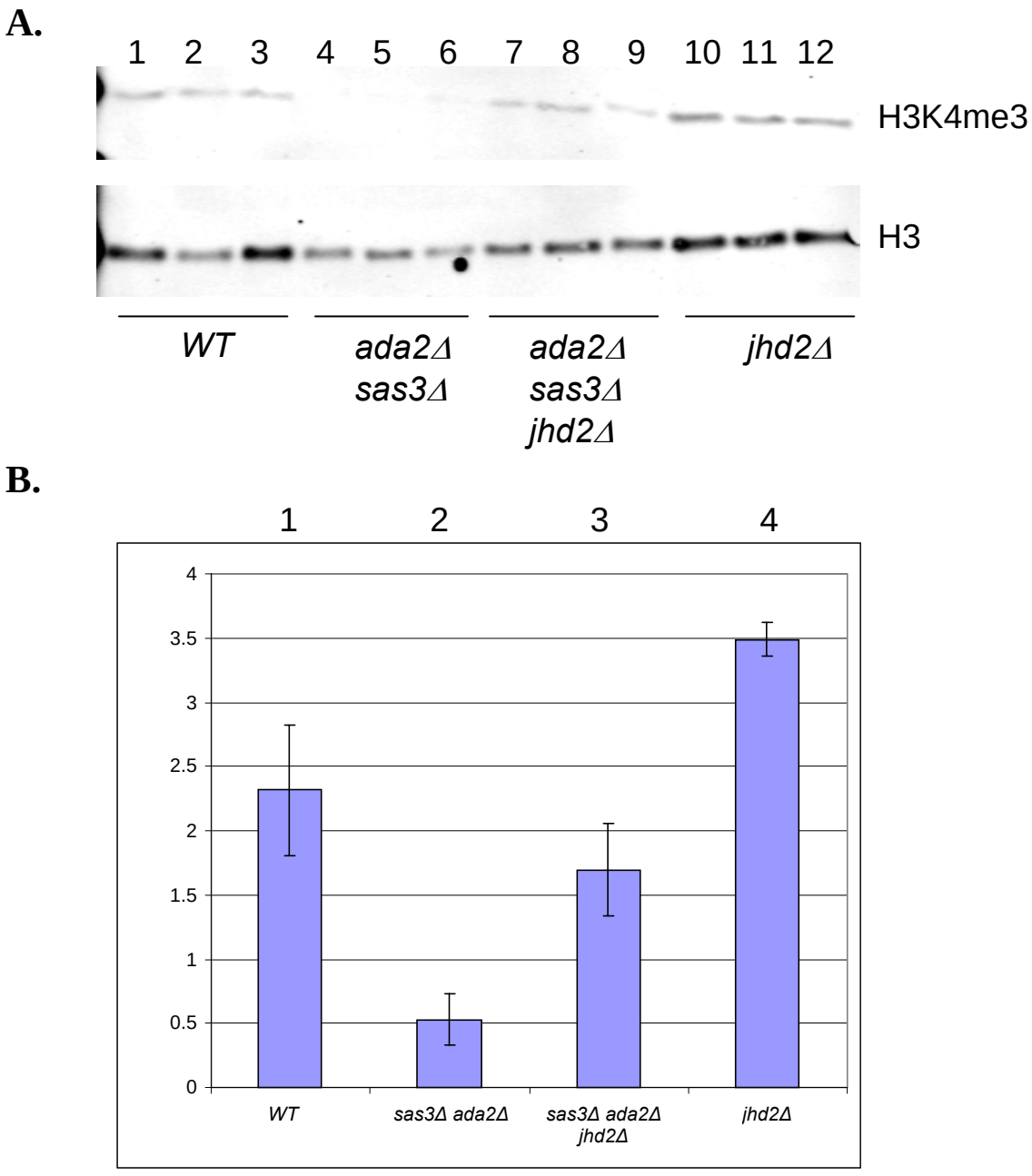


Figure 3.4 Deletion of JHD2 rescues loss of H3K4me3 resulting from deletion of ADA2 and SAS3.

(A) Whole cell extracts of wild type yeast, and yeast lacking either the histone acetyltransferases Ada2 and Sas3, the histone demethylase Jhd2, or all three. Two identical western blots were performed using the extract. One blot was probed with anti-H3K4me3 while the other was probed with anti-H3. Each extract was prepared from an independent culture. (B) Average relative intensities of anti-H3K4me3 signal divided by anti-H3 signal and multiplied by 10, expressed in arbitrary units. Error bars are standard error.

regulated by acetylation of H3K14ac which protects H3K4me3 from the demethylase Jhd2 by an as of yet uncharacterized mechanism.

3.2 The role played by the methyl-lysine binding domains of Isw1b in localization of Isw1b to chromatin

ISW1 is a non-essential gene in yeast that encodes the ATP-dependent nucleosome-remodeling protein Isw1. This protein positions nucleosomes at the 5' and 3' end of genes by sliding them along the DNA in order to insure proper nucleosome spacing for transcriptional activation or repression (Mellor and Morillon. 2003). Most of the observed modifications carried out by Isw1 seem to be subtle, specifically localized events needed for maximum gene regulation efficiency (Mellor and Morillon. 2003). To date Isw1 has been identified in three different complexes; Isw1a, Isw1b, and independent Isw1 (Vary *et. al.* 2003; Mueller and Bryk. 2007). Only Isw1b contains putative MBDs, which are located in the subunits Ioc2 and Ioc4. It has been shown that Isw1 localization is dependent on histone H3 methylation (Santos-Rosa, *et. al.* 2003; Morillon *et al*. 2005), thus it is likely that the MBDs are important for chromatin interactions, and thus proper function, of the Isw1b complex.

Ioc2 contains a non-canonical Plant Homeo Domain (PHD) finger. A subset of PHD finger proteins recognize methylated lysines by means of an aromatic cage, which is held together by the zinc finger (Peña *et. al.* 2006). The PHD finger of Ioc2 is well conserved however it is missing two of the cystines required to coordinate the first zinc ion of the finger. This non-canonical domain is often left out of studies involving PHD fingers and is thus not well characterized, even though it possesses the conserved Tryptophan residue required to form an aromatic cage (Fig. 3.5) as well as a critical

aspartic acid that specifies binding of many fingers to methylated lysine 4 of histone H3. In order to see if this domain is important for the function of Isw1b, *ioc2D574A* was created (D574 is the conserved aspartic acid indicated by a diamond in Fig. 3.5). The highly conserved aspartic acid has been shown to be essential for the interaction of multiple PHD finger proteins with H3K4me mimicking peptides (Shi *et. al.* 2007). Thus this mutation should prevent Ioc2 from being properly localized to methylated chromatin provided that the PHD finger is responsible for said localization.

Ioc4 on the other hand, contains a Proline Tryptophan Tryptophan Proline (PWWP) domain (Fig. 3.6). This domain has been recently shown to also recognize methylated lysines though an aromatic cage (Wang *et. al.* 2009). To investigate the importance of this domain, *ioc4P21A* was created. The mutated proline 21 is the most highly conserved residue in the domain, and should abolish its function (Fig. 3.6). If either the PWWP domain of Ioc4 or the PHD finger of Ioc2 is important for the function of the Isw1b complex, then the mutations should mimic phenotypes caused by loss of Isw1b function.

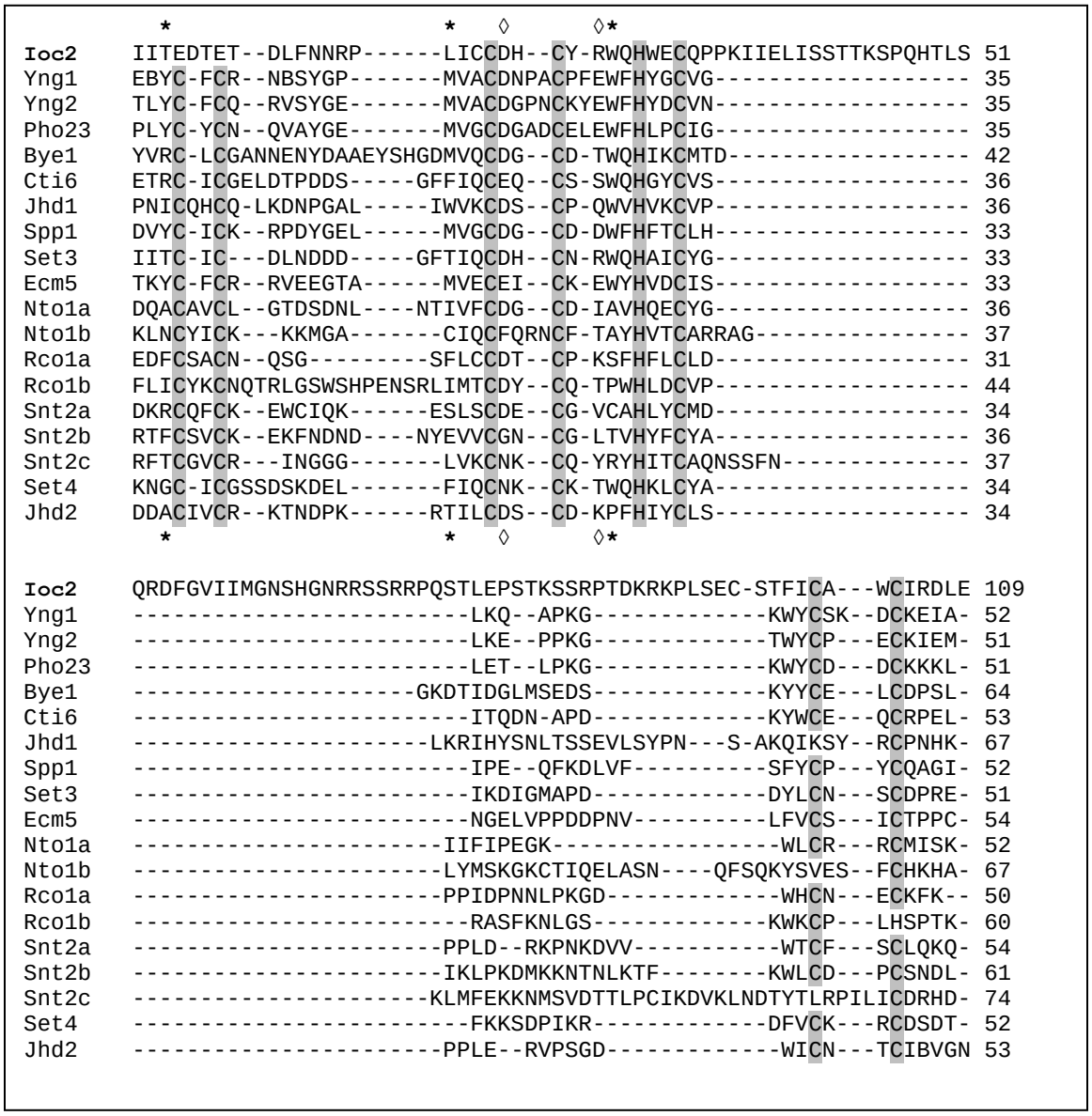


Figure 3.5 Alignment of PHD fingers from Saccharomyces cerevisiae.

The 19 PHD fingers of *Saccharomyces cerevisiae* were aligned using ClustalW2. Residues that coordinate zinc ions and maintain the finger structure are highlighted. Residues that form an aromatic cage to interact with H3K4 are starred. Residues essential for interaction with H3R2 and aid H3K4me binding are marked with diamonds. Nto1, Rco1, and Snt2 contain multiple PHD fingers which are differentiated by lowercase letters a-c.

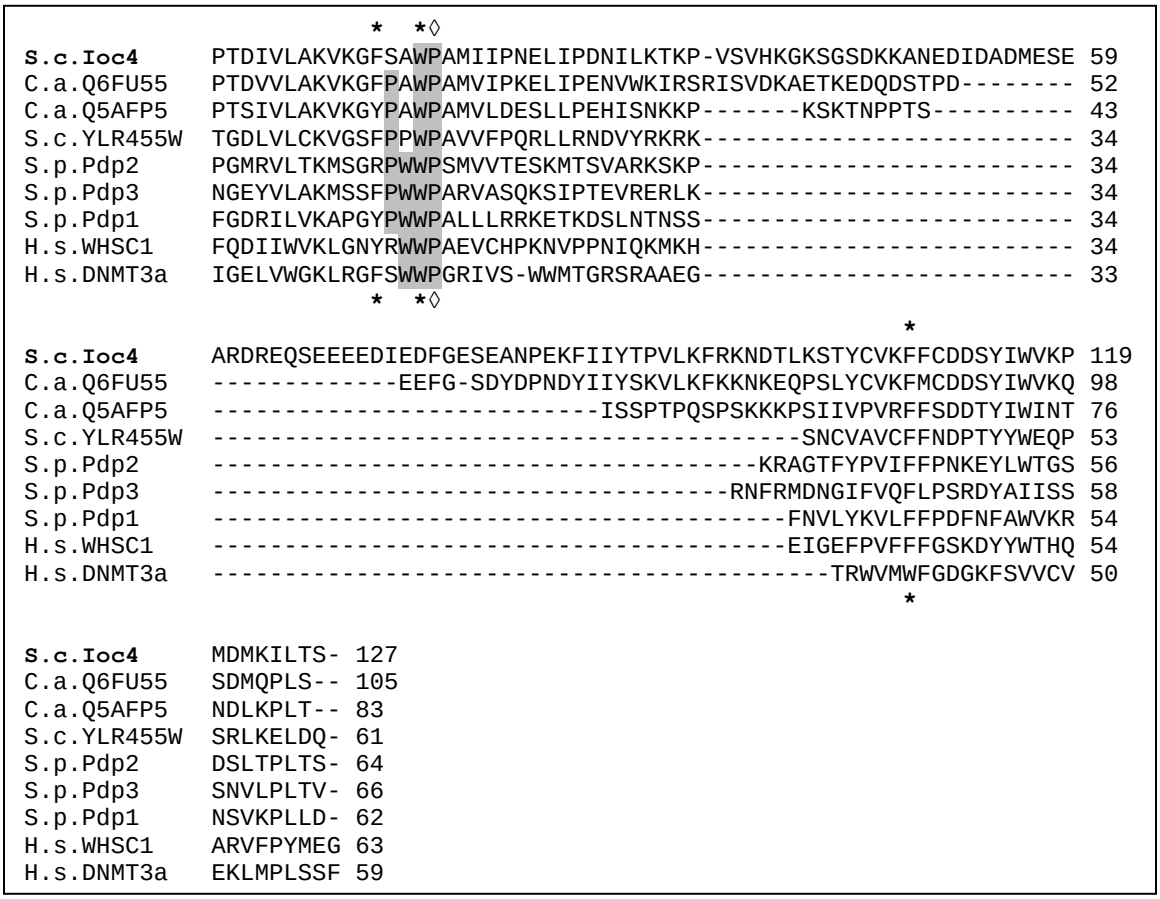


Figure 3.6 Alignment of PWWP domains.

Alignment of all the PWWP domains of *Saccharomyces cerevisiae, Schizosaccharomyces pombe,* and *Candida albicans* as well as 2 PWWP domains from *Homo sapiens*. The highlighted region is the PWWP motif. The residues that make up the aromatic cage for methyl-lysine binding are starred. The diamond indicates the proline that was mutated to an alanine for the experiments in this thesis.

3.2.1 Phenotype analysis of Isw1 methyl-lysine binding mutants

As an ATP-dependent chromatin-remodeling protein, Isw1 must be properly localized to its chromatin substrate in order to carry out its physiological role. Thus any perturbation of the mechanisms involved in localizing Isw1 would have a similar affect as inhibiting Isw1's biological function. To investigate the roles played by the MBDs of Isw1b on its localization, we either mutated the domains, or deleted the genes that encode the domain-bearing subunits, and assayed for Isw1b function by phenotypic analysis.

There are several published phenotypes for lack of Isw1 function including temperature sensitivity (Pinskya *et. al.* 2009), synthetic lethality with *yng2*Δ, and synthetic temperature sensitivity in a *chd1Δ isw2Δ* strain (Tsukiyama *et. al.* 1999). Unfortunately these phenotypes could not be reproduced in our strain background or in strains generated by the *Saccharomyces* deletion project. We also tried phenotypes for galactose auxotrophy (Kundu *et. al.* 2007), and enhanced galactose auxotrophy in the presence of ethidium bromide, to no avail. Furthermore, due to the availability of already prepared media, we tested *isw1Δ* strains for sensitivity to the drugs antimycine A and a combination of the iron limiting drugs bathophenanthrolin and bathocuprocine di-sulffonic acid, neither of which resulted in a phenotype.

Another more promising phenotype we attempted to test for was sensitivity to the drug amantadine hydrochloride. This phenotype was found by the Boone lab in a massive drug screen in which the entire yeast deletion set was pooled into a single culture, and then split into two. One culture was grown in the presence of a drug, and the other in the presence of di-methyl-sulfoxide (DMSO) as a negative control. After competitive growth in these conditions, PCR was used to amplify barcodes that are unique to each deletion

strain. The PCR products from each bar code were then hybridized to a microarray to assay for relative amounts of product. Comparisons of the amount of product generated from the culture grown with drug to that of the culture grown with DMSO were made to identify strains that showed sensitivity to the drug of interest relative to the other deletion strains in the culture. This procedure was performed with 82 different chemicals (Parsons *et. al.* 2006). Under these competitive growth conditions deletion of genes encoding the entire Isw1b complex, *ISW1*, *IOC2*, and *IOC4*, were found to result in sensitivity to the drug amantadine hydrochloride. Furthermore, an *ioc3Δ* mutant, which lacks a part of the Isw1a complex, was not sensitive suggesting the phenotype was specific to loss of Isw1b. Additionally mutation of many genes involved in the methylation of H3K4, such as *BRE1, PAF1, SDC1, SWD1,*and *SWD3* (Miller, *et. al.* 2001; Lee *et. al.* 2007)*,* also resulted in sensitivity to amantadine hydrochloride suggesting that Isw1b and H3K4 methylation function in the same pathway (Fig. 3.7). However, when *isw1Δ, ioc2Δ,* and *ioc4Δ* strains are grown alone in amantadine hydrochloride, no phenotype is detected using both solid and liquid media, despite the fact that identical strains were used. Our inability to recapitulate the phenotypes observed by Parsons *et al*., is therefore presumably due to differences in experimental design. While we attempted to reproduce the culture conditions used, we were unable to mimic the competitive effect of co-culturing with the entire deletion set, which may amplify minor drug sensitivities.

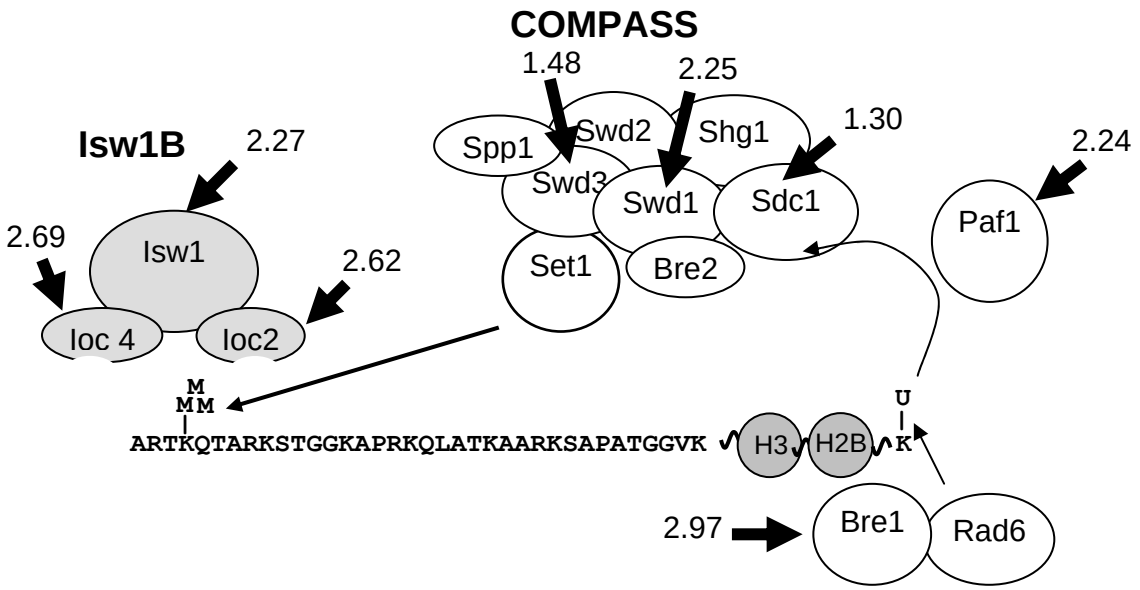


Figure 3.7 The Isw1b complex and the H3K4me pathway are required for resistance to the drug amantadine hydrochloride.

A partial model of the pathway responsible for methylation of H3K4. Set1 of the COMPASS complex is dependent on Paf1 as well as ubiqutination of H2BK123 for methylation of H3K4 *in vivo*. Bre1 and Rad6 are required for ubiquitination of H2BK123. Numbers indicate amantadine HCl sensitivity as described in Parsons *et. al.* 2006. Out of 4108 strains tested, only 269 deletions strains scored greater than 1.

3.2.2 Chromatin imunoprecipitation analysis of Isw1 methyl-lysine binding mutants

After attempting to investigate Isw1b localization by assaying for an Isw1-specific phenotype, we decided to assay for Isw1b localization directly using chromatin imunoprecipitation (ChIP). We used the 3'end of the active *MET16* gene as our target, for it has been shown that the Isw1b complex specifically ChIPs there while Isw1a does not (Morillon *et. al.* 2003). This allowes us to investigate if the MBDs of the Isw1b complex playes a role in the localization of the complex to chromatin. Figure 4.1 contains schematics of our interpretation of the data and may help clarify the results.

3.2.2.1 Design of strains and expression of constructs

In order to ensure that we were ChIPing Isw1b and not one of the other forms of Isw1, we decided not to tag Isw1 itself but one of the other Isw1b specific subunits. Since Ioc2 and Ioc4 are the only other subunits of Isw1b and they each contain a MBD that must be mutated, there is no uninvolved subunit of the Isw1b complex that can easily be used as a surrogate for tagging Isw1. As such we decided to tag one of either Ioc2 or Ioc4 with six repeats of the influenza hemagglutinin-HA-epitope antigen (6HA), while manipulating the other subunit. If our manipulation of one of the Ioc subunits, by either mutating the MBD or deleting the encoding gene, diminished Isw1b's ability to interact with chromatin, then the ChIP signal of the other 6HA tagged Ioc subunit should be diminished, assuming that the subunits can only bind chromatin when associated with Isw1b complex. In order to perform all the necessary ChIP experiments we set up the following system. First the strain *ioc2Δ ioc4Δ* was created as an empty parent strain. We then created the following integrating plasmids; *pIOC2.406*, *pioc2D574A.406*, *pIOC2-6HA.406*, *pIOC4.405*, *pioc4P21A.405*, *pIOC4-6HA.405*, *pioc4P21A-6HA.405*. By

integrating an Ioc2 encoding plasmid and an Ioc4 encoding plasmid from this set into our empty parent strain we could create any combination of tagged, untagged, mutated, or wild type Isw1b complex that we needed (Table 3-2). For experiments requiring null alleles of either *IOC2* or *IOC4* we integrated empty pRS406 or pRS405 respectively (Sikorski and Heiter. 1989). Although the strains used in all of the following experiments carrying deletions of both the endogenous *IOC2* and *IOC4* loci, in many cases we have rendered these strains *IOC2* or *IOC4* through integration of a wild type *IOC2* or *IOC4* at *ura3* or *leu2* respectively. For the sake of clarity, unless explicitly described as containing tagged, null, or mutated allele all strains are wild type for *IOC2* or *IOC4*. To demonstrate that the 6HA tag, does not affect the stability or expression of the tagged Isw1b subunits, we performed a western blot of the strains normalized by wet cell weight using an anti-HA antibody as a probe. The results in Figure 3.8 show that Ioc2-6HA, Ioc4-6HA, and Ioc4P21A-6HA are all stably expressed.

Once the strains were obtained, we had to address another important question before proceeding with ChIP. What if the MBDs played a structural role in the Isw1b complex, and their disruption leads to destabilization of the complex? To test the stability of the Isw1b complex in each of our above strains we tagged the Isw1 protein in each strain with the tandem affinity purification (TAP) tag, thus allowing us to purify the Isw1 containing complexes and probe for intact Isw1b using the 6HA tag on the other Isw1b subunits. This Isw1b complex stability data, when compared with the ChIP data yielded some very interesting results that are discussed in the following sections.

Table 3.2 Constructed strains for designer Isw1b complexes.

Parent Strain *ioc2Δ ioc4Δ*		
Integrated Vectors		**Effective Genotype**
ura3::pIOC2.406	*leu2::pIOC4.405*	Untagged *WT*
ura3::pIOC2-6HA.406	*leu2::pIOC4.405*	*IOC2-6HA, IOC4*
ura3::pIOC2-6HA.406	*leu2::pRS405*	*IOC2-6HA, ioc4Δ*
ura3::pIOC2-6HA.406	*leu2::p.ioc4P21A.405*	*IOC2-6HA, ioc4P21A*
ura3::pIOC2.406	*leu2::pIOC4-6HA.405*	*IOC4-6HA, IOC2*
ura3::pRS406	*leu2::pIOC4-6HA.405*	*IOC4-6HA, ioc2Δ*
ura3::pIOC2D574A.406	*leu2::pIOC4-6HA.405*	*IOC4-6HA, ioc2D574A*
ura3::pIOC2.406	*leu2::p.ioc4P21A-6HA.405*	*ioc4P21A-6HA, IOC2*
ura3::pRS406	*leu2::p.ioc4P21A-6HA.405*	*ioc4P21A-6HA, ioc2Δ*
ura3::pIOC2D574A.406	*leu2::p.ioc4P21A-6HA.405*	*ioc4P21A-6HA, ioc2D574A*

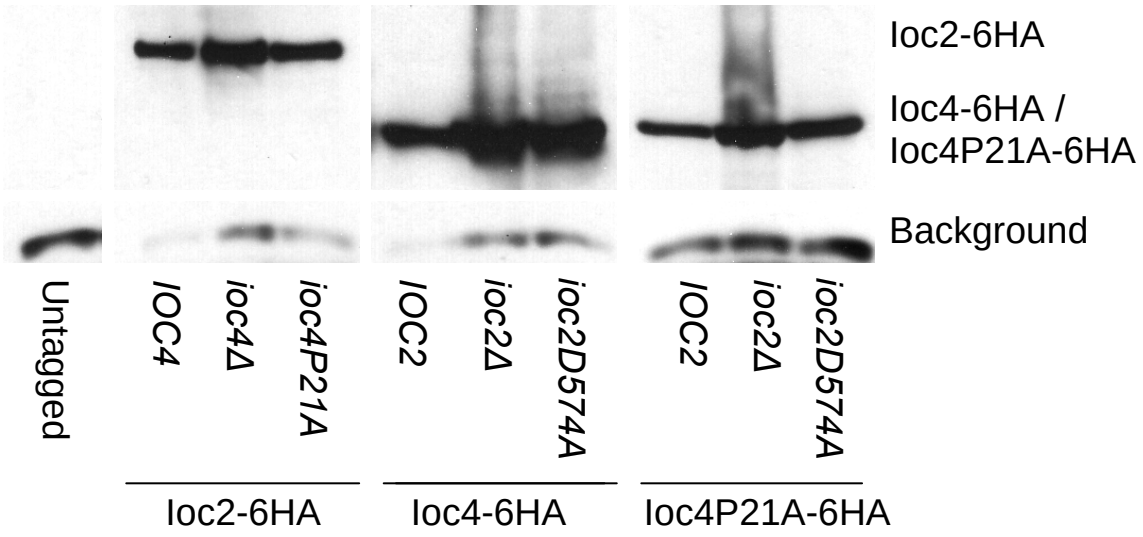


Figure 3.8 Expression of 6HA tagged Ioc2, Ioc4 and Ioc4P21A.

Western blot analysis of whole cell extracts from each of the strains described in Table 2 using an anti-HA antibody. Cultures were grown in synthetic media lacking methionine to ensure expression of tagged proteins in culture conditions used for ChIP. A non-specific background band that ran with the dye-front was included as an approximate loading control.

3.2.2.2 The PHD finger of Ioc2

The Ioc2 subunit of the Isw1b complex contains a putative PHD finger MBD. To investigate the role that this domain plays in the localization of the Isw1b complex we created strains of yeast lacking the Ioc2 subunit, or expressing an Ioc2 subunit with an alanine substitution of D574, a residue important for methyl-lysine recognition by other PHD fingers (Fig. 3.5, the aspartic acid indicated with a diamond). These strains were used for co-imunoprecipitation studies with TAP tagged Isw1 in order to assess the role of the PHD finger in the stability of the Isw1b complex, as well as ChIP experiments to assess the role played by the PHD finger in localizing Isw1b to the 3' end of the active *MET16* gene.

When Isw1-TAP is purified from a strain expressing Ioc4-6HA, an anti-HA western blot detects a 100 kDa band corresponding to Ioc4-6HA (Fig. 3.9B. lane 2.). However, in *ioc2*□ strains, this 100kDa band is absent (Fig. 3.9B. lane 3). The fact that Ioc4-6HA is still stably expressed in an *ioc2Δ* strain (Fig. 3.8), indicates a requirement for Ioc2 in maintaining the association of Ioc4 with Isw1. In order to determine if the PHD finger's putative methyl-binding ability is required for Isw1b stability, the same co-imunoprecipitation was performed with a strain expressing mutant Ioc2D574A. The mutant Ioc2D574A subunit did not disrupt the ability of Isw1-TAP to co-purify with Ioc4-6HA (Fig. 3.9B. lane 4), suggesting that the putative methyl-lysine binding ability of Ioc2 is not required for Isw1b complex stability.

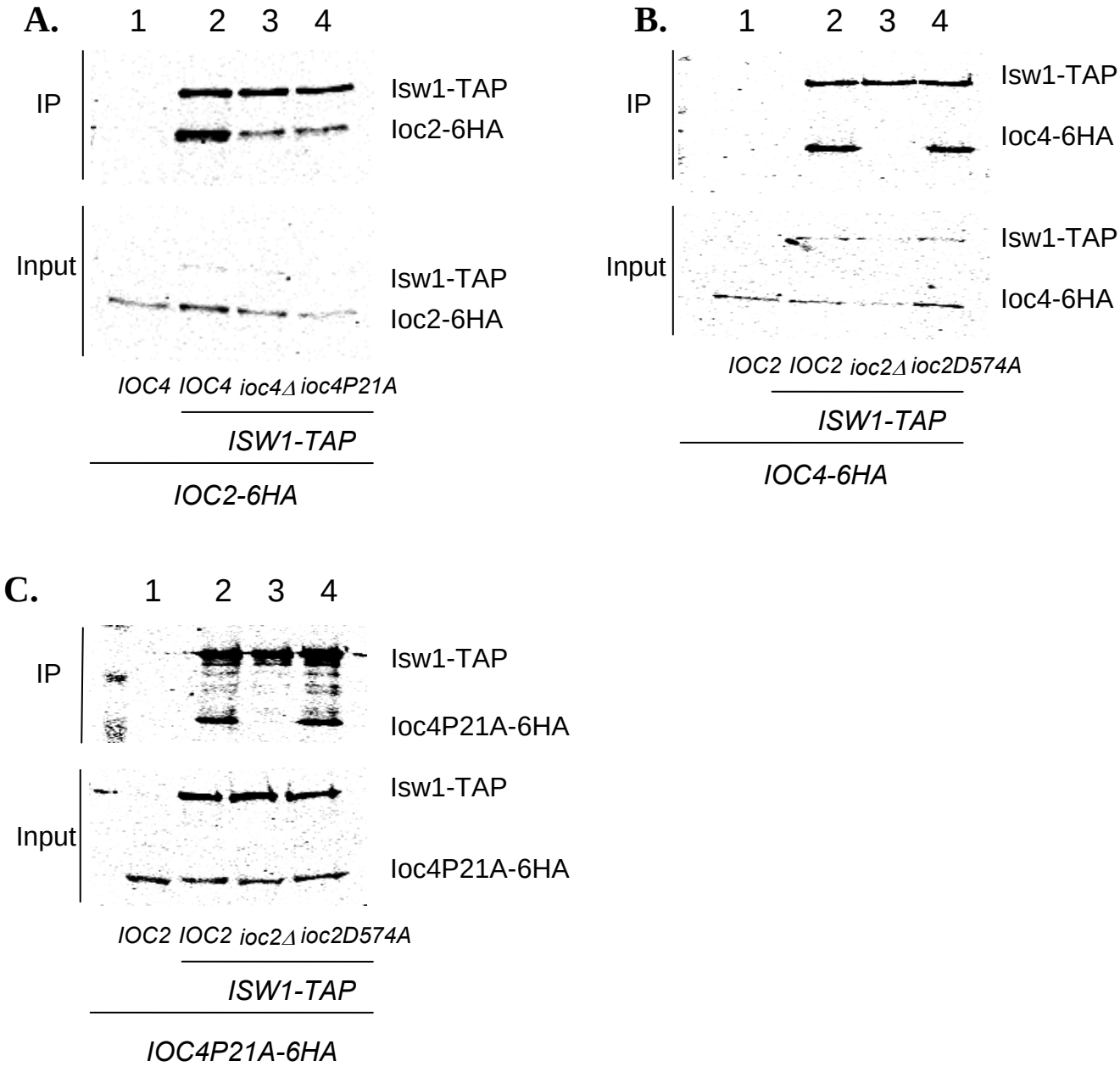


Figure 3.9 Complex stability of Isw1b determined by purification of TAP tagged Isw1 with 6HA tagged Ioc subunits.

Isw1 was TAP tagged in each of the strains described in table 2. The genotypes given represent the effective genotype as explained in table 2. Isw1-TAP was then purified from whole cell extracts using IgG resin and subject to western blot using anti-HA antibody. The protein A component of the TAP tag interacts with the detection antibodies giving a ~200 kDa band. (A) Ioc2-6HA co-purified with Isw1-TAP in strains expressing various forms of Ioc4. (B) Ioc4-6HA co-purified with Isw1-TAP in strains expressing various forms of Ioc2. (C) Ioc4P21A-6HA co-purified with Isw1-TAP in strains expressing various forms of Ioc2.

Once the role of Ioc2 in complex stability was determined we moved on to investigating the role Ioc2 and its PHD finger play in localizing Isw1b to the 3' end of *MET16*. This was done by ChIPing Ioc4-6HA in strains expressing either wild type Ioc2, no Ioc2, or mutant Ioc2D574A, and comparing the ChIP signal to that of an untagged strain. In a wild type *IOC2* strain, the Ioc4-6HA ChIP signal was ~11 fold stronger than that of the untagged strain (Fig. 3.10, compare lanes 1 and 4), indicating a successful ChIP. Deletion of *IOC2* resulted in considerably less signal, only ~3.5 fold stronger than untagged (Fig. 3.10, compare lanes 2 and 4) suggesting that Ioc2 is required for maximal binding of Isw1b to chromatin. These results support the conclusion that the Ioc2 subunit plays an important role in localizing the Isw1b complex to *MET16*. It is important to note that, even though a significant portion of the Ioc4-6HA ChIP signal is lost in an *ioc2Δ* strain, there is still significantly more signal observed than from the untagged strain. The relevance of this data will be highlighted when discussing the role of the Ioc4 PWWP domain in a later section.

To determine whether the putative MBD of Ioc2 is required for the interaction of Isw1b with *MET16*, we repeated the Ioc4-HA ChIP using the Ioc2D574A mutant. The results indicate that D574 of Ioc2 does not play a role in the localization of the Isw1b complex as ChIP of Ioc4-6HA is similar in both Ioc2 expressing strains, and strains expressing mutant Ioc2D574A (Fig. 3.10 lanes 1 and 3). Thus, while the Ioc2 subunit is important for localizing Isw1b to the 3' end of *MET16*, the putative H3K4 MBD of this protein is dispensable.

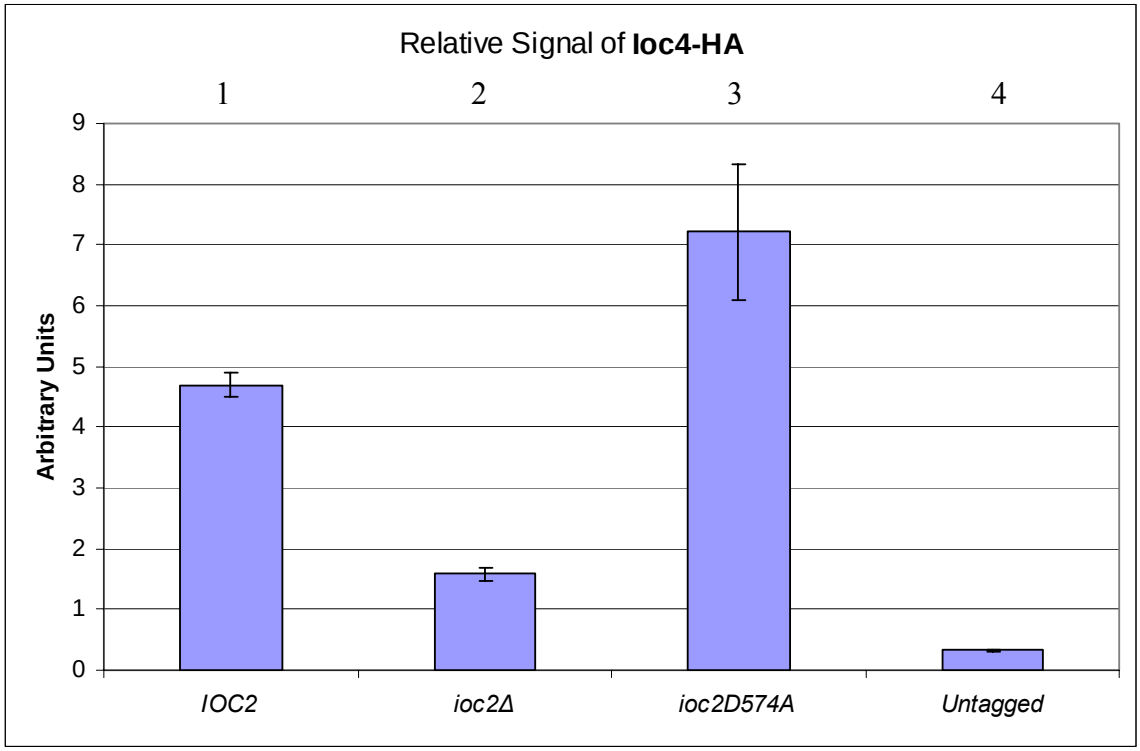


Figure 3.10 Ioc4 requires Ioc2 for full chromatin-binding activity

Chromatin Imunoprecipitation of Ioc4-HA from the strain *ioc2Δ ioc4Δ pIOC4-HA* transformed with plasmids encoding the indicated forms of Ioc2, or empty vector for the *ioc2Δ*. Untagged strains contained p*IOC4* instead of p*IOC4-HA*. Cultures were grown in synthetic media lacking methionine in order to activate the *MET16* gene. PCR primers specific to the 3' end of the active *MET16* gene were used to detect an interaction with chromatin. Each data point represents average percent ChIP relative to input from three independent yeast cultures performed in parallel and expressed in arbitrary units. Error bars indicate standard error.

The requirement of Ioc2 for Ioc4-chromatin interactions could reflect its role in Isw1b complex stability. In other words, it may be Ioc4 that is responsible for Isw1b-chromatin interactions and it is the loss of the Ioc4-Isw1 interaction in an *ioc2Δ* strain that disrupts the targeting of Ioc4 to DNA. Alternatively, it is possible that a yet unidentified motif in Ioc2 is mediating the interaction of Isw1 with chromatin and this motif is not disrupted in the Ioc2D574A mutant. To investigate further, we tested whether Ioc2 could bind chromatin independently of Isw1 or Ioc4. We found that deletion of *IOC4* disrupts the interaction of Ioc2 with Isw1 (Fig. 3.9A. lane 3), despite the fact that Ioc2 is still stably expressed (Fig. 3.8). When Ioc2-6HA is ChIPed from strains expressing wild type Ioc4 a significantly stronger signal is obtained than that from untagged strains indicating a successful ChIP (Fig. 3.11 lanes 1 and 4). However when the same is done in an *ioc4Δ* strain, where Ioc2 does not associate with Isw1b, the ChIP signal for Ioc2-6HA is significantly stronger than that from strains expressing wild type Ioc4 (Fig. 3.11 lanes 2 and 1)! These results indicate that either, Ioc2 is indeed able to bind chromatin independently of the remaining subunits of Isw1, or the remaining residual Ioc2-Isw1 complex in the *ioc4Δ* strain has greatly enhanced chromatin binding properties. Either way we have demonstrated that Ioc2-chromatin interaction is negatively regulated by Ioc4. A similar result is seen in strains expressing mutant Ioc4P21A (Fig. 3.11 lane 3), although the difference from an *IOC4* strain are not statistically significant. Furthermore, the partial loss of Ioc4-6HA ChIP observed in *ioc2Δ* mutants is consistent with the possibility that Ioc2 has chromatin binding ability (Fig. 3.10 lane 2). In conclusion our results suggest that Ioc2 can bind chromatin, although this activity is independent of the putative H3K4 MBD.

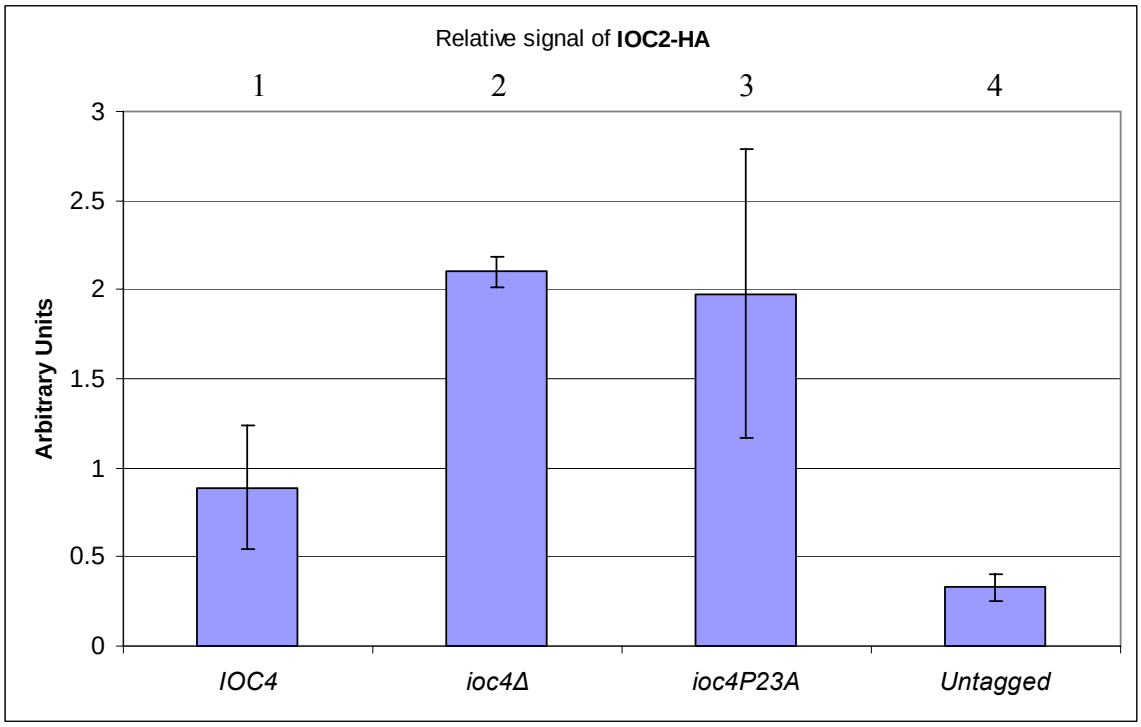


Figure 3.11 The presence of Ioc4 inhibits the ability of Ioc2 to bind chromatin

Chromatin Imunoprecipitation of Ioc2-HA from the strain *ioc2Δ ioc4Δ pIOC2-HA* transformed with plasmids encoding the indicated forms of Ioc4, or empty vector for the *ioc4Δ*. Untagged strains contained p*IOC2* instead of p*IOC2-HA*. Cultures were grown in synthetic media lacking methionine to activate the *MET16* gene. PCR primers specific to the 3' end of the active *MET16* gene was used to detect an interaction with chromatin. Each data point represents three independent whole cell extracts ChIPed in parallel. Error bars indicate standard error.

3.2.2.3 The PWWP domain of Ioc4

The Ioc4 subunit of the Isw1b complex contains a putative PWWP. The fact that Ioc4 can be detected at *MET16* when not associated with Isw1b in an *ioc2Δ* strain suggests that this protein also possesses chromatin-binding ability (Fig. 3.10 compare lanes 2 and 4). To investigate the role that the PWWP domain plays in the localization of the Isw1b complex we created strains of yeast lacking the Ioc4 subunit, or expressing an Ioc4 subunit with a P21A point mutation that changes the most highly conserved residue in the PWWP domain (Fig. 3.6 The proline indicated by a diamond). These strains were used with TAP tagged Isw1, as well as for ChIP experiments, in order to analyze the role played by the PWWP domain in localizing Isw1b to the 3' end of the active *MET16* gene.

To investigate the role that Ioc4 and its PWWP domain play in the stability of the Isw1b complex, we purified Isw1 from a strain expressing Ioc2-6HA. An anti-HA western blot of the purified material detected a 120 kDa band corresponding to Ioc2-6HA, demonstrating that an interaction between Ioc2 and Isw1 can be detected using this approach (Fig. 3.9A. lane 2). However in strains lacking Ioc4 or expressing Ioc4P21A, there is a reduction in the strength of the 120 kDa Ioc2-6HA signal, indicating that both Ioc4 and its PWWP domain are required for stability of the Isw1b complex (Fig. 3.9A. lanes 3-4). Interestingly, western blot of a similar purification of Isw1-TAP from a strain expressing Ioc4P21A-6HA generates a strong 100 kDa band corresponding to Ioc4P21A-6HA (Fig. 3.9C. lane 2). This indicates that the mutant Ioc4P21A still associates with Isw1b, even though the mutation disrupts the association of Ioc2 with Isw1b. Thus it is evident that the PWWP domain of Ioc4 is required for the association of Ioc2 with the Isw1b complex but not for the association of Ioc4 with Isw1.

To see how mutating the PWWP domain of Ioc4 affects the ability of the Isw1b complex to localize to chromatin we performed two separate ChIPs using three strains, an untagged strain, an Ioc4-6HA tagged strain, and an Ioc4P21A-6HA strain. First, the ability of Ioc4-6HA to localize to the 3' end of *MET16* was compared to the untagged strain (Fig. 3.12A.). Second, the ChIP of Ioc4P21A-6HA was compared to the untagged strain (Fig. 3.12B.). These comparisons indicate that Ioc4-6HA gives a ChIP signal ~15 fold stronger than untagged Ioc4, whereas mutant Ioc4P21A-6HA gives a ChIP signal statistically similar to untagged Ioc4, suggesting that the interaction of Ioc4-6HA with the 3' end of *MET16* is dependent on the Ioc4 PWWP domain. A significant part of this loss of localization is presumably due to the disruption of the Isw1b complex. As discussed earlier, mutation of Ioc4 to Ioc4P21A disrupts the association of Ioc2 with Isw1b (Fig. 3.9A.). When the Isw1b loses the chromatin-binding activity of Ioc2, Ioc4 will not properly localize to the 3' end of *MET16* (Fig. 3.10 lane 2). Furthermore we have seen that mutant Ioc4P21A prevents incorporation of Ioc2 in the Isw1b complex (Fig. 3.9C.), and Ioc4P21A does not localize to the 3' end of *MET16* (Fig. 3.12B.) suggesting that Ioc2 is essential for proper Isw1b localization.

Despite the essential nature of Ioc2 for localization of the Isw1b complex, experiments done in strains lacking Ioc2 revealed another role for the PWWP domain of Ioc4. As before, two ChIP experiments were performed, one comparing Ioc4-6HA to untagged (Fig. 3.13A.), and one comparing Ioc4P21A-6HA to untagged (Fig. 3.13B.), only this time the tagged strains did not express any Ioc2. Because loss of *IOC2* disrupts the interaction of Ioc4 with Isw1, these experiments analyzed the binding of Ioc4 to chromatin independently of the other subunits of Isw1b. A trend similar to that of

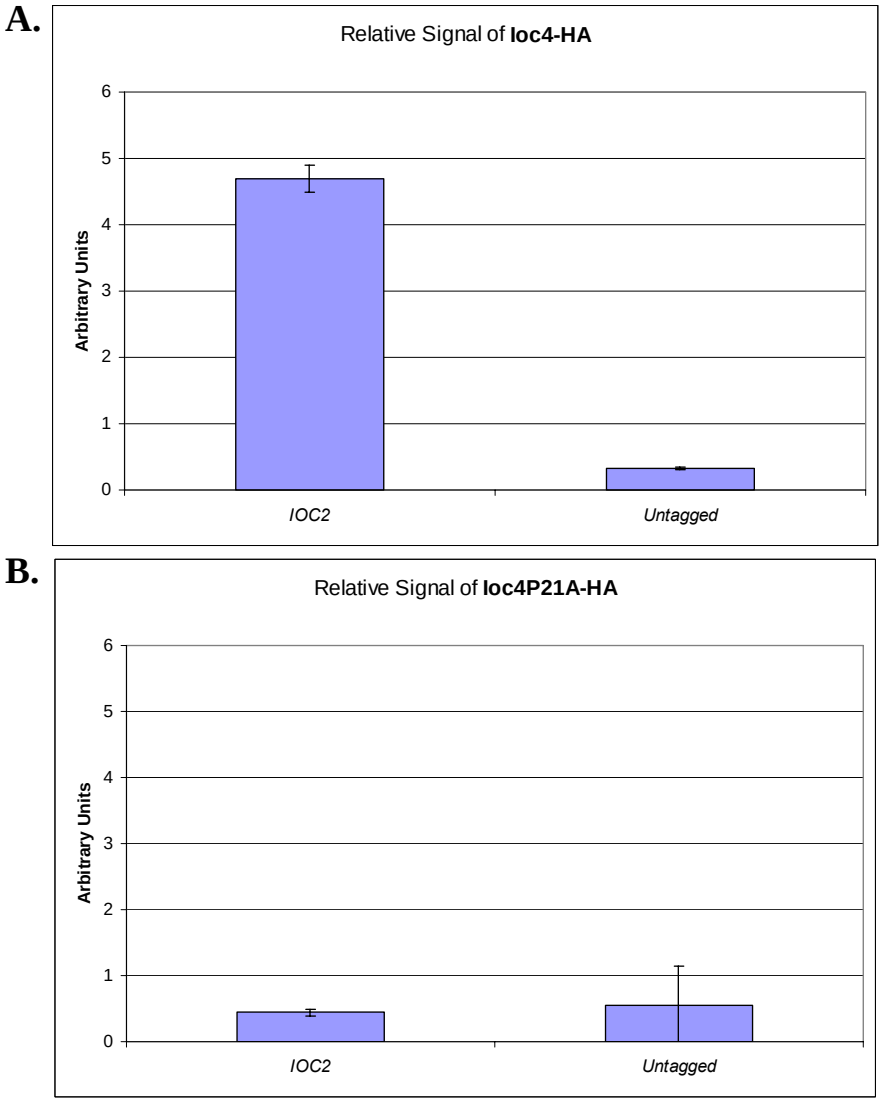
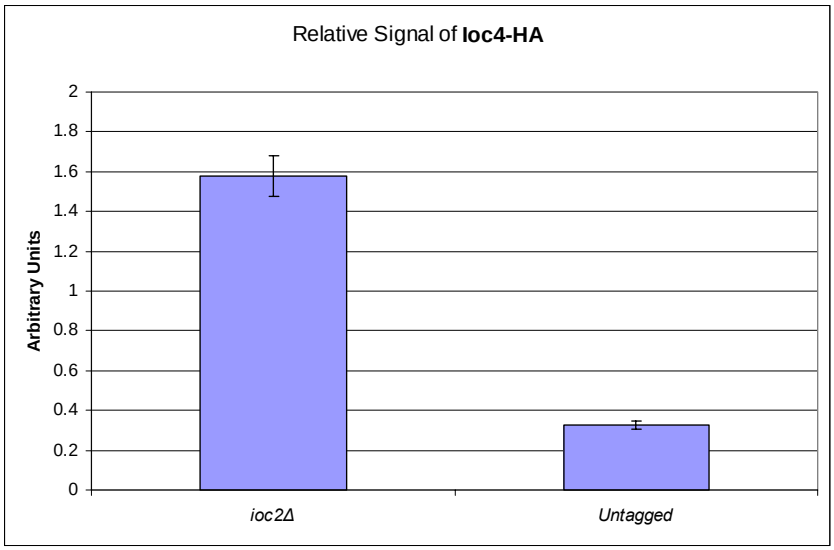


Figure 3.12 Ioc4 requires its PWWP domain to interact with chromatin.

ChIP of 6HA tagged Ioc4 or Ioc4P21A expressed from integrated plasmids in an *Δioc2Δioc4* strain expressing wild type Ioc2 also from an integrated plasmid. Untagged strains express wild type Ioc4 from an integrated plasmid. Cultures were grown in synthetic media lacking methionine to activate the *MET16* gene. Primers specific to the 3' end of the active *MET16* gene were used to detect an interaction with chromatin. Each data point represents three whole cell extracts that were ChIPed in parallel. Signal is arbitrary units obtained by dividing amount of DNA in IP by the amount of DNA in input. Error bars represent standard error.

A.



B.

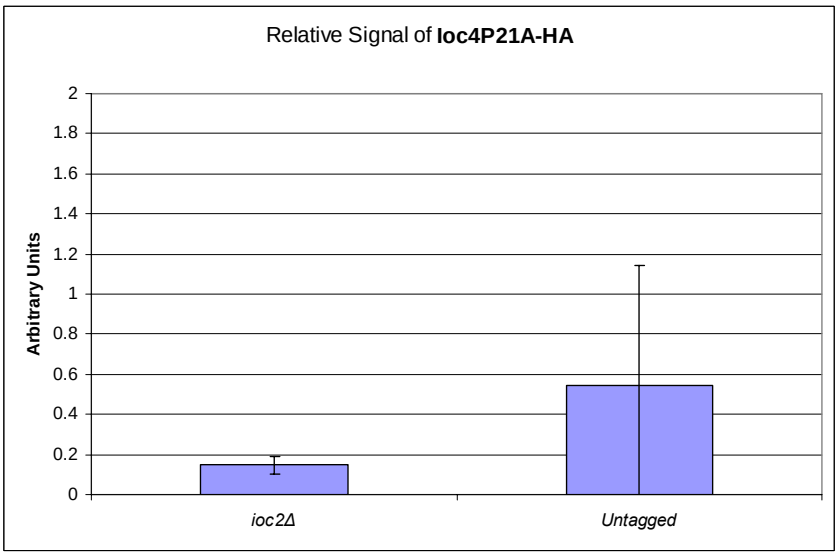


Figure 3.13 Ioc4 requires its PWWP domain to interact with chromatin in an IOC2-independent manner.

ChIP of 6HA tagged Ioc4 or Ioc4P21A expressed from integrated plasmids in an *Δioc2Δioc4* strain background. Untagged strains express wild type Ioc4 from an integrated plasmid. Cultures were grown in synthetic media lacking methionine to activate the *MET16* gene. Primers specific to the 3' end of the active *MET16* gene were used to detect an interaction with chromatin. Each data point represents three whole cell extracts that were ChIPed in parallel. Signal is arbitrary units obtained by dividing amount of DNA in IP by amount of DNA in input. Error bars represent standard error.

wild type *IOC2* strains, where Ioc4-6HA has a stronger ChIP signal than the untagged strain, but mutant Ioc4P21A-6HA has a similar signal as the untagged strain. Again, this suggests that the PWWP domain is important in localizing Ioc4 to the 3' end of *MET16*, though this time the difference in ChIP signal cannot be attributed to a loss of Ioc2, as both tagged strains are lacking that subunit.

Utilizing co-purification with TAP tagged Isw1 to test for complex stability, and ChIP to the 3' end of *MET16* to test for complex localization to chromatin we have demonstrated two roles for the PWWP domain of Ioc4. Firstly the domain is required for association of Ioc2 into Isw1b (Fig. 3.9A.), which is required for proper localization of Isw1b to the 3' end of *MET16* (Fig. 3.10 lane 2). Secondly the PWWP domain of Ioc4 is required for the Ioc4 subunit to interact with the 3' end of *MET16* in an Ioc2-independent manner (Fig. 3.13). It is evident that the PWWP domain is an important part of the Isw1b complex, and plays a role in localizing Isw1b to chromatin.

3.2.3 Dependence of Isw1b localization on Histone Methylation

Isw1 has been shown to associate with chromatin in an H3K4me-dependent manner (Santos-Rosa *et. al.* 2003; Morillon *et al*. 2005). This is one of the reasons we chose Isw1b as a model complex for histone methylation-dependent recruitment. However, the mechanism responsible for the dependence of Isw1-localization on H3K4me is still under investigation. The results discussed so far suggest that the Ioc2 and Ioc4 subunits of the Isw1b complex, which contain putative MBDs, play a role in Isw1b localization and may be responsible for the observed dependence of Isw1 on

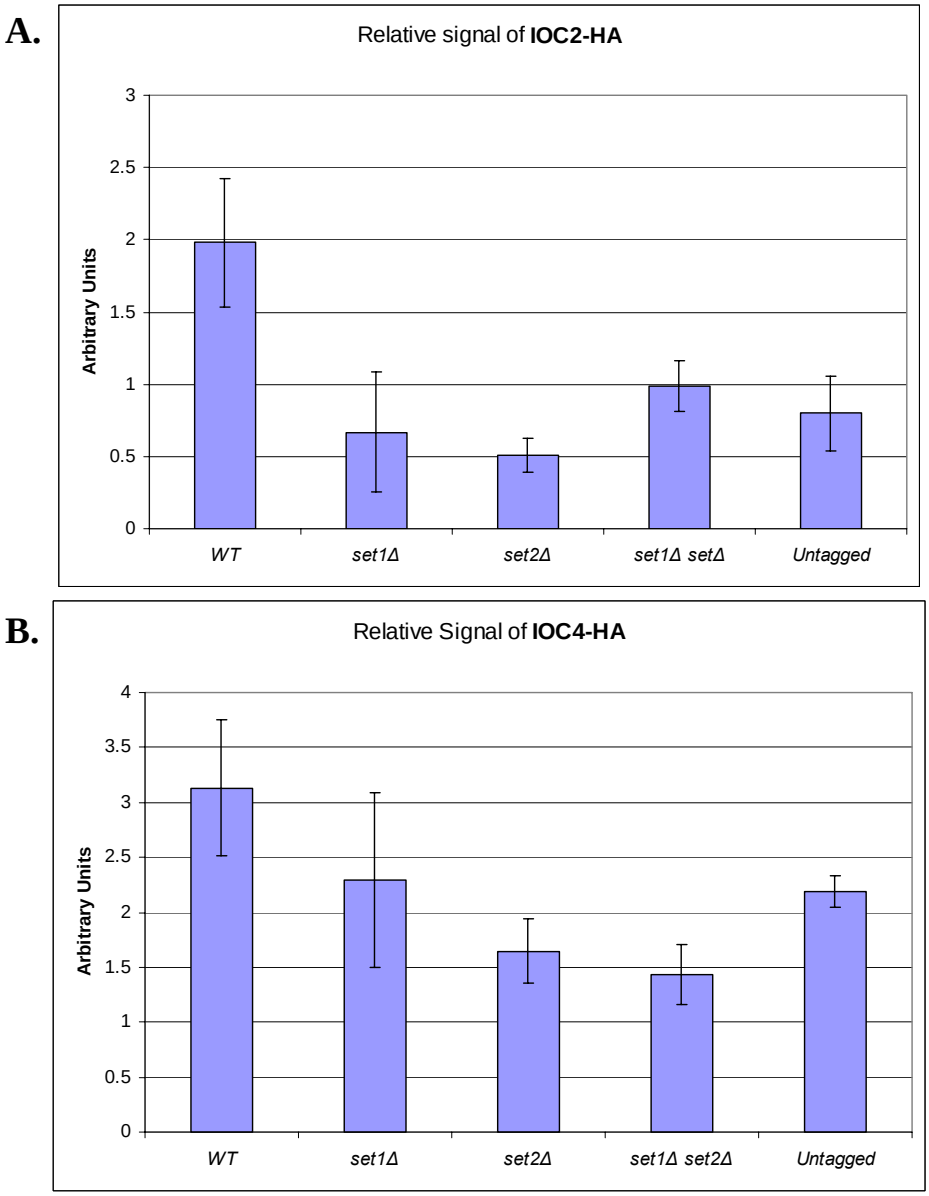


Figure 3.14 Removing histone tail methylation by deletion of SET1 and SET2 prevents Ioc2 and Ioc4 from localizing to chromatin.

ChIP of 6HA tagged (A) Ioc2 and (B) Ioc4 in strains lacking the histone methyltransferases Set1 and Set2. Cultures were grown in synthetic media lacking methionine to activate the *MET16* gene. Primers specific to the 3' end of the active *MET16* gene were used to detect an interaction with chromatin. Each data point represents the average of three whole cell extracts that were ChIPed in parallel. Error bars are standard error.

H3K4me for chromatin interaction. However, a direct interaction between the MBDs and methylated lysines on histones has yet to be observed.

If the observed dependency of Isw1 on H3K4me for chromatin binding is a function of the Isw1b complex, then one would expect the other subunits of the Isw1b complex to also be dependent on H3K4me for chromatin interaction. To test whether or not Ioc2 and Ioc4 rely on the presence of methylated histones to interact with *MET16*, we integrated plasmids expressing either Ioc2-6HA or Ioc4-6HA into strains deficient in various forms of histone methylation and performed ChIP to check for interactions with the 3' end of *MET16*. As mentioned earlier there are three histone methyltransferases in *S. cerevisiae*, two of which are responsible for methylating the N-terminal tails of histone H3: Set1 and Set2. A *set1Δ* strain lacks all H3K4 methylation, while a *set2Δ* strain lacks all H3K36 methylation, and a double *set1Δ set2Δ* strain lacks all histone tail methylation. ChIP of Ioc2-6HA revealed that loss of any H3 methylation results in loss of Ioc2-6HA localization to the 3' end of *MET16* (Fig. 3.13A.). This result is interesting as mutant Ioc2D574A, which lacks the putative H3K4me-binding activity, did not disrupt Isw1b localization at all (Fig. 3.10 lane 3). ChIP of Ioc4-6HA gives similar results to that of Ioc2, demonstrating a dependence on both H3K4 methylation and H3K36 methylation (Fig. 3.13B.). Now that we have demonstrated the dependence of the Ioc subunits on methylated histones for localization to chromatin, the next step would be to show a direct biochemical interaction between each subunit and histone H3 that is dependent on both the methylation state of the histone, and the presence of a functional MBD in the Ioc subunit.

Chapter 4 – Discussion

4.1 Cross-talk between H3K4me3 and H3K14ac

4.1.1 Acetylation of H3K14 is required for H3K4me3

Tri-methylation of histone H3K4 is a highly conserved mark associated with gene activity. It correlates with the promoter and 5' end of active genes in every organism that it has been observed in. Though not essential for the survival of yeast, deletion of the H3K4 tri-methyltransferase-encoding *trithorax* group (*TrxG*) genes in metazoans is embryonically lethal (Milne *et. al.* 2002). As a result of the significance of these methyltransferases, and the strong correlation between H3K4me3 and active transcription, a great deal of research has gone into understanding the regulation of this mark. This study adds a new component to that pathway, by identifying cross talk between H3K4 methylation and H3K14ac mediated by the newly characterized histone demethylase Jhd2.

The dependence of H3K4me3 on the H3K14 residue was first observed in yeast by the Shilatifard lab (Nakanishi *et al.* 2009). They demonstrated that mutating the fourteenth lysine of histone H3 to an alanine *in vivo* resulted in a global loss of the H3K4me3 mark from whole cell extracts. This interaction is interesting considering that H3K14 is a well known site of acetylation, and this acetylation co-localizes with H3K4me3. We were able to demonstrate that it is the acetylation of H3K14 that is required for H3K4me3 and not K14 itself. This was done by abolishing H3K14 acetylation through the deletion of the acetyltransferase-encoding gene, *SAS3*,

accompanied by the disruption of Gcn5-mediated acetylation through the deletion of *ADA2*.

4.1.2 Acetylation of H3K14 protects H3K4me3 from demethylation by Jhd2

The link between H3K4 methylation and H3K14 acetylation may function through a couple of different mechanisms. For one, H3K4 methylation by Set1 is dependent on transcription. Transcription is stimulated by histone acetylation. Thus, H3K14 acetylation could be an upstream requirement for transcription-dependent H3K4 methylation. Alternately, H3K14 acetylation may protect the local chromatin from demethylation by a histone demethylase. Because mutation of lysine 14 does not result in phenotypes consistent with a transcription defect, transcription is not likely responsible for the observed link. We therefore tested the possibility that histone acetylation prevents removal of H3K4me3 by a histone demethylase.

If histone acetylation blocks the activity of a histone demethylase, then the loss of the H3K4 demethylase should restore H3K4me3 levels in cells lacking H3K14ac. The first demethylase we tested was Jhd2, as it is the strongest H3K4-specific demethylase in yeast (Tu *et. al.* 2007). Using simple genetics and assaying for global H3K4me3 by western blot, we showed that the majority of H3K4me3 is lost when H3K14ac is absent, but rescued when *JHD2* is deleted. This indicates that acetylation of H3K14 protects H3K4me3 from demethylation by Jhd2.

A discrepancy between this study and previous research was the significant increase in global H3K4me3 levels that we observed in *jhd2*Δ strains. Two studies of Jhd2 as a demethylase were unable to detect such a change (Tu *et. al.* 2006; Mersmen *et. al.* 2009). However, the Zhang lab did manage to show a clear increase of global

H3K4me3 levels in a *jhd2*Δ strain of *S. cerevisiae* (Liang *et. al.* 2007). It is difficult to explain why different labs see different results with similar experiments. Perhaps it has to do with the antibodies used, or the sensitivity of the western blot. Regardless, the 51% global increase of H3K4me3 in the *jhd2*Δ strain as compared to wild type, cannot account for the observed 221% increase of H3K4me3 signal in the *ada2*Δ *sas3*Δ *jhd2*Δ strain as compared to the *ada2*Δ *sas3*Δ strain.

4.1.3 How does H3K14ac protect H3K4me3 from demethylation by Jhd2?

There are two possible explanations for how H3K14ac prevents removal of H3K4me3 by Jhd2. First, Jhd2 may specifically interact with an unmodified H3K14 effectively recruiting this protein to the H3 tail. If this is the case then although loss of the H3 HATs should result in increased Jhd2 binding and decreased H3K4me3, mutation of H3K14 should not. We found that replacing lysine 14 with either arginine, or glutamine had the same effect on histone methylation suggesting that this simple model is incorrect. However, one caveat is that H3K14ac may cause a conformational change to the H3 tail that prevents docking of Jhd2. Indeed it has been previously shown that acetylation of the H4 tail increases the alpha-helicity of the tail, effectively shortening the tail (Wang *et.al.* 2000). If the H3 tail undergoes a similar "shortening" upon acetylation of H3K14, this may weaken the interaction of Jhd2 with chromatin. Consistent with this hypothesis, any acetylation-induced structural change in the H3 tail would be unique to H3K14ac and could not be recapitulated by any other amino acid at this site.

The second explanation for how H3K14ac protects H3K4me3 from Jhd2 is that a protein that specifically recognizes H3K14ac blocks access of Jhd2 to the chromatin. Since this protein could not bind H3K14R or H3K14Q, these mutations would result in

the same loss of methylation as disruption of the H3-specific HATs. The only domain known to selectively bind acetylated lysines is the bromodomain. If a bromodomain-containing protein binds H3K14ac and blocks the access of Jhd2 to H3K4me3, then mutation of the bromodomain should result in the same loss of H3K4me3 as observed in a H3K14 mutant. There are 11 bromodomain-containing proteins in yeast and none were identified in screens for genes required for H3K4me3. However, since many bromodomain-containing proteins are essential for cell growth, and thus were not tested, this hypothesis remains a possibility.

4.1.4 Jhd2 only appears to demethylate tri-methylated H3

Our results differ noticeably from the Shilatifard lab's in that we could still detect a small amount of H3K4me3 in our lysine mutants while the Shilatifard lab could not. We attributed this to the fact that our anti-H3K4me3 antibody cross-reacts with H3K4me2. Though this did not hinder the interpretation of the data, it does help bring up an interesting point. Why does Jhd2 only demethylate tri-methyl H3 and not di-methyl H3? It is possible that Jhd2 functions as part of a complex and some other regulatory protein inhibits the full demethylase activity of Jhd2 until the right conditions are met. Testing of this possibility will have to wait until the physical interacting partners of Jhd2 and the other histone demethylases are identified.

4.1.5 The histone demethylases of *S. cerevisiae* contain putative methyl-lysine binding domains

One last interesting observation about Jhd2 that warrants further investigation is its PHD finger. In fact, three of the five histone demethylases in yeast, Jhd1, Jhd2, and Ecm5, have a PHD finger, and the other two have a similar zinc finger structure. The role of these putative MBDs located in demethylases remains mostly uninvestigated. Do they play a role in substrate recognition? Or do they mediate histone cross-talk by targeting these histone demethylases to different methyl marks than the ones that their JmjC domains specifically remove? While the PHD finger of Jhd2 does not bind methylated or unmethylated H3 tail peptides, the PHD fingers of Jhd1 and Ecm5 specifically interact with H3K4me3 and H3K36me3 respectively (Shi *et. al.* 2007). However since deletion of *JHD1* or *ECM5* does not result in significant changes in either methylation mark, the relevance of this data is unclear. ChIP and co-localization studies would be an excellent technique to help elucidate the potential role of these domains. Additionally being able to observe the localization of Jhd2 would allow one to see if H3K14ac protects chromatin by physically excluding the demethylase from these regions. Even though some of the physiological roles of histone demethylases are beginning to be unraveled, there are still many questions to be answered in the new and exciting field of histone demethylases.

4.1.6 Conclusions

H3K4me3 is arguably the most highly used indicator of transcriptional activity. However, how H3K4me3 is removed upon transcription repression has not been characterized. In this work we have shown for the first time how the cell mediates erasure of this hallmark of transcriptional activity, through the actions of the histone demethylase

Jhd2. Moreover, the observed sensitivity of Jhd2 to H3K14ac, may help explain why disruption of other HDMs results in such minor changes in histone methylation.

4.2. Methyl-lysine binding domains of Isw1b

The methylation mark on histones carries out its downstream effects by means of binding domains that recognize the methylated histone. These MBDs are found in proteins and protein complexes with chromatin-modifying functions. The MBDs are responsible for localizing the chromatin-modifying proteins to the regions of chromatin that bear the methylation mark specific to that MBD. With many different MBDs each specifically targeted to a small set of the many different histone methylation marks, histone methylation and MBDs have a remarkable capacity for regulation of chromatin-modifying activities.

Isw1 is the catalytic subunit of multiple ATP-dependent chromatin-remodeling complexes, and is highly conserved in eukaryotes. Isw1 has numerous functions in the cell including both activation and repression of different genes. Regulation of Isw1 activity appears to be achieved, in part, through incorporation into protein complexes. One such complex is Isw1b, which contains a PHD finger in the subunit Ioc2, and a PWWP domain in the subunit Ioc4. We used ChIP to investigate the roles that these MBDs play in the localization of Isw1b to chromatin, and our data suggest that Ioc4's PWWP domain is indeed a MBD that localizes Isw1b. We also found that the Ioc2 subunit was very important for localization of Isw1b, but our mutation of its PHD finger did not have any noticeable effects. For a schematic summary of our results see figure 4.1.

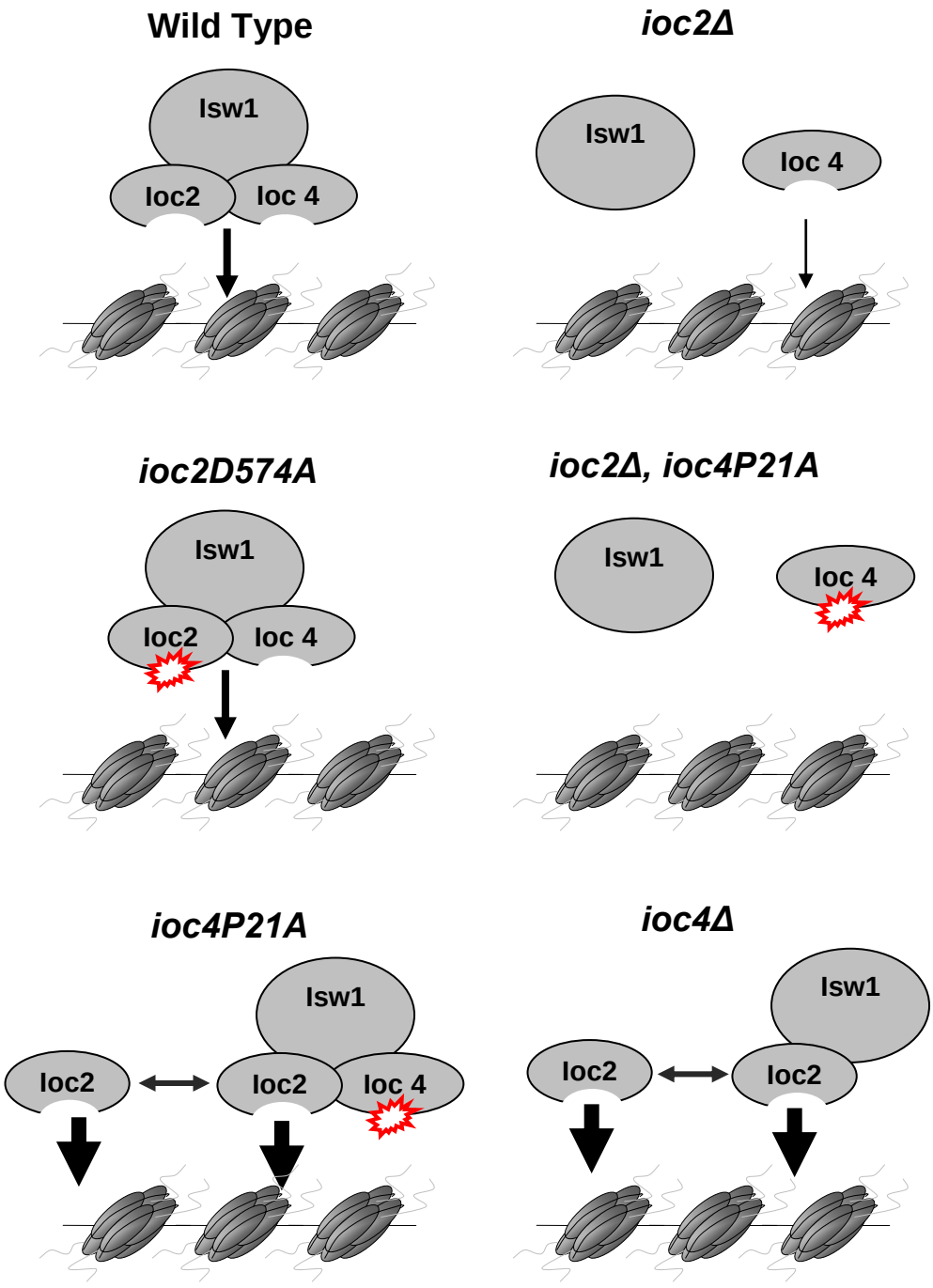


Figure 4.1 Isw1b complexes binding to methylated chromatin

Schematic representation of Isw1b-chromatin interactions of various mutant strains based on our results. The circles on the chromatin represent histone methylation. Black arrows indicate chromatin binding affinity with thicker arrows representing greater affinity. Grey arrows indicate that Ioc2 is only partially associated with Isw1.

4.2.1 The PHD finger of Ioc2 in Isw1 complex stability and localization

Ioc2 was found to be essential for the formation of the Isw1b complex and important for its proper localization. This was clearly indicated by the inability of Ioc4-6HA to co-purify with Isw1-TAP in an *ioc2*Δ strain, as well as the inability of Ioc4-6HA to fully localize to chromatin in an *ioc2*Δ mutant. However, our D574A mutation of the PHD finger of Ioc2 had no noticeable affect on either Isw1b complex integrity or Ioc4-chromatin interaction. In fact Ioc4-6HA seemed to localize to chromatin better after the PHD finger was mutated. The simplest interpretation of this data is that Ioc2's role is to incorporate Ioc4 into the Isw1b complex in a PHD finger-independent manner and the PHD finger is not required for localizing Isw1b, at least in the context of *MET16*. An alternate explanation is that the conserved aspartic acid, though important in other PHD fingers, is not an essential residue for chromatin binding in the non-canonical PHD finger of Ioc2. If so we may have obtained different results had we mutated the conserved tryptophan in the methyl-binding cage.

Deletion of *IOC2* results in decreased ChIP of Ioc4-6HA suggesting that while the Ioc2 PHD finger is dispensable for Isw1b-chromatin interaction, full length Ioc2 is not. This may indicate that there is an uncharacterized chromatin-binding domain in Ioc2 that has not yet been discovered. If so deletion mapping may reveal such a domain. Alternatively, Ioc2 may be important for Isw1b-chromatin interactions because it bridges the chromatin binding abilities of Isw1 and Ioc4. Isw1 contains two domains, the SANT and SLIDE domains, which have been shown to bind chromatin (Pinskaya *et al*., 2009). By linking the chromatin-binding abilities of Isw1 and Ioc4 (described below) Ioc2 may

enhance the binding of Isw1b to chromatin. Whether this is the primary role of Ioc2, or whether it contacts chromatin directly, cannot be differentiated with our data.

4.2.2 Ioc4 negatively regulates the interaction of Ioc2 with chromatin

Fascinatingly, Ioc2-6HA localization to chromatin is enhanced in the absence of Ioc4, even though loss of Ioc4 results in a significant reduction of Ioc2 association with Isw1 (Fig. 3.11; Fig. 3.9A). This suggests that Ioc4 negatively regulates Ioc2's ability to associate with chromatin. This regulation may be direct, in that the presence of Ioc4 in the Isw1b complex alters the chromatin-binding ability of Ioc2 or Isw1. This assumes that Isw1b has chromatin-binding activity that is not disrupted by our D574A mutation of the Ioc2 PHD finger. If this is the case, whatever Isw1-Ioc2 complex that remains in an *ioc4*Δ strain would have to have greatly increased binding affinity for chromatin as compared to intact Isw1b complex.

Another explanation for enhanced Ioc2 ChIP in an *ioc4*Δ mutant is that Ioc2 has a function that is independent of Isw1b. We have shown that in an *ioc4*Δ mutant there is less Ioc2 associated with Isw1. Dissociation of Ioc2 from the Isw1b complex in an *ioc4*Δ strain may result in the accumulation of Isw1b-independent Ioc2, which may bind chromatin better than Isw1b-associated Ioc2. There is support for existence of Isw1b-independent Ioc2, evident in the initial identification of Ioc2 (Vary *et. al.* 2003). When myc tagged Ioc2 was purified, there were two elution peaks, one large peak for Isw1b, and a smaller uncharacterized peak, which may be the independent Ioc2. Additionally an Isw1b-independent form of Ioc2 may explain why Ioc2 has a putative MBD that does not appear to be important for the localization of Isw1b. It would be interesting to see what

the effect of mutating the PHD finger of Ioc2 would be on its localization in an *ioc4*Δ strain.

4.2.3 The PWWP domain of Ioc4 in Isw1b complex stability and localization

Ioc4 is important for the stability of the Isw1b complex, as seen by reduced co-purification of Ioc2-6HA with Isw1-TAP in an *ioc4*Δ strain. However, there is still some association of Ioc2-6HA with Isw1 suggesting that the interaction is not entirely dependent on Ioc4. What is interesting though is that the PWWP domain of Ioc4 is also needed for Isw1 to fully co-purify with Ioc2-6HA, yet mutation of the PWWP domain does not inhibit the interaction between Ioc4 and Isw1. It would appear that the PWWP domain of Ioc4 is important for the incorporation of Ioc2 into the Isw1b complex, though not entirely necessary, as some Ioc2-Isw1 interaction persists.

The PWWP domain of Ioc4 is essential for localization of Ioc4 to chromatin. We first showed this in the context of wild type Isw1b, where mutation of Ioc4's PWWP domain prevents ChIP of Isw1b. To ensure that this loss of ChIP was not due to dissociation of the Isw1b complex we repeated the experiment in an *ioc2*Δ strain. In this strain the Isw1b complex is completely dissociated, yet some Ioc4-6HA still interacts with chromatin. However, this Isw1b-independent interaction is dependent on the PWWP domain. Thus we have conclusively shown that the PWWP domain is required for Ioc4 localization to chromatin, though we must note that this interaction is considerably weaker then that of wild type Isw1b.

4.2.4 Isw1b is dependent on both H3K4 and H3K36 methylation for localization

Using ChIP we have provided evidence that the Isw1b complex is dependent on both H3K4 methylation and H3K36 methylation for localization to chromatin. Even though we have not demonstrated a direct biochemical interaction between the subunits of Isw1b and methylated histones, we have shown that *SET1* and *SET2* are required for ChIP of both Ioc2-6HA and Ioc4-6HA, thus supporting the idea of an interaction between Isw1b and methylated histone H3. The fact that both *set1*Δ and *set2*Δ strains, which are lacking H3K4 methylation and H3K36 methylation respectively, show similar losses of Isw1b-chromatin interaction strongly suggests that Isw1b is recognizing two methylation marks co-operatively, and both are needed for full localization of Isw1b. Likely candidates are H3K36me3 and H3K4me1, which both tend to localize at the 3' end of actively transcribed genes. Furthermore, co-operative binding is also suggested by the relatively weak binding of Ioc4's PWWP domain to chromatin when not in the context of Isw1b. Unless incorporation of Ioc4 into Isw1b drastically alters its binding affinity, some other interaction must be aiding in the localization of Isw1b. Perhaps our D564A mutation of Ioc2 does not affect its chromatin binding ability and Ioc2 is contributing to the interaction. Alternatively, Isw1 itself plays a role in binding chromatin through its SANT and SLIDE domains. It may be that Ioc4's PWWP domain has relatively little impact on the binding of Isw1b to chromatin, but the additional binding affinity it does provide is what preferentially targets Isw1b to the 3' end of *MET16* as apposed to somewhere else that Isw1 may be needed. Or perhaps the combination of a couple weak interactions, including the PWWP domain of Ioc4, creates a strong multivalent interaction that is specific to certain combinations of methylation marks.

4.2.5 Conclusion

Methyl-lysine binding domains have been the focus of intense study due to their complex regulatory roles, yet the PWWP domain has just recently been identified as one. Here we not only identify the second PWWP domain required for chromatin localization, but show how it is involved in localizing the highly conserved Isw1 protein to specific regions of chromatin. Our data also suggests that Ioc2 may be involved in a chromatin binding activity that is inhibited by its association with Ioc4. Whether this involves Isw1, and whether the Ioc2 PHD finger is involved, is still unknown but may warrant investigation. Overall, this research has shed some light on the vast regulatory network of histone methylation in yeast.

References

Anderson, J., and J. Widom. 2001. Poly(dA-dT) promoter elements increase the equilibrium accessibility of nucleosomal DNA target sites. *Mol. Cell Biol.* **11:** 3830

Ausubel, F. M. 1987. Current protocols in molecular biology. Published by Greene Pub. Associates and Wiley-Interscience: J. Wiley, New York.

Barski, A., Cuddapag, S., Cui, K., Roh, T., Schones, D., Wang, Z., Wei, G., Chepelev, I., and K. Zhao. 2007. High-resolution profiling of histone methyaltions in the human genome. *Cell* **129:** 823

Bhaumik, S., Smith, E., and A. Shilatifard. 2007. Covalent modifications of histones during development and disease pathogenesis. *Nature Struct. Mol. Biol.* **14:** 1008

Boeke, J.D., Lacroute, F., G.R. Flink. 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarbocylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol. Gen. Genet.* **197:** 345-346

Brownell, J., Zhou, J., Ranalli, T., Kobayashi, R, Edmondson, D., Roth, S., and D. Allis. 1996. *Cell* **84:** 843

Bustin, M. 2001. Chromatin unfolding and activation by HMGN chromosomal proteins. *Trend. Biochem. Sciences* **26:** 431

Chang, B., Chen, Y., Zhao, Y., and R. Bruick. 2007. JMJD6 Is a Histone Arginine Demethylase. *Science* **318:** 444

Choi. J., Grimes, D.E., Rowe, K.M., L.J. Howe. 2008. Acetylation of Rsc4p by Gcn5p Is Essential in the Absence of histone H3 Acetylation. *Mol. Cell Biol.* **28:** 6967-6972

Dehé, P. *et. al.* 2005. Protein Interactions within the Set1 Complex and Their Roles in the Regulation of Histone 3 Lysine 4 Methylation. *J. Bio. Chem.* **281:** 35404

Dorigo, B., Schalch, T., Kulangara, A., Duda, S., Schroeder, R., and T. Richmond. 2004. Nucleosome Arrays Reveal the Two-Start Organization of the Chromatin Fiber. *Science* **306:** 1571

Dover, J., Schneider, J., Tawiah-Boateng, M., Wood, A., Dean, K., Johnston, M., and A. Shilatifard. 2002. Methylation of histone H3 by COMPASS requires ubiquitination of histone H2B by Rad6. *J. Bio. Chem.* **277:** 28368

Eissenberg, J., James, T., Foster-Hartnett, D., Hartnett, T., Ngan, V., and S. Elgin. 1990. Mutation in a heterochromatin-specific chromosomal protein is associated with suppression of poistion-effect variegation in *Drosophila melanogaster*. *Proce. Nat. Acad. Science* **87:** 9923

Erkina, T., Zou, Y., Freeling, S., Vorobyev, V., and A. Erkine. 2010. Functional interplay between chromatin remodeling complexes RSC, SWI/SNF and ISW1 in regulation of yeast heat shock genes. *Nucleic Acids Research* **38:** 1441

Fischle, W., Wang, Y, and D. Allis. 2003. Histone and Chromatin Cross-talk. *Curr. Opin. Cell Bio.* **15:** 172

Flaus, A., and T. Owen-Hughes. 2002. Mechanisms for Nucleosome Mobilization. *Biopolymers* **68:** 563

Frederiks, F., Tzouros, M., Oudgenoeg, G., van Welsem T., Fornerod, M., Krijgsveld, J., and van F. Leeuen. 2008. Nonprocessive methylation by Dot1 leads to functional redundancy of histone H3K79 methylation states. *Nature Struct. Mol. Bio.* **15:** 550

Goldstein, A., and J. McCusker. 1999. Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae. *Yeast* **15:** 1541

Guccione, E. *et al.* 2007. Methylation of histone H3R2 by PRMT6 and H3K4 by an MLL complex are mutually exclusive. *Nature* **449:** 933-937

Hassan, A., Prochasson, P., Neely, K., Galasinski, S., Chandy, M., Carrozza, M., and J. Workman. 2002. Function and selectivity of bromodomains in anchoring chromatin-modifying complexes to promoter nucleosomes. *Cell* **111:** 285

Hebbes, T., Thorne, A., and C. Crane-Robinson. 1988. A direct link between core histone acetylation and transcriptionally active chromatin. *EMBO J.* **5:** 1395

Horn, P., and C. Peterson. 2002. Molecular biology. Chromatin higher order folding--wrapping up transcription. *Science* **297:** 1824

Howe, L., Auston, D., Grant, P., John, S., Cook, R.G., Workman, J.L., and L. Pillus. 2001. Histone H3 specific acetyltransferases are essential for cell cycle progression. *Genes and Dev.* **15:** 3144-3154

Jacobs, S., and S. Khorasanizadeh. 2002. Structure of HP1 Chromodomain Bound to a Lysine 9-Methylated Histone H3 Tail. *Science* **295:** 2080

Kan, P., Caterino, T., and J. Hayes. 2009 The H4 Tail Domain Participates in Intra- and Internucleosome Interactions with Protein and DNA during Folding and Olibomerization of Nucleosome Arrays. *Mol. Cell. Bio.* **29:** 538

Kelly D.E., Lamb, D.C., S.L. Kelly. 2001. Genome-wide generation of yeast gene deletion strains. *Comp. Funct. Genomics* **2:** 236-242

Kirmizis, A. *et al.* 2007. Arginine methylation at histone H3R2 controsl deposition of H3K4 trimethylation. *Nature* **449:** 928-932

Koning, L., Corpet, A., Haber, J., and G. Almouzni. 2007. Histone chaperones: an escort network regulating histone traffic. *Nat. Struct. Mol. Bio.* **14:** 997

Kornberg, R. 1974. Chromatin Structure: A Repeating Unit of Histones and DNA. *Science* **184:** 868

Kouzarides, T. 2007 Chromatin Modifications and Their Function. *Cell* **128:** 693

Krogan, J., Kim, M., Tong, A., Golshani, A., Cagney, G., Canadien,V., Richards, D., Beattie, B., Emili, A., Boone, C., Shilatifard, A., Buratowski, S., and J. Greenblatt. 2003 Methylation of histone H3 by Set2 is *Saccharomyces cerevisiae*is linked to transcriptional elongation by RNA polymerase II.. *Mol. Cell Biol.* **23:** 4207

Krogan, N., Dover, J., Wood, A., Schneider, J., Heidt, J., Boateng, M., Dean, K., Ryan, O., Golshani, A., Johnston, M., Greenblatt, J., A. Shilatifard. 2003. The Paf1 Complex is Required for Histone H3 Methylation by COMPASS and Dot1p: Linking Transcriptional Elongation to Histone Methylation. *Mol. Cell* **11:** 721

Kundu, S., Horn, P., and C. Peterson. 2007. SWI/SNF is required for transcriptional memory at the yeast *GAL* gene cluster. *Genes Dev.* **21:** 997

Lan, F., Collins, R., Cegli, R., Alpatov, R., Horton, J., Shi, X., Gozani, O., Cheng, X., and Y. Shi. 2007. Recognition of unmethylated histone H3 lysine 4 links BHC80 to LSD1-mediated gene repression. *Nature* **448:** 718

Lee, J., Shukla, A., Schneider, J., Swanson, S., Washburn, M., Florens, L., Bhaumik, S., and A. Shilatifard. 2007. Histone Crosstalk between H2B Monoubiquitination and H3 Methylation Mediated by COMPASS. *Cell* **131:** 1084

Li, B., Gogol, M,. Carey, M., Lee, D., Seidel, C., and J. Workman. 2007. Combined actionof PHD and chromo domains directs the Rpd3S HDAC to transcribed chromatin. *Science* **316:** 1050

Liang, G., Klose, R., Gardner, K., and Y. Zhang. 2007. Yeast Jhd2p is a histone H3 Lys4 demethylase. *Nature Struct. Mol. Bio.* **14:** 243

Lindstrom, K., Vary, J., Parthun, M., Delrow, J., and T. Tsukiyama. 2006. Isw1 functions in Parallel with the NuA4 and Swr1 Complexes in Stress-Induced Gene Rerpession. *Mol. Cell. Biol.* **26:** 16

Longtine, M.S., McKenzie III, A., Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., Philippsen, P., and J.A. Pringle. 1998. Additional Modules for Versatile and Economical PCR-based Gene Deletion and Modification in *Saccharomyces cerevisiae*. *Yeast* **14:** 953-961

Lorch, Y., LaPointe, J., and R. Kornberg. 1987 Nucleosomes inhibit the initiation of transcription but allow chain elongation with the displacement of histones. *Cell* **49:** 203

Luger, K., Mäder, A., Richmond, R., Sargent, D., and T. Richmond. 1997. Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* **389:** 251

MacDonald, V.E., L.J. Howe. 2009. Histone acetylation: where to go and how to get there. *Epigenetics* **4:** 139-43

Martin, D., Baetz, K., Shi, X., Walter, K., MacDonald, V., Wlodarski, M., Goazni, O., Hieter, P., and L. Howe. 2006. The Yng1p plant homeodomain finger is a methyl-histone binding module that recognizes lysine 4-methylated histone H3. *Mol. Cell Biol.* **26:** 7871

Martin, D., Grimes, D.,Baetz, K., and L. Howe. 2006. Methylation of Histone H3 Mediates the Association of the NuA3 Histone Acetyltransferase with Chromatin. *Mol. Cell. Biol.* **26:** 3018-3028.

Mellor, J. 2006. It takes a PHD to Read the Histone Code. *Cell* **126:** 22

Mellor, J., and A. Morillon. 2004. ISW1 complexes in *Saccharomyces cerevisiae. Biochimica et Biophysica Acta.* **1677:** 100

Mersman, D., Du, H., Fingerman, I., South, P., and S. Briggs. 2009. Polyubiquitination of the demethylase Jhd2 controls histone methylation and gene expression. *Genes & Dev.* **23:** 951

Miller, T., Krogan, J., Dover, H., Erdjument-Bromage, P., Tempst, J., Greenblatt, J., and A. Shilatifard. 2001. COMPASS: a complex of proteins associated with a throthorax-related SET domain protein. *Proc. Natl. Acad. Sci. USA* **98:** 12902

Milne, T., Briggs, S., Brock, H., Martin, M., Gibbs, D., Allis, D., and J. Hess. 2002. MLL Targets SET Domain Methyltransferase Activity to *Hox* Gene Promoters. *Mol. Cell* **10:** 1107

Morillon, A., Karabetsou, M., O'Sullivan, J., Kent, N., Proudfoot, N., and J. Mellor. 2003. Isw1 Chromatin Remodeling ATPase Coordinates Transcription Elongation and Termination by RNA Polymerase II. *Cell* **115:** 425

Morrilon, A., Karabetsou, N., Nair, A., and J. Mellor. 2005. Dynamic Lysine Methylation on Histone H3 Defines the Regulatory Phase of Gene Transcription. *Mol. Cell* **18:** 723

Mueller, J., and M. Bryk. 2007. Isw1 Acts Independently of the Isw1a and Isw1b Complexes in Regulating Transcriptional Silencing at The Ribosomal DNA Locus in *Saccharomyces cerevisiae*. *J. Mol. Biol.* **371:** 1

Nakanishi, S., Sanderson, B., Delventhal, K., Bradford, W., Staehling-Hampton, K., and A. Shilatifard. 2008. A comprehensive library of histone mutants identifies nucleosomal residues required for H3K4 methylation. *Nat. Struct. Mol. Biol.* **15:** 881

Ng, H., Feng, Q., Wang, H., Erdjument-Bromage, H., Tempst, P., Zhang, Y., and K. Struhl. 2002. Lysine methylation within the globular domain of histone H3 by Dot1 is important for telomeric silencing and Sir protein association. *Genes & Dev.* **16:** 1518

Ng, H.H., Robert, F., Young, R.A., and K. Struhl. 2003. Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. *Mol. Cell* **11:** 709-19

Nightingale, K., Gendreizig, S., White, D., Bradbury, C., Hollfelder, F., and B. Turner. 2007. Cross-talk between Histone Modifications in Response to Histone Deacetylase Inhibitors. *J. Bio. Chem.* **282:** 4408

Parsons, A.B. *et. al.* 2006. Exploring the Mode-of-Action of Bioactive Compounds by Chemical-Genetic Profiling in Yeast. *Cell* **126:** 611-625

Peña, P.V., Davrazou, F., Shi, X., Walter, K., Verkhusha, V.V., Gozani, O., Zhao, R., and T.G. Kutateladze. 2006. Molecular mechanism of histone H3K4me3 recognition by plant homeodomain of ING2. *Nature* **442:** 100-103

Pinskaya, M., Nair, A., Clynes, D., Morillon, A., and J. Mellor. 2009. Nucleosome Remodeling and Transcriptional Repression are Distinct Functions of Isw1 in *Saccharomyces cerevisiae*. *Mol. Cell. Bio.* **29:** 2419

Pokholok, D.K., *et al.* 2005. Genome-wide Map of Nucleosome Acetylation and Methylation in Yeast. *Cell* **122:** 517-527

Puig, O., Caspary, F., Rigaut, G., Rutz, B., Bouveret, E., Bragado-Nilsson, E., Wilm, M., and B. Séraphin. 2001. The Tandem Affinith Purification (TAP) Method: A General Procedure of Protein Complex Purification. *Methods* **24:** 216-229

Santos-Rosa, H., Schneider, R., Bernstein, B., Karabetsou, N., Morillon, A., Weise, C., Schreiber, S., Mellor, J., and T. Kouzarides. 2003. Methylation of histone H3 K4 mediates association of the Isw1 ATPase with chromatin. *Mol. Cell* **12:** 1325

Sarma, K., and D. Reinberg.2005 Histone Variants Meet Their Match. *Nature Rev. Mol. Cell Biol.* **6:** 139

Schneider, R., Bannister, A., Myers, F., Thorne, A., Crane-Robinson, C., and T. Kouzarides. 2003. Histone H3 lysine 4 methylation patterns in higher eukaryotic genes. *Nat. Cell Biol.* **6:** 73

Schübeler, D., MacAlpine, D., Scalzo, D., Wirbelauer, C., Kooperberg, C., Leeuwen, F., Gottschling, D., O'Neill, L., Turner, B., Delrow, J., Bell, S., and M. Groudine. 2004. The histone modification pattern of active genes revealed through genome-wide chromatin analysis of higher eukaryote. *Genes Dev.* **18:** 1263

Shi, X. *et. al.* 2007. Proteiome-wide Analysis in *Saccharomyces cerevisiae* Identifies Several PHD Fingers as Novel Direct and Selective Binding Modules of Histone H3 Methylated at Either Lysine4 or Lysine 36. *J. BIol. Chem.* **282:** 2450-2455

Shi, Y., Lan, F., Matson, C., Mulligan, P., Whetstine, J., Cole, P., Casero, R., and Y. Shi. 2004. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **119:** 941

Sikorski, R., and P. Hieter. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* **122:** 19

Smith, B., and J. Denu. 2009. Chemical mechanisms of histone lysine and arginine modifications. *Biochimica et Biophysica Acta.* **1799:** 45

Taverna, S., Li, H., Ruthenburg, A., Allis, D., and D. Patel. 2007. How chromatin-binding modules interpret histone modifications: lessons from professional pocketpickers. *Nat. Struct. Mol. Biol.* **14:** 1025

Thoma, F., Koller, T., and A. Klug. 1979. Involvement of histone H1 in the organization of the nucleosome and of the salt-dependent superstructures of chromatin. *J. Cell. Biol.* **83:** 403

Trojer, P., and D. Reinberg. 2007. Facultative Heterochromatin: Is there a Distinctive Molecular Signature? *Mol. Cell* **28:** 1

Tsukada, Y., Fang, J., Erdjument-Bromage, H., Warren, M., Borchers, C., Tempst, P., and Y. Zhang. 2006. Histone demethylation by a family of JmjC domain-containing proteins. *Nature* **439:** 811

Tsukiyama, T., Palmer, J., Landel, C.C., Shiloach, J., and C. Wu. 1999. Characterization of the Imitation of Switch subfamily of ATP-dependent chromatin-remodeling factors in *Saccharomyces cerevisiae*. *Genes and Dev.* **13:** 686-697

Tu, S., Bulloch, E., Yang, L., Ren, C., Huang, W., Hsu, P., Chen, C., Liao, C., Yu, H., Lo, W., Freitas, M., and Ming-Daw Tsai. 2007. Identification of Histone Demethylases in *Saccharomyces cerevisiae. J. Biol. Chem.* **282:** 14262-14271

Vary, J.C., Gangaraju, V.K., Qin, J., Landel, C.C., Kooperberg, C., Bartholomew, B., and T. Tsukiyama. 2003. Yeast Isw1p Forms Two Separable Complexes In Vivo. *Mol. and Cellular Bio.* **23:** 80-91

Wang, X., and J. Hayes. 2008. Acetylation mimics within individual core histone tail domains indicate distinct roles in regulating the stability of higher-order chromatin structure. *Mol. Cell. Bio.* **28:** 227

Wang, X., Moor, S., Laszchzak, M., and J. Ausio. 2000. Acetylation Increases the α-Helical Content of the Histone Tails of the Nucleosome. *J. Biol. Chem.* **275:** 35013

Wang, Z., Schones, D., and K. Zhao. 2009. Characterization of human epigenomes. *Curr. Opin. Genet. Dev.* **19:** 127

Wood, A., Shukla, A., Schneider, J., Lee, J.S., Stanton, J., Dzuiba, T., Swanson, S., Florens, L., Washburn, M.P., Wyrick, J., Bhaumic, S. R., and A. Shilatifard. 2007. Ctk Complex-Mediated Regulation of Histone Methylation by COMPASS *Mol. Cell. Bio.* **27:** 709-720

Workman, J., and R. Kingston. 1998. Alteration of nucleosome structure as a mechanism of transcriptional regulation. *Annu. Rev. Biochem.* **67:** 545

Wyce, A., Xiao, T., Whelan, K., Kosman, C., Walter, W., Erick, D., Hughes, T., Krogan N., Strahl, B., and S. Berger. 2007. H2B ubiquitiylation acts as a barrier to Ctk1 nucleosomal recruitment prior to removal by Ubp8 within SAGA-related complex. *Mol. Cell* **27:** 275

Yamada, T., Fischle, W., Sugiyama, T., Allis, C., and S. Grewal. 2005. The nucleation and maintenance of heterochromatin by a histone deacetylase in fission yeast. *Mol. Cell* **20:** 173

Zhang, K., Tang, H., Huang, L., Blankenship, J., Jones, P., Xiang, F., Yau, P., and A. Burlingame. 2002. Identification of acetylation and methylation sites on histone H3 from chicken erythrocytes by high-accuaracy matrix-assisted laser desorption ionization-time-of-flight, matrix-assisted laser desorption ionization-postsource decay, and nanoelectrospray ionization tandem mass spectrometry. *Anal Biochem*. **306:** 259

Zhang, X., Bernatavichute, Y., Cokus, S., Pellegrini, M., and S. Jacobsen. 2009. Genome-wide analysis of mono-, di-, and trimethylation of histone H3 lysine 4 in Arabidosis thaliana. *Genome Biol.* **10:** 562