INVESTIGATION INTO THE ROLE AND REGULATION OF HISTONE LYSINE
METHYLATION IN THE YEAST SACCHAROMYCES CEREVISIAE

by

Vicki Elizabeth MacDonald

B.Sc., The University of British Columbia, 2005

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

in

THE FACULTY OF GRADUATE STUDIES

(Genetics)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

December 2011

© Vicki Elizabeth MacDonald, 2011
ABSTRACT

Post-translational modification of histones is one way that chromatin structure is altered to modulate transcription. Histone modifications serve as “marks” on chromatin, which are recognized by specific proteins that have a direct effect on chromatin structure. Lysine methylation is particularly complex and the function and regulation of all lysine methylation marks is not fully understood.

Histone H3 lysine 4 tri-methylation (H3K4me3) correlates with patterns of histone acetylation and transcriptional activation. Jhd2 has been identified as the major H3K4me3 histone demethylase (HDM) in S. cerevisiae, but its mechanism during transcriptional repression is unknown. This thesis demonstrates that acetylation of H3 negatively regulates demethylation of H3K4me3 by Jhd2 and provides evidence of crosstalk between lysine acetylation and H3K4me3. Additionally, we provide a potential mechanism for removal of H3K4me3 following gene repression.

Several methyl-lysine binding domains have been identified. Isw1 is found as the catalytic subunit of three ATP-dependent chromatin remodelling complexes in S. cerevisiae. These complexes function at different regions of the genome, but the mechanism behind this patterning is uncharacterized. One of these complexes,
Isw1b, localizes to the mid- and 3' regions of genes in a pattern similar to H3K36me3. We demonstrate that the Isw1b subunit, loc4, interacts with H3K36me3 through its PWPP domain. This provides a targeting mechanism for Isw1b to specific areas of the genome.

NuA3 is a histone acetyltransferase (HAT) complex that has also been demonstrated to interact with H3K36me3. Like, Isw1b, NuA3 contains an auxiliary protein that contains a PWPP domain, Nto2. Our data demonstrates that the Nto2 PWPP domain is the best candidate for NuA3 interaction with H3K36me3, however a direct interaction remains to be shown. We also demonstrate that both H3K4me3 and H3K36me3 are required for interaction of NuA3 with chromatin; thus, the subunits in NuA3 may undergo bivalent interaction with chromatin.

These findings provide insight into the regulation of H3K4me3 and the function of H3K36me3 during normal cellular function. H3K4me3 is regulated through crosstalk with lysine acetylation and H3K36me3 targets other chromatin modification complexes to specific areas of the genome.
PREFACE

Chapter 2: Vicki MacDonald designed and performed all research and wrote the paper. Ian Johnson created some of the strains used for this study. Julia Schulze assisted with experimental protocol for ChIP-on-chip, performed the data analysis, helped create figures based on the ChIP-on-chip data and edited the manuscript. Michael Kobor provided the facilities for ChIP-on-chip and edited the manuscript. LeAnn Howe helped design research and edited the manuscript.

Chapter 3: Vicki MacDonald designed and performed all research and wrote the manuscript. Ian Johnson created some strains and clones used for this study. Julia Schulze assisted with experimental protocol for ChIP-on-chip, performed the data analysis, helped create figures based on the ChIP-on-chip data and edited the manuscript. Michael Kobor provided the facilities for ChIP-on-chip and edited the manuscript. LeAnn Howe created some clones, designed research and edited the manuscript.

Chapter 4: Vicki MacDonald designed and performed all research and wrote the manuscript. LeAnn Howe designed research and edited the manuscript.
TABLE OF CONTENTS

ABSTRACT ........................................................................................................................................... ii
PREFACE .............................................................................................................................................. iv
TABLE OF CONTENTS .......................................................................................................................... v
LIST OF TABLES ..................................................................................................................................... viii
LIST OF FIGURES .................................................................................................................................... ix
LIST OF ABBREVIATIONS ...................................................................................................................... xi
NOMENCLATURE ................................................................................................................................... xiii
ACKNOWLEDGEMENTS ......................................................................................................................... xiv
DEDICATION ........................................................................................................................................... xv
CHAPTER 1. INTRODUCTION .................................................................................................................... 1
    1.1 CHROMATIN ................................................................................................................................. 1
    1.2 HISTONE MODIFICATIONS ............................................................................................................ 4
    1.3 HISTONE LYSINE ACETYLATION .................................................................................................. 8
    1.4 HISTONE LYSINE METHYLATION ............................................................................................... 12
    1.5 HISTONE LYSINE DEMETHYLATION ............................................................................................ 14
    1.6 METHYL-LYSINE BINDING DOMAINS ....................................................................................... 16
    1.7 PUTATIVE H3K36ME BINDING DOMAINS ................................................................................ 17
    1.8 CHAPTER SUMMARIES ................................................................................................................ 19

CHAPTER 2. HISTONE H3 ACETYLATION NEGATIVELY REGULATES DEMETHYLATION OF HISTONE H3K4 BY THE JMJC DOMAIN PROTEIN, JHD2 ................................................................................................................................. 21
    2.1 INTRODUCTION ............................................................................................................................. 21
    2.2 MATERIALS AND METHODS ........................................................................................................ 24
        2.2.1 Yeast strains and plasmids ....................................................................................................... 24
        2.2.2 Western blot analysis ............................................................................................................. 24
        2.2.3 Chromatin immunoprecipitation qPCR and ChIP-on-chip ....................................................... 25
        2.2.4 Expression microarray analysis ............................................................................................... 27
        2.2.5 Chromatin association assay .................................................................................................. 28
        2.2.6 Jhd2 purification and peptide demethylase assays ................................................................. 28
    2.3 RESULTS AND DISCUSSION ....................................................................................................... 29
2.3.1 Histone H3-specific HATs are required for maintenance of H3K4me3 levels. ................................................................. 29
2.3.2 Loss of H3K4me3 is not due to transcriptional defects in ada2Δ sas3Δ strains ......................................................................... 39
2.3.3 Loss of histone H3 acetylation promotes H3K4 demethylation by Jhd2. ............................................................................ 42
2.3.4 Histone H3 acetylation negatively regulates demethylation by Jhd2 in vitro ................................................................. 48
2.4 CONCLUSIONS .................................................................................................................. 50

CHAPTER 3. THE PWWP DOMAIN OF IOC4 BINDS TO TRI-METHYLATED LYSINE 36 OF HISTONE H3 ........................................................................................................ 54
3.1 INTRODUCTION ........................................................................................................... 54
3.2 METHODS AND MATERIALS ...................................................................................... 59
3.2.1 Yeast strains and plasmids ..................................................................................... 59
3.2.2 Chromatin immunoprecipitation and ChIP-on-chip .............................................. 61
3.2.3 Modified TAP tag immunoprecipitation (IP) and enrichment assays ...................... 62
3.2.4 In vitro binding assay .............................................................................................. 63
3.3 RESULTS AND DISCUSSION ..................................................................................... 64
3.3.1 Ioc4 is bound throughout the coding region of genes ......................................... 64
3.3.2 Histone H3 lysine 36 methylation is required for Ioc4 association with chromatin .............................................................................. 66
3.3.3 The PWWP domain is required for Isw1b association with chromatin .................. 69
3.3.4 Ioc4 preferentially interacts with histones which are methylated at lysine 36. ............................................................................ 72
3.4 CONCLUSIONS ........................................................................................................... 76

CHAPTER 4. THE PWWP DOMAIN OF NTO2 IS A PUTATIVE H3K36ME3 INTERACTION DOMAIN ........................................................................................................ 78
4.1 INTRODUCTION ........................................................................................................... 78
4.2 MATERIALS AND METHODS ..................................................................................... 83
4.2.1 Yeast strains and plasmids ............................................................... 83
4.2.2 Co-immunoprecipitations ................................................................. 86
4.2.3 Chromatin immunoprecipitation ...................................................... 87
4.2.4 Modified TAP tag immunoprecipitation ........................................... 88
4.3 RESULTS AND DISCUSSION ................................................................ 88
  4.3.1 Deletion of TAF14, EAF, and YLR455w show a sas3Δ phenotype in a yng1ΔPHD background ................................................................. 88
  4.3.2 Nto2 is not required for stable association of Eaf6 with Sas3 .... 95
  4.3.3 Eaf6 is required for stable association of Nto2 with Sas3 .......... 97
  4.3.4 Set1, Set2, and Eaf6 are required for Sas3 interaction with chromatin ................................................................. 99
  4.3.5 Nto2 interacts with histones ............................................................ 102
4.4 CONCLUSIONS ..................................................................................... 104

CHAPTER 5. CONCLUSIONS AND DISCUSSION ......................................... 108
  5.1 CHAPTER FINDINGS ............................................................................ 109
  5.2 GENERAL DISCUSSION ....................................................................... 111
  5.3 FUTURE DIRECTIONS ......................................................................... 116

REFERENCES ............................................................................................ 120
### LIST OF TABLES

Table 1.1: Yeast HATs and their substrates .......................................................... 9
Table 2.1: Yeast strains used in this study .............................................................. 24
Table 2.2: Antibodies used for ChIP and western blot analysis ............................ 25
Table 2.3: Primers used for ChIP analysis ............................................................. 27
Table 3.1: Yeast strains used in this study .............................................................. 60
Table 3.2: Antibodies used in this study ............................................................... 62
Table 4.1 Yeast strains used in this study .............................................................. 84
Table 4.2: Antibodies used in this study ............................................................... 86
LIST OF FIGURES

Figure 1.1: Post-translational modifications of human histones ................. 5
Figure 1.2: Patterns of modifications across an active gene ......................... 11
Figure 2.1: Deletion of SET1 does not affect levels of lysine 14 acetylation .... 30
Figure 2.2: Deletion of single HAT mutants does not affect levels of H3K4me3 on histone H3 ................................................................. 32
Figure 2.3: Deletion of ADA2 and SAS3 disrupts acetylation on the histone H3 tail ....................................................................................... 34
Figure 2.4: Loss of H3-specific histone acetyltransferases results in decreased levels of H3K4me3 .............................................................. 35
Figure 2.5: Deletion of ADA2 and SAS3 results in loss of H3K4me3 at several loci ....................................................................................... 36
Figure 2.6: Deletion of ADA2 and SAS3 results in loss of H3K4me3 at loci that only show marginal loss of H3K4me3 by ChIP-on chip .......... 38
Figure 2.7: Expression array analysis provides replicable data sets .............. 40
Figure 2.8: Deletion of ada2Δ sas3Δ does not cause transcriptional defects ... 41
Figure 2.9: Deletion of JHD2 rescues the H3K4me3 defect in cells lacking H3 acetylation ............................................................................ 44
Figure 2.10: Deletion of JHD2 does not rescue ada2Δ sas3Δ phenotypes ....... 46
Figure 2.11: Correlation between replicates of full genome expression profiles ......................................................................................... 47
Figure 2.12: Histone H3 acetylation negatively regulates demethylation but not binding by Jhd2 in vitro ......................................................... 49
Figure 2.13: Model of control of histone acetylation and methylation on the H3 tail ....................................................................................... 52
Table 3.2: Antibodies used in this study ....................................................... 62
Figure 3.1: Deletion of IOC2 results in destabilization of the Isw1b complex, whereas deletion of IOC4 has no effect on complex integrity .......... 65
Figure 3.2: Ioc4 is found within the coding region and the 3’ end of ORFs ...... 66
Figure 3.3: The HMT Set2 and H3K36 are required for Ioc4 association with chromatin ............................................................................. 68
Figure 3.4: The PWWP domain of Ioc4 is required for Ioc4’s interaction with chromatin. .................................................................................................................. 71
Figure 3.5: Ioc4 preferentially binds histones that are tri-methylated at H3K36 .............................................................................................................................................................................. 74
Figure 3.6: Isw1b preferentially interacts with H3K36 methylated histones..... 75
Figure 3.7: Schematic diagram of the architecture of Isw1b.......................... 77
Figure 4.1: A genetic screen to identify NuA3 complex components as potential H3K36me3 interactors shows that Nto1 shows a sas3Δ phenotype due to loss of Sas3 from the cell.................................................................................................................. 89
Figure 4.2: A genetic screen to identify NuA3 complex components as potential H3K36me3 interactors shows that disruption of TAF14 also results in loss of Sas3 from the cell. .................................................................................................................. 92
Figure 4.3: A genetic screen to identify NuA3 complex components as potential H3K36me3 interactors indicates that deletion of either EAF6 or NTO2 shows a sas3Δ phenotype. .......................................................................................................................................................................................... 94
Figure 4.4: Deletion of NTO2 does not disrupt NuA3 complex stability........ 96
Figure 4.5: Deletion of EAF6 results in loss of Nto2 from the NuA3 complex but does not affect Taf14 incorporation.................................................................................................................................................................................. 98
Figure 4.6: SET1, SET2, Yng1PHD finger and EAF6 are all required for Sas3 interaction with chromatin at the 5’ end of COX10. ................................................................. 101
Figure 4.7: Nto2 co-immunoprecipitates with histones..................................... 103
Figure 4.8: Schematic representation of the NuA3 complex interacting with the histone H3 tail. .................................................................................................................................................................................. 107
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-FOA</td>
<td>5-fluoroorotic acid</td>
</tr>
<tr>
<td>ac</td>
<td>Acetylated</td>
</tr>
<tr>
<td>ARS</td>
<td>Autonomously replicating sequence</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine tri phosphate</td>
</tr>
<tr>
<td>ChIP</td>
<td>Chromatin immunoprecipitation</td>
</tr>
<tr>
<td>ChIP-on-chip</td>
<td>ChIP followed by microarray</td>
</tr>
<tr>
<td>ChIP-qPCR</td>
<td>Chromatin immunoprecipitation followed by quantitative PCR</td>
</tr>
<tr>
<td>ChIP-seq</td>
<td>ChIP followed by high throughput sequencing</td>
</tr>
<tr>
<td>COMPASS</td>
<td>Complex of proteins associated with set1</td>
</tr>
<tr>
<td>CTD</td>
<td>C-terminal domain</td>
</tr>
<tr>
<td>H2BK123</td>
<td>Histone HTB lysine 123</td>
</tr>
<tr>
<td>H3K14</td>
<td>Histone H3 lysine 14</td>
</tr>
<tr>
<td>H3K18</td>
<td>Histone H3 lysine 18</td>
</tr>
<tr>
<td>H3K36</td>
<td>Histone H3 lysine 36</td>
</tr>
<tr>
<td>H3K4</td>
<td>Histone H3 lysine 4</td>
</tr>
<tr>
<td>H3K9</td>
<td>Histone H3 lysine 9</td>
</tr>
<tr>
<td>H3S10</td>
<td>Histone H3 serine 10</td>
</tr>
<tr>
<td>H4K20</td>
<td>Histone H4 lysine 20</td>
</tr>
<tr>
<td>HAT</td>
<td>Histone acetyltransferase</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>HDM</td>
<td>Histone demethylase</td>
</tr>
<tr>
<td>HMT</td>
<td>Histone methyltransferase</td>
</tr>
<tr>
<td>JmjC</td>
<td>Jumonji C</td>
</tr>
<tr>
<td>JmjN</td>
<td>Jumonji N</td>
</tr>
<tr>
<td>MBT</td>
<td>Malignant brain tumour</td>
</tr>
<tr>
<td>me1</td>
<td>Mono-methylated</td>
</tr>
<tr>
<td>me2</td>
<td>Di-methylated</td>
</tr>
<tr>
<td>me3</td>
<td>Tri-methylated</td>
</tr>
<tr>
<td>MORF</td>
<td>MOZ related factor</td>
</tr>
<tr>
<td>MOZ</td>
<td>Monocytic leukemic zinc finger protein</td>
</tr>
<tr>
<td>MYST</td>
<td>MOZ-YBF2(SAS3)-SAS2-TIP60</td>
</tr>
<tr>
<td>NuA3</td>
<td>Nucleosome acetyltransferase 3</td>
</tr>
<tr>
<td>NuA4</td>
<td>Nucleosome acetyltransferase 4</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PAF</td>
<td>Polymerase II associated factor</td>
</tr>
<tr>
<td>ph</td>
<td>Phosphorylation</td>
</tr>
<tr>
<td>PHD</td>
<td>Plant homeodomain</td>
</tr>
<tr>
<td>PTMs</td>
<td>Post translational modifications</td>
</tr>
<tr>
<td>PWWP</td>
<td>Proline-tryptophan-tryptophan-proline</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RNAPII</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>RSC</td>
<td>Remodels structure of chromatin</td>
</tr>
<tr>
<td>SAGA</td>
<td>Spt-Ada-Gcn5-acetyltransferase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>TBP</td>
<td>TATA binding protein</td>
</tr>
<tr>
<td>TELVII-L</td>
<td>Left arm of telomere seven</td>
</tr>
<tr>
<td>TES</td>
<td>Transcriptional end site</td>
</tr>
<tr>
<td>TSS</td>
<td>Transcriptional start site</td>
</tr>
<tr>
<td>ub</td>
<td>Ubiquitylation</td>
</tr>
<tr>
<td>WCE</td>
<td>Whole cell extract</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>α</td>
<td>Alpha – signifies anti</td>
</tr>
<tr>
<td>Δ</td>
<td>Delta – signifies deletion</td>
</tr>
</tbody>
</table>
NOMENCLATURE

This body of work follows conventional designations for genetics symbols and protein products. Dominant alleles of wild type loci are designated in upper case, italicized letters (ie. $JHD2$) and mutant genes are designated with lower case italicized letters (ie. $jhd2$). Gene deletions are indicated with a Greek ‘delta’ symbol following the gene name (ie. $jhd2\Delta$ indicated a deletion of the JHD2 locus). In the case where a portion of the locus has been deleted this is indicated after the delta sign (ie. $jhd2\Delta\text{PHD}$ indicates a deletion of the PHD finger domain of $JHD2$). The symbol :: followed by the gene name of an auxotrophic marker indicated that the locus has been replaced by insertion of that auxotrophic marker (ie. $jhd2::\text{HIS3MX6}$ indicates that $JHD2$ was replaced with the $\text{HIS3MX6}$ gene). Protein products are designated with only the first letter capitalized and no italics (ie. Jhd2 is the protein product of the $JHD2$ locus).
ACKNOWLEDGEMENTS

I would like to thank all Howe Lab members - past and present. I especially would like to thank Dr. LeAnn Howe for bringing me into her lab, giving me guidance and support and providing me with helpful critiques. You have been a wonderful mentor and I could not have asked for a better experience as a PhD student. Also thank you especially to Adam Chruscicki, Jennifer Choi, and Ben Martin for their friendship, helpful critiques, assistance and for always keeping me on my toes. I will miss you guys. I also thank our lab assistant and my wonderful friend Erynne for keeping the lab operating with an endless supply of dishes, media and buffers.

Thank you to everyone in the Kobor Lab making me feel welcomed to your lab. A special thanks to both Dr. Julia Schulze and Alice Wang for their assistance with the ChIP-on-chip protocol and to Julia for her help and expertise with data analysis. Thank you to all the professors of the Molecular Epigenetics Group for their help and support particularly Hugh Brock for all his advice. Thank you to my committee members, Dr. Christopher Loewen, Dr. Michael Kobor and Dr. Carolyn Brown for taking the time to listen to my research and providing insight and encouragement throughout the years.

Most of all, I would like to thank my family and friends for their support and for not complaining (too much) about my status as a perpetual student. I would not be where I am today without the love and support of both my parents and my grandparents. I especially want to thank Steven Maltby for always supporting me and loving me. I am so lucky to have always had you there for advice on grad school and to pick me up when I’m feeling down or stressed.

xiv
DEDICATION

I would like to dedicate this thesis to my loving family, especially my grandparents and parents whose love and support has brought me to where I am today. I love you all.

And to the memory of my “other father,” Derek Walker, who shortly before I commenced on this journey lost his battle with cancer. People like you are the reason I continue to do what I do.
CHAPTER 1. INTRODUCTION

1.1 CHROMATIN

The genetic information required for the creation and function of every organism is carried in each cell as DNA. Typically each eukaryotic cell has several billion base pairs of DNA, which are split into smaller linear segments known as chromosomes. In a single human cell, if all the chromosomes were placed end to end the total length of DNA would be over 2m in length (Peterson and Laniel 2004). For this reason, the cell requires an efficient means of organizing DNA into the very small space of the nucleus where it is stored. This organization occurs through packaging of DNA into a highly ordered structure known as chromatin. Chromatin is a combination of DNA, histones and non-histone proteins and its structure plays an important role in the regulation of numerous cell processes including transcription, DNA replication and repair.

The basic unit of chromatin is the nucleosome core particle, which is comprised of 147 base pairs of DNA wrapped around the globular domains of two copies each of histones H2A, H2B, H3 and H4 (Luger et al. 1997). Protruding from this core particle are the unstructured histone amino terminal tails, which are the primary site for histone post-translational modifications (PTMs) (Peterson and Laniel 2004) (see section 1.2). From this basic unit of the nucleosome, chromatin is further condensed into what is known as “higher order structure”. The first level of higher order structure is believed to be the 30nm fiber. Nucleosomes are connected by varying lengths of linker DNA depending on the species and the cell type within a
single organism (Woodcock and Ghosh 2010). Associated with the linker DNA are linker histones (such as histone H1), which have been shown to contribute to formation of higher order chromatin (Bednar et al. 1998, Thoma et al. 1979).

Higher order chromatin structure may also be formed through intra- and internucleosomal interactions. Arguably, Histone H4 has the most impact on chromatin structure, and several studies have investigated the role of the histone H4 tail, which is thought to interact with an acidic patch on the surface of the globular domain of histone H2A and contribute to the formation of the 30nm fiber (Dorigo et al. 2003, Kan et al. 2009, Shogren-Knaak et al. 2006, Sinha and Shogren-Knaak 2010). Beyond the 30nm fiber there are higher order structures of chromatin, the most obvious being the metaphase chromosome, which is compacted 10,000-20,000 fold and can be seen under a light microscope. However, there is little agreement in the field as to whether there are distinct levels of higher order of chromatin structure and if there is, which of these are bona fide structures (reviewed in (Woodcock and Ghosh 2010)).

Chromatin has traditionally been separated into two types of structures: heterochromatin and euchromatin. Heterochromatin was originally identified as regions of the nucleus that stain darkly with basic dyes and euchromatin as the lightly staining regions. More recently the word euchromatin has become synonymous with actively transcribed regions of the genome, whereas heterochromatin refers to non-transcribed regions, regardless of their staining

2
characteristics. Even in early chromatin research, there was recognition that euchromatic regions are structured with nucleosomes, which pose a barrier to transcription and other DNA-modification processes; thus, it was hypothesized that there must be some mechanism to overcome this barrier and create a dynamic chromatin environment (Lorch et al. 1987). How this dynamic environment is achieved within the cell has been the focus of many studies, particularly in the last two decades.

Several processes are known to affect chromatin structure. One example is incorporation of histone variants, which can alter structure of the nucleosome and either ease or hinder the ability of cellular machinery to disrupt histone/DNA contacts (Angelov et al. 2003, Angelov et al. 2004, Morrison and Shen 2009). Additionally, there are chromatin remodelling complexes, most of which use the energy of adenosine triphosphate (ATP) to destabilize the nucleosome/DNA contacts, resulting in sliding of nucleosomes along DNA or removal of histone proteins altogether (Hargreaves and Crabtree 2011). Another level of regulation is through the amino terminal tails of histones, which are exposed on the surface of the nucleosome and serve as the main site of histone PTMs (Peterson and Laniel 2004). PTMs, such as acetylation and methylation, have been proposed to act as docking sites for various chromatin remodelling complexes; thus, they also play a role in modifying chromatin structure (Peterson and Laniel 2004). In some eukaryotes, DNA methylation also contributes to chromatin structure by either directly preventing cellular machinery access to DNA or by acting as a target for histone modification complexes, which in
turn affect chromatin structure (Miranda and Jones 2007). These mechanisms work together to form a complex regulatory network that maintains a dynamic chromatin environment.

1.2 HISTONE MODIFICATIONS

Histones are post-translationally modified with numerous modifications such as methylation, acetylation, phosphorylation, ubiquitylation, and sumoylation. Most of these modifications occur on the amino-terminal tails, but there are some modifications occurring within the histone globular domains (and C terminal tails of H2A and H2B) (Figure 1.1). It had been suspected for many years (Allfrey et al. 1964) that there was a link between histone acetylation and transcription; however, the molecular basis for this link was unclear until the discovery that Gcn5, a transcriptional activator, was also a histone acetyltransferase (HAT) (Brownell et al. 1996). This finding lead to an explosion of research focussed on histone modifications and their link to transcriptional activity. Since this initial discovery, the development of ChIP-on-chip (chromatin immunoprecipitation followed by hybridization to microarrays) and more recently, ChIP-seq (ChIP followed by high throughput sequencing), has allowed study of the patterns of histone modifications across the genome (for example (Barski et al. 2007, Kurdistani et al. 2004, Liu et al. 2005, Pokholok et al. 2005, Schubeler et al. 2004, Sinha et al. 2006, Wang et al. 2008)).
Figure 1.1: Post-translational modifications of human histones

Schematic representation of histone amino-terminal tails in humans. A black (*) indicates modifications that are conserved in *S. cerevisiae*, a red (*) followed by a number indicates the modified residue in yeast. Modified from (Bhaumik et al. 2007).
Several studies have analyzed patterns of histone acetylation and methylation in the yeast *S. cerevisiae*, showing a correlation between transcriptional activity, acetylation and methylation (Kurdistani et al. 2004, Pokholok et al. 2005). In addition, a number of studies have demonstrated that different modifications occur in specific regions of actively transcribed loci (Kurdistani et al. 2004, Pokholok et al. 2005). For example, histone H3 acetylation (H3ac) and histone H3 lysine 4 trimethylation (H3K4me3) were found at the 5’ end of active loci, whereas histone H3 lysine 36 tri-methylation H3K36me3 was found at the coding region and the 3’ ends (Pokholok et al. 2005). Similar studies have also been done in mammals, fission yeast, plants and flies and the similarities between species is striking, demonstrating the evolutionary importance of these modifications (Barski et al. 2007, Schubeler et al. 2004, Sinha et al. 2006, Wang et al. 2008). Histone PTMs are not only correlated with transcriptional activation, but also with transcriptional repression, such as histone H3 lysine 9 methylation (H3K9me3) (Hublitz et al. 2009).

As an added layer of complexity, some histone residues serve as the site of more than one modification. Several lysine residues can be either acetylated, methylated or ubiquitinated, but only one modification can exist at any given point; thus, the idea of histone “crosstalk” emerged (Suganuma and Workman 2008). At the simplest level, histone crosstalk can refer to two marks existing on the same residue (Suganuma and Workman 2008). For example, H3K4 can be either methylated or acetylated; however, both cannot exist together (Guillemette et al. 2011). A more complex level of crosstalk refers to modifications on different histone
residues interacting with each other. A well characterized example of one histone modification stimulating the placement of another is phosphorylation of serine 10 on histone H3 phosphorylated (H3S10ph) by the kinase Snf1 (Lo et al. 2001). This phosphorylation promotes acetylation of lysine 14 by Gcn5 and together, these two modifications enhance the interaction of the 14-3-3 proteins with histone H3 during gene activation (Lo et al. 2001). Conversely, one histone modification can also lead to the removal of another. Set2 is the histone methyltransferase (HMT) that methylates H3K36me (Strahl et al. 2002). H3K36me serves as a target for the Rpd3S histone deacetylase complex (HDAC) complex; thus, it serves to target deacetylation (Carrozza et al. 2005, Joshi and Struhl 2005, Keogh et al. 2005). Another well established example of crosstalk is between histone H2B lysine 123 ubiquitination (H2BK123ub) and H3K4 methylation, where several labs have demonstrated that the H2B conjugating enzyme, Rad6, is required for placement of H3K4me3 by the COMPASS (Complex of Proteins Associated with Set1) complex and placement of H3K79me by Dot1 (Dover et al. 2002, Sun and Allis 2002).

Histone crosstalk can also occur when multiple histone modification enzymes exist in the same complex. The ubiquitin protease, Ubp8, is a member of the SAGA (Spt-Ada-Gcn5-Acetyltransferase) complex which also contains the HAT, Gcn5 (Henry et al. 2003). Upon recruitment of SAGA to chromatin, Ubp8 deubiquitylates histone H2B allowing the kinase, Ctk1 to associate with the C-terminal domain (CTD) of RNA polymerase II (RNAPII) (Henry et al. 2003). Ctk1 phosphorylates serine 2 of the CTD, allowing Set2 to associate with serine 2 and methylate lysine 36
(Krogan et al. 2003b). This chain reaction of histone modifications serves as an example of how histone modifications can act in concert to facilitate transcription. There are other examples of crosstalk between modifications, and in particular my thesis focuses on the crosstalk between histone H3 lysine acetylation and methylation.

1.3 HISTONE LYSINE ACETYLATION

Nearly 50 years ago, Allfrey and colleagues recognized that histones were not only both acetylated and methylated, but that lysine acetylation was positively correlated with transcription (Allfrey et al. 1964). Since then, genome-wide studies have confirmed this observation providing evidence that lysine acetylation is a hallmark of actively transcribed genes and is enriched primarily at the promoters of genes (Kurdistani et al. 2004, Liu et al. 2005, Pokholok et al. 2005, Schubeler et al. 2004, Wang et al. 2008). These studies also demonstrate that histone acetylation is positively correlated with transcription rates (Liu et al. 2005, Pokholok et al. 2005, Schubeler et al. 2004, Wang et al. 2008). In addition to promoter acetylation, low levels of some histone acetylation marks exist throughout the open reading frames of actively transcribed genes (global acetylation) (Kurdistani et al. 2004, Liu et al. 2005, Pokholok et al. 2005, Schubeler et al. 2004, Wang et al. 2008).

Lysine acetylation is placed by HAT complexes, which are multi-subunit complexes consisting of a catalytic subunit and several auxiliary subunits required for targeting and enzymatic activity. There have been at least nine HAT complexes
identified in the yeast *S. cerevisiae*, most of which preferentially acetylate histone H3 or H4 tails (Table 1.1 and (Allard et al. 1999, Grant et al. 1997, Howe et al. 2001, Lee and Workman 2007, Sutton et al. 2003, Winkler et al. 2002)). Many HATs have overlapping targets. For example in *S. cerevisiae* Gcn5, the catalytic subunit of at least three HAT complexes (SAGA, SLIK/SALSA and ADA) and Sas3, the catalytic subunit of the HAT complex (NuA3 – Nucleosome acetyltransferase 3), both preferentially acetylate H3K14 (Grant et al. 1997, Howe et al. 2001, Kuo et al. 1996). These two HATS also have non-overlapping targets, as Gcn5 also acetylates H3K9 and H3K18 and Sas3 acetylates H3K23 (Grant et al. 1997, Howe et al. 2001).

**Table 1.1: Yeast HATs and their substrates**

<table>
<thead>
<tr>
<th>HAT</th>
<th>Target histone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elp3</td>
<td>H3K14, H4K8</td>
</tr>
<tr>
<td>Esa1</td>
<td>H4 K5,8,12,16</td>
</tr>
<tr>
<td></td>
<td>H2A K4,7</td>
</tr>
<tr>
<td></td>
<td>H2A.Z K14</td>
</tr>
<tr>
<td>Gcn5</td>
<td>H2B K11,16</td>
</tr>
<tr>
<td></td>
<td>H3 K9,14,18,23,27</td>
</tr>
<tr>
<td></td>
<td>H2A.Z K14</td>
</tr>
<tr>
<td>Hat1</td>
<td>H4 K5,12</td>
</tr>
<tr>
<td>Rtt109</td>
<td>H3 K56, K9</td>
</tr>
<tr>
<td>Sas2</td>
<td>H4 K16</td>
</tr>
<tr>
<td>Sas3</td>
<td>H3 K14,23</td>
</tr>
</tbody>
</table>

Histone acetylation occurs primarily at gene promoters, where it serves a role in transcription initiation; however, some histone acetylation marks are found throughout the transcribed regions of genes (Figure 1.2 and reviewed in (MacDonald and Howe 2009)). In yeast, SAGA and NuA4 (Nucleosome acetyltransferase 4) are
the two main HAT complexes involved in transcription initiation through promoter targeted acetylation (Durant and Pugh 2007, Robert et al. 2004). Both complexes facilitate transcription initiation by recruiting TATA binding protein (TBP); however, they do so in slightly different ways. Histone acetylation by Gcn5 as part of SAGA may recruit RSC (Remodels the structure of chromatin) which uses the energy of ATP to disrupt nucleosome-DNA contacts, thus, facilitating transcription activation (Ferreira et al. 2007, Kasten et al. 2004, Workman 2006). Acetylation of histone H4 by NuA4 may have a more direct role in transcriptional activation by acting as a binding site for components of general transcription factors, such as TFIID (Matangkasombut and Buratowski 2003).

In addition to promoter-targeted acetylation, global acetylation exists throughout the open reading frames of genes (Figure 1.2 and (Vogelauer et al. 2000, Wang et al. 2008)). In activated T-cells, H3K14ac, H3K23ac and H4K12ac, were found at peak levels at the promoter region but, unlike other histone acetylation marks, were also found throughout the transcribed regions (Wang et al. 2008). The function of global acetylation and how it is targeted is largely unknown; however, there have been a handful of studies that suggest some HAT complexes are able to interact with RNAPII or with the histone methylation left after RNAPII passes through the open reading frame (Cho et al. 1998, Martin et al. 2006b, Taverna et al. 2006).
Histone acetylation is removed by histone deacetylase complexes (HDACs), which, like HATs, exist as multi-subunit complexes. HDACs are also targeted to promoters through interaction with DNA-bound transcriptional repressors, as well as acting in a non-specific global manner (Kadosh and Struhl 1998, Rundlett et al. 1998, Vogelauer et al. 2000). A good example is the HDAC Rpd3, which is targeted to promoters via interaction with the transcriptional repressor, Ume6 (Kadosh and Struhl 1998, Rundlett et al. 1998). Rpd3 has also been shown to have activity throughout the coding regions of genes (global deacetylation) (Vogelauer et al. 2000). Hypoacetylation is correlated with transcriptional repression and consistent
with this, deletion of HDACs has been shown to cause increased transcription rates (Vogelauer et al. 2000). For example, expression at the *PHO5* locus can be increased under repressive conditions (high phosphate) in an *RPD3* deletion strain (Vogelauer et al. 2000).

### 1.4 HISTONE LYSINE METHYLATION

In yeast, histone H3 tails can be methylated at lysines 4, 36 and 79 by the histone methyltransferases (HMTs) Set1, Set2 and Dot1 respectively. The H3K4 HMT Set1 functions as part of COMPASS (Complex of Proteins Associated with Set1), which, in addition to Set1, contains several auxiliary proteins required for Set1 to catalyze different methylation states (Miller et al. 2001, Morillon et al. 2005, Schneider et al. 2005). Genome-wide studies show that H3K4me3, like histone acetylation, is primarily found at the 5' region of transcriptionally active genes (Figure 1.2 and (Barski et al. 2007, Bernstein et al. 2005, Liu et al. 2005, Pokholok et al. 2005, Schubeler et al. 2004). This localization is presumably due to the interaction of COMPASS with an early elongating form of RNAPII (Krogan et al. 2003a, Ng et al. 2003). This interaction is dependent on the kinase Kin28, which phosphorylates serine 5 of the CTD of RNAPII and on the PAF (polymerase II associated factor) complex, which serves as a molecular bridge between the two complexes (Krogan et al. 2003a, Ng et al. 2003). During the late elongation stage of transcription, RNAPII becomes phosphorylated at serine 2 by the kinase, Ctk1, and the H3K36 HMT, Set2, is then able to interact with the CTD (Krogan et al. 2003b, Li et al. 2003, Xiao et al. 2003). The PAF complex was also found to be important for this interaction;
however, unlike the interaction of Set1 with RNAPII, the interaction of Set2 with RNAPII appears to be direct (Krogan et al. 2003b).

Unlike Set1, the H3K36 HMT, Set2, is not known to associate with any auxiliary proteins. H3K36me is found throughout the coding region and towards the 3’ end of genes, is not found at the 5’ regions or promoters, and is likely involved in negative regulation of transcription elongation (Figure 1.2 and (Barski et al. 2007, Pokholok et al. 2005)). The HDAC complex Rpd3S was shown to interact with H3K36me3 via one of its auxiliary components Eaf3, presumably resulting in deacetylation of nucleosomes and re-compaction of chromatin (Carrozza et al. 2005, Keogh et al. 2005). Consistent with this, Set2 and Rpd3S are required to prevent cryptic transcription of genes such as FLO8 and STE11 (Carrozza et al. 2005, Joshi and Struhl 2005, Keogh et al. 2005). These genes have TATA consensus sequences within their coding regions that are normally repressed, presumably due to nucleosome positioning (cryptic promoters). These cryptic promoters can become active in mutants which fail to reassemble chromatin after passage of RNAPII, resulting in the production of short spurious transcripts (Kaplan et al. 2003). More recent studies, however, suggest that Set2 is not required for Rpd3 association with chromatin and the effects seen in prior studies are likely due to the requirement of Set2 for Rpd3 catalytic activity (Drouin et al. 2010).
1.5 HISTONE LYSINE DEMETHYLATION

Methylation of lysine 4 and 79 persists for a significant time after gene repression, originally leading some to speculate that histone methylation, unlike histone acetylation, is a permanent modification (Katan-Khaykovich and Struhl 2005, Ng et al. 2003). However, in recent years two classes of lysine-specific HDMs have been identified: amine oxidases, such as LSD1, and the JmjC (Jumonji C)-domain containing HDMs. All *S. cerevisiae* HDMs are JmjC containing HDMs. The JmjC class of HDMs is the only class of HDMs that have been shown to remove all three states of methylation and they represent the largest class of HDMs (Klose et al. 2006). JmjC domain-containing demethylases can be split further into seven subfamilies, at least three of which have representative proteins in *S. cerevisiae*. These include the JHDM1 family, the JHDM3/JMJD2 family and the JARID1 family of demethylases.

The JHDM1 family of demethylases, along with their JmjC domain, contain a PHD (plant homeodomain) finger (Tsukada et al. 2006). In higher eukaryotes this family also contains a CXXC-zinc finger and F-box domain; however, the only *S. cerevisiae* member of this family, Jhd1, does not contain either of these two domains (Tu et al. 2007). *S. cerevisiae* have two members of the JHDM3/JMJD2 family, Rph1 and Gis1. This family of demethylases is characterized by having both a JmjC and JmjN domain as well as a PHD finger and Tudor domain (Klose et al. 2006). Mass spectrophotometry of Jhd1, Rph1, and Gis1 mutants demonstrated that each is capable of demethylating H3K36 (Tu et al. 2007). Further studies suggest that
Rph1 is capable of demethylating di- (me2) and tri- (me3) methylation of histone H3 lysine 36 (H3K36me2/3) whereas Jhd1 can only demethylate H3K36me3 (Kim and Buratowski 2007, Klose et al. 2007). Little is known about Gis1’s demethylase specificity.

The JARID1 family of demethylases is characterized by a JmjC, JmjN, AT-rich interactive, C5HC2 zinc finger, and PHD finger domain (Klose et al. 2006). In *S. cerevisiae*, the lone member of the JARID1 family of demethylases that has been identified is the lysine 4 demethylase, Jhd2 (Tu et al. 2007). Jhd2 was identified as a H3K4me3 demethylase that is capable of demethylating H3K4 *in vivo* (Liang et al. 2007, Seward et al. 2007, Tu et al. 2007) and *in vitro* (Ingvarsdottir et al. 2007). A subsequent study demonstrated that the CCR4/NOT complex is responsible for polyubiquitin-mediated degradation of Jhd2 (Mersman et al. 2009). While that study provided a mechanism for Jhd2 regulation during active transcription, they did not account for the mechanism behind loss of methylation upon transcriptional repression. Another study found that the JmjN domain was required for protein stability and that the PHD finger was necessary for Jhd2 interaction with chromatin (Huang et al. 2010). Although a great deal of work has been done investigating Jhd2, little is known about how it is targeted to specific regions of the genome and how its activity is regulated during transcriptional repression.
One striking observation from previous studies on HDMs is that disruption of most demethylases results in minimal or no increase in global methylation (Tu et al. 2007). Consistent with this, normal levels of Jhd2 in the cell are undetectable by western blot analysis, suggesting that HDMs are under some form of regulation (Mersman et al. 2009). The body of work focussed on HDMs is still small; thus, we still have a poor understanding of their mechanism and regulation compared to other histone-modifying enzymes. This is exemplified by the fact that to date no HDM for H3K79 methylation has been identified.

1.6 METHYL-LYSINE BINDING DOMAINS

As previously mentioned, one function of histone modifications, such as methylation, is to act as a docking sites for other proteins, which then have a direct effect on chromatin structure. This is evident by the large number of domains which specifically interact with methylated lysines including chromodomains, tudor, MBT (malignant brain tumour) domains, WD40, PWWP (proline, tryptophan, tryptophan, proline), and PHD finger domains (Maurer-Stroh et al. 2003). All but the PHD finger domain are members of the Tudor domain “Royal Family” (Maurer-Stroh et al. 2003). Methyl-lysine binding domains are characterized by hydrophobic cavities, made up of aromatic residues, which are capable of binding chromatin either through methylated histones or methylated DNA (Maurer-Stroh et al. 2003, Taverna et al. 2006).
The PWWP domain is a poorly conserved domain that is characterized by a central core proline-tryptophan-tryptophan-proline motif and is found in proteins involved in transcription regulation (Stec et al. 2000). It has long been suspected that the PWWP domain is a methyl-lysine binding domain. Original studies depicted the PWWP of Dnmt3a as a DNA methylation binding protein (Qiu et al. 2002); however, recent studies have demonstrated that PWWP domain containing proteins in *Schizosaccharomyces pombe* (Wang et al. 2009c) and humans (Dhayalan et al. 2010, Vezzoli et al. 2010, Wu et al. 2011) are capable of interaction with methyl-lysines, particularly H3K36me3. This suggests that the PWWP domain may be capable of interaction with both DNA and histone methylation.

1.7 PUTATIVE H3K36ME BINDING DOMAINS

The majority of methyl-lysine binding domains identified to date recognize lysine 4 methylation on histone H3 (H3K4me). Some methyl-lysine binding domains which primarily recognize other methyl marks (such as the PHD finger which is primarily an H3K4me3 binding domain) have been shown to be capable of binding H3K36me in vitro (Shi et al. 2007), but there is little or weak evidence for this in vivo (Saksouk et al. 2009). The PWWP domain is emerging as an H3K36me3 recognition domain (Dhayalan et al. 2010, Vezzoli et al. 2010, Wu et al. 2011). Two complexes in yeast which contain PWWP domains are the NuA3 HAT complex and the Isw1b chromatin remodelling complex.
The NuA3 HAT complex contains the catalytic subunit Sas3 and is responsible for acetylation of lysines 14 and 23 on the histone H3 tail (Howe et al. 2001). Previous work on NuA3 shows that interaction of this HAT complex is dependent on both H3K4me3 and H3K36me3 (Martin et al. 2006b). Consistent with this, lysine 14 and 23 are found throughout the coding regions of genes and NuA3 is found not only at the 5’ region of genes but also throughout the mid and 3’ regions (Taverna et al. 2006, Wang et al. 2008). Subsequent studies showed that the PHD finger of the Yng1 subunit was responsible for the interaction of H3K4me3 and that the N-terminus of Yng1 interacts with unmodified histones (Chruscicki et al. 2010, Martin et al. 2006a, Taverna et al. 2006).

To date it remains unknown how this complex interacts with H3K36me3; however, the existence of an H3K36me3 binding domain would explain how NuA3 is targeted to the mid and 3’ regions. Nto2 is one of the auxiliary subunits in NuA3 and contains a putative H3K36me binding domain - a PWWP domain. If the PWWP domain of Nto2 interacts with H3K36me3 it could explain how NuA3 is targeted to the H3K36me3 and why its target substrates, lysines 14 and 23, are found throughout the open reading frames of genes. However to date an H3K36me3 interaction domain in NuA3 has not been identified.

Isw1b is one of three Isw1-containing chromatin remodelling complexes in yeast. It has a role in facilitating transcription elongation and termination by interacting with the coding regions of genes (Morillon et al. 2003). The majority of
studies on Isw1b have focussed on its function; however, there is little known about how it is targeted to particular areas of the genome. Isw1b is found throughout the mid and 3' regions of genes in a pattern that is similar to that of H3K36me3 (Morillon et al. 2003, Pokholok et al. 2005). Like NuA3, Isw1b contains a putative H3K36me3 binding protein, loc4, which has a PWWP domain (Vary et al. 2003). Interaction of this domain with H3K36me may represent a targeting mechanism for the Isw1b complex.

1.8 CHAPTER SUMMARIES

Histone H3 acetylation and H3K4me3 co-localize at the promoter and 5' regions of actively transcribed genes, suggesting that one of these two marks may be functionally dependent on the other (Pokholok et al. 2005). In Chapter 2 we hypothesized that H3 acetylation would be dependent on H3K4me3 because the two major H3 HATs are found in complexes that associate with H3K4me3 (Pray-Grant et al. 2005, Shi et al. 2006, Vermeulen et al. 2010). Surprisingly, we show that the opposite is true and that H3K4me3 is dependent on H3 acetylation. Jhd2 is a JmjC domain protein that has recently been identified as the major H3K4me3 HDM in S. cerevisiae (Liang et al. 2007, Seward et al. 2007, Tu et al. 2007). However, deletion of JHD2 does not result in a significant increase in H3K4me3 and the role of it plays in mediating demethylation of this modification is unknown (Ingvarsdottir et al. 2007, Tu et al. 2007). In this study we show that acetylation of H3 negatively regulates demethylation of H3K4me3 by Jhd2, providing a possible mechanism for removal of H3K4me3 following gene repression. Furthermore, we demonstrate that this
protection is due to negative regulation of Jhd2 demethylase activity by H3 acetylation.

The PWWP domain is a poorly conserved domain that is shown to bind H3K36me3 in higher eukaryotes (Dhayalan et al. 2010, Vezzoli et al. 2010). The chromatin remodelling complex Isw1b and the HAT complex NuA3 both contain a PWWP domain containing protein, loc4 and Ylr455w respectively, as one of the auxiliary subunits and both have been shown to have chromatin interaction patterns that correlate with H3K36me3 patterns (Morillon et al. 2003, Taverna et al. 2006). This led us to hypothesize that the PWWP domain is an H3K36me3 interaction domain in *S. cerevisiae*.

In **Chapter 3** we demonstrate that the loc4 is localized to genes in a pattern similar to H3K36me3. We also demonstrate that loc4 interaction with chromatin is dependent on both lysine 36 and Set2 and on critical residues within the PWWP domain. Taken together, we have found a mechanism for targeting of Isw1b to the mid-coding region and 3’ end of open reading frames (ORFs).

In **Chapter 4**, we demonstrate that the uncharacterized ORF, Ylr455w, is a stable component of the NuA3 complex. Genetic and biochemical assays suggest that Ylr455w is a possible H3K36me3 binding protein. In addition to this, we provide some insight into the architecture of the NuA3 complex. We also propose a name for Ylr455w, Nto2 (NuA3 Open Reading Frame 2).
CHAPTER 2. HISTONE H3 ACETYLATION NEGATIVELY REGULATES DEMETHYLATION OF HISTONE H3K4 BY THE JMJC DOMAIN PROTEIN, JHD2

2.1 INTRODUCTION

Lysine methylation is distinctive among histone modifications as it is found in three states, mono- (me1), di- (me2) or tri- (me3) methylation. Recent studies have revealed that different methylation states can have implications in different biological processes. For example, H4K20me3 is associated with constitutive heterochromatin (Kourmouli et al. 2004, Schotta et al. 2004), whereas, H4K20me2 was plays a role in DNA repair (Greeson et al. 2008). To add to this complexity, lysine methylation has been correlated with both transcriptional activation and repression. While H3K4me3 is a hallmark of transcriptional activation (Pinskaya et al. 2009), H3K9me3 is associated with transcriptional repression (Hublitz et al. 2009).

In yeast, all three methylation states of H3K4 are catalyzed by the histone methyltransferase (HMT) Set1 as part of the COMPASS complex (Miller et al. 2001). COMPASS contains Set1 as well as several auxiliary proteins required for Set1 to catalyze the transition from mono- to di- and di- to tri-methylation, suggesting that diverse states of methylation are differentially regulated (Morillon et al. 2005, Schneider et al. 2005). Genome-wide studies show that H3K4me3 is primarily found at the 5’ regions of transcriptionally active genes, presumably due to the interaction of COMPASS with an elongating form of RNA polymerase II (Krogan et al. 2003b, Ng et al. 2003).

*A version of Chapter 2 is in preparation for submission. Maltby, VE*, Schulze, J., Chruscicki, A., Johnson, I., Kobor, MS., Howe, LJ. *Author formerly known as MacDonald, VE.
Following gene repression, H3K4me3 levels persist after loss of both RNA polymerase II and Set1 localization, leading some to speculate that histone methylation is not actively removed (Katan-Khaykovich and Struhl 2005, Ng et al. 2003). However, the dynamic nature of lysine methylation has become apparent with the discovery of HDMs. Two classes of lysine-specific HDMs have been identified: amine oxidases, such as LSD1, and the JmjC (Jumonji C) domain-containing demethylases. JmjC-containing demethylases are of particular interest, as they are the only class of demethylases that have been shown to remove all three states of methylation (Klose et al. 2006). This class of demethylases can be split further into several subfamilies including the evolutionarily-conserved JARID1 family of demethylases, which is characterized not only by a JmjC domain, but also JmjN, AT-rich interactive, C5HC2 zinc finger, and PHD finger domains (Klose et al. 2006). In the yeast, *S. cerevisiae*, the lone member of the JARID1 family of demethylases to be identified is Jhd2.

Previous studies have identified Jhd2 as a H3K4me3 demethylase capable of demethylating H3K4 *in vivo* (Liang et al. 2007, Seward et al. 2007, Tu et al. 2007) and *in vitro* (Ingvarsdottir et al. 2007). While several studies have focussed on the identification and substrate specificity of Jhd2, little is known about how it is targeted to specific regions of the genome or how its enzymatic activity is regulated. Repression of the *GAL1*, *SUC2*, and *INO1* genes is accompanied by loss of H3K4me3, which is dependent on *JHD2* (Huang et al. 2010, Ingvarsdottir et al. 2007). Additionally, over-expression of *JHD2* results in loss of H3K4me3 (Liang et al.
2007, Seward et al. 2007, Tu et al. 2007); however, deletion of *JHD2* does not result in a major increase of H3K4me3 in bulk histones, indicating that, under normal conditions, Jhd2 is unable to demethylate the majority of H3K4me3 in the cell (Ingvarsdottir et al. 2007, Tu et al. 2007).

In addition to H3K4me3 localization at the 5' ends of active genes, histone H3 acetylation is also present (Liu et al. 2005, Pokholok et al. 2005, Wang et al. 2008), which in yeast is primarily added by two histone acetyltransferases (HATs), Gcn5 and Sas3, as part of multi-protein complexes (John et al. 2000). These two HATs redundantly acetylate a preferred site (H3K14); however, other acetyl marks, such as H3K23ac are thought to co-localize with H3K4me3 also (Wang et al. 2008). The co-localization of H3K4me3 and H3 acetylation suggests that one may be functionally dependent on the other. Gcn5 and Sas3 are found in complexes that specifically interact with H3K4me3, suggesting that histone H3 methylation is required for establishment of H3 acetylation (Pray-Grant et al. 2005, Shi et al. 2006, Vermeulen et al. 2010). These data led us to hypothesize that histone H3 acetylation is dependent on H3K4me3. Surprisingly, we found that the reciprocal was true: H3K4me3 is dependent on histone H3 acetylation. Moreover, we show that this relationship is due to the negative regulation of Jhd2 demethylation by H3 acetylation. These results establish a mechanism behind the correlation between H3 acetylation and H3K4 methylation, and explain why H3K4me3 is susceptible to demethylation only after gene inactivation.
2.2 MATERIALS AND METHODS

2.2.1 Yeast strains and plasmids

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

Table 2.1: Yeast strains used in this study

<table>
<thead>
<tr>
<th>Yeast strain</th>
<th>Mating type</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>YLH101</td>
<td>Mat a</td>
<td>hisΔ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63</td>
</tr>
<tr>
<td>Yi37</td>
<td>Mat a</td>
<td>hisΔ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>jhd2::KANMX6</td>
</tr>
<tr>
<td>Yi51</td>
<td>Mat a</td>
<td>hisΔ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ada2::HIS3 sas3::HISMX6 jhd2::NATMX4</td>
</tr>
<tr>
<td>YLH106</td>
<td>Mat a</td>
<td>hisΔ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ada2::HIS3 sas3::HISMX6</td>
</tr>
<tr>
<td>YLH144</td>
<td>Mat a</td>
<td>hisΔ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ada2::HIS3 sas3::HISMX6</td>
</tr>
<tr>
<td>YLH146</td>
<td>Mat a</td>
<td>hisΔ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63</td>
</tr>
</tbody>
</table>

2.2.2 Western blot analysis

Whole cell extracts were analyzed by SDS PAGE and western blotting with antibodies listed in Table 2.2 followed by fluorescence detection and quantification using the Licor Odyssey System. Standard deviation was calculated using at least three biological replicates.
Table 2.2: Antibodies used for ChIP and western blot analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Cat number/notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>αH3</td>
<td>GenScript</td>
<td>Rabbit polyclonal - raised to yeast specific antigen CKDIKLARRLRGERS</td>
</tr>
<tr>
<td>αH3K4me3</td>
<td>Abcam</td>
<td>ab1012</td>
</tr>
<tr>
<td>Goat anti-mouse (800)</td>
<td>Licor</td>
<td>926-32221</td>
</tr>
<tr>
<td>Goat anti-rabbit (680)</td>
<td>Licor</td>
<td>926-32210</td>
</tr>
<tr>
<td>Goat anti-rat (800)</td>
<td>Licor</td>
<td>926-32219</td>
</tr>
<tr>
<td>αHA-HRP</td>
<td>Roche Diagnostics</td>
<td>High affinity 3F10 clone 12013819001 (antigen YPYDVPDYA)</td>
</tr>
<tr>
<td>Rabbit Immunoglobulin G (IgG)</td>
<td>Chemicon</td>
<td>PP64</td>
</tr>
<tr>
<td>αHA</td>
<td>Roche Diagnostics</td>
<td>High affinity 3F10 clone 11867423001 (antigen YPYDVPDYA)</td>
</tr>
<tr>
<td>αK14ac</td>
<td>GenScript</td>
<td>Order ID 72638-1</td>
</tr>
</tbody>
</table>

2.2.3 Chromatin immunoprecipitation qPCR and ChIP-on-chip

Chromatin immunoprecipitation (ChIP) and genome wide location analyses were performed as previously described (Schulze et al. 2009a). Cells for both assays were processed as previously described (Schulze et al. 2009a) with the exception that cells for ChIP-qPCR were grown in smaller volumes. Antibodies used for immunoprecipitations are listed in Table 2.2.

In ChIP-qPCR assays, after reversal of crosslinking and DNA purification, immunoprecipitated and input DNA were amplified using an ABI StepOne thermocycler using the primer pairs listed in table 2.3. Each PCR reaction consisted of 13.5 μl ddH₂O, PCR buffer (20 mM Tris-Cl, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄ and 0.1% TX-100), 0.2 mM dNTPs, 1.6 mM of each primer, and 1 μl of a
1:1000 dilution of SYBR green. PCRs went through a program of 94°C for 2 min followed by 40 cycles of 94°C 30 s, 58°C 1 min, 72°C 30 sec. Percent IP values were calculated and are shown relative to WT.

For ChIP-on-chip, immunoprecipitated and input DNA was amplified to 1µg of aRNA using T7 RNA polymerase in two rounds. Samples were labelled with biotin and input and immunoprecipitated samples were hybridized to two affymetrix 1.0R S. cerevisiae microarrays. Data was analyzed as previously described (Schulze et al. 2009a). The 5'end of genes were defined as 300 base-pairs downstream of the transcription start sites and classified as “enriched” if all probes in these regions were above the enrichment cutoff of 1.5. The average H3K4me3 profile was plotted similar to a previous approach (Mayer et al. 2010), where all known transcripts were grouped into five length classes: very short (<750) bp, short (750 to 1500 bp), medium (1500 to 2250), long (2250 to 3000), and very long (3000 to 3750). The five groups comprised 542, 1859, 1266, 631, 291 transcripts respectively. All transcripts in each group were partitioned into 150bp bins and the average enrichment values were calculated and plotted. A list of all transcription start and end sites for 4868 transcripts was provided by Harm van Bakel (Timothy Hughes’ lab, University of Toronto).
Table 2.3: Primers used for ChIP analysis

Primer name indicates position relative to ATG. “s” indicates a sense strand primer “a” indicates anti-sense primer.

<table>
<thead>
<tr>
<th>Name</th>
<th>Number</th>
<th>Sequence 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH2 s+47</td>
<td>918</td>
<td>CGGCAAGTTGGAGCATAAGG</td>
</tr>
<tr>
<td>ADH2 a+238</td>
<td>920</td>
<td>CGTTTTCAACCCATGCCGAC</td>
</tr>
<tr>
<td>CDC8 s+33</td>
<td>936</td>
<td>GGATTGGATAGGACTGGTAAAACC</td>
</tr>
<tr>
<td>CDC8 a+210</td>
<td>937</td>
<td>CGAAACAAGAGGTGAATTGCCTG</td>
</tr>
<tr>
<td>CSE1 s+32</td>
<td>926</td>
<td>GGCCGAATCAGTTATTGCTTCTAC</td>
</tr>
<tr>
<td>CSE1 a+250</td>
<td>927</td>
<td>CTACGTTTGGACCCGGCAGC</td>
</tr>
<tr>
<td>PMA1 s+85</td>
<td>848</td>
<td>CTTACGATGACGCTGCATC</td>
</tr>
<tr>
<td>PMA1 a+232</td>
<td>849</td>
<td>CTTCTGGAACTGTTATAGC</td>
</tr>
<tr>
<td>YCF1 s+45</td>
<td>932</td>
<td>CCTGAAGGGTTTGGACCTATATC</td>
</tr>
<tr>
<td>YCF1 a+246</td>
<td>933</td>
<td>CTCCCAACAGAACTAGTGCATC</td>
</tr>
<tr>
<td>YPP1 s+59</td>
<td>934</td>
<td>CGACCAAGCGCTAAGAGCG</td>
</tr>
<tr>
<td>YPP1 a+270</td>
<td>935</td>
<td>CGGATAGTCATTCAACTCCTCC</td>
</tr>
<tr>
<td>TDH2 s+20</td>
<td>1023</td>
<td>CGGTTTCCGTTAGAATCCTGTA</td>
</tr>
<tr>
<td>TDH2 a+184</td>
<td>1024</td>
<td>CATCGTGGGAATACTCACCAG</td>
</tr>
<tr>
<td>ARR1 s+27</td>
<td>1025</td>
<td>GCCAGGAAGCCTCCACTTAC</td>
</tr>
<tr>
<td>ARR1 a+166</td>
<td>1026</td>
<td>CATTTTACCTAGGTTTACGAGC</td>
</tr>
<tr>
<td>FUI1 s+40</td>
<td>1031</td>
<td>CAATGAAGGAGCAGACTATACC</td>
</tr>
<tr>
<td>FUI1 a+207</td>
<td>1032</td>
<td>CACCTTTTCGTCAATCTCAGG</td>
</tr>
<tr>
<td>IPK1 s+33</td>
<td>1033</td>
<td>CTGATTGATTAGGTTGATCCTACG</td>
</tr>
<tr>
<td>IPK1 a+199</td>
<td>1034</td>
<td>CGACGTCAATCAGATACATCG</td>
</tr>
</tbody>
</table>

2.2.4 Expression microarray analysis

RNA was isolated by hot phenol extraction (Ausubel et al. 1987). Poly(A⁺) RNA was amplified and fragmented using the Message Amp III kit (Ambion) as per manufacturer’s instructions. Hybridization, wash and staining of the samples were performed using the GeneChip® Hybridization, Wash, and Stain Kit (Affymetrix) as per the manufacturer’s instructions. Samples were hybridized to a GeneChIP® Yeast Genome 2.0 (Affymetrix 900553) and scanned with the Gene Chip Scanner 3000 7G (Affymetrix). Expression data was extracted using Expression Console™ Software (Affymetrix) with MAS5.0 Statistical algorithm. Representative probe sets
that showed greater than two-fold changes relative to wild type were chosen for GO term and transcriptional frequency class analysis.

### 2.2.5 Chromatin association assay

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

### 2.2.6 Jhd2 purification and peptide demethylase assays.

Jhd2 was over-expressed from pBG1805 (Thermo Scientific Open Biosystems Yeast ORF Collection) in a caf1Δ strain by growth overnight in raffinose followed by a four hour induction with galactose. Caf1 is a member of the CCR4/NOT complex, which is responsible for polyubiquitin-mediated degradation of Jhd2 (Mersman et al. 2009). Over-expression of Jhd2 in a caf1Δ strain results in higher yields of total Jhd2 (data not shown). Cell extracts were prepared by bead beating in 50 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Tween 20, and Jhd2 was immobilized on Dynabeads (Invitrogen: M-370 epoxy) cross-linked to rabbit IgG as per manufacturer’s instructions. The purified protein was liberated by treatment with
PreScission Protease (GE Healthcare) and subjected to demethylase assays using the Demethylase (Jumonji-type) Activity Assay Kit (Cayman Chemical) and synthetic peptides corresponding to the first 23 amino acids of the histone H3 tail (Anaspec).

2.3 RESULTS AND DISCUSSION

2.3.1 Histone H3-specific HATs are required for maintenance of H3K4me3 levels.

Histone H3 acetylation and H3K4me3 co-localize at the 5’ end of actively transcribed genes (Liu et al. 2005, Pokholok et al. 2005, Wang et al. 2008); therefore, we questioned whether there is “crosstalk” between these two modifications. To test this we used quantitative western blotting of S. cerevisiae whole cell extracts to analyze the level of each mark in the absence of the enzyme(s) that catalyzes the other. While multiple HAT complexes contain H3K4me3-binding proteins (Martin et al. 2006a, Pray-Grant et al. 2005, Shi et al. 2006, Vermeulen et al. 2010), deletion of SET1, which encodes the sole H3K4 HMT in yeast, does not result in a decrease in H3K14ac (the preferred target of both Gcn5 and Sas3 in vitro) (Grant et al. 1999, Howe et al. 2001) levels in bulk histones (Figure 2.1 A-B).
Figure 2.1: Deletion of \textit{SET1} does not affect levels of lysine 14 acetylation.

Whole cell extracts (WCE) from the indicated strains were subjected to western blot analysis with antibodies against H3K14ac and histone H3. A) Graphic representation of quantified western blot. Values for H3K14ac are normalized for histone H3 and shown relative to wild type levels. Error bars indicate the mean +/- SEM from at least three independent experiments. B) Representative western blot image.
We next tested whether disruption of H3-specific HAT activity resulted in decreased levels of H3K4me3. In *S. cerevisiae*, Gcn5 and Sas3 are the two HATs responsible for the bulk of H3 acetylation so, we measured levels of H3K4me3 in strains containing a deletion of *GCN5* or *SAS3*. Each of these mutations resulted in only marginal changes of H3K4me levels (Figure 2.2 A-B). To confirm that our antibody was specific for the tri-methylated state and not other methylation states, we determined antibody specificity by peptide competition assays (Figure 2.2 C). Together, these results suggest that H3K4me3 is not required for H3K14ac and that deletion of either of the two primary histone H3 HATs does not disrupt lysine methylation.
Figure 2.2: Deletion of single HAT mutants does not affect levels of H3K4me3 on histone H3.

Whole cell extracts from the indicated strains were subjected to western blot analysis with antibodies against H3K4me3 (red), H3K14ac (blue), and H3 (for normalization). A) Graphic representation of quantified western blot. Values for each modification are normalized for histone H3 and shown relative to WT levels. Error bars indicate the mean +/- SEM from at least three independent experiments. B) Representative western blot image. C) Competition assays using modified peptides in WT and set1Δ strains indicate that the antibody used for western blots analysis, ChIP and ChIP-on-chip studies is specific for H3K4me3 only.
Given that many residues on the H3 tail are acetylated by redundant HAT complexes, we reasoned that if H3 tail methylation is dependent on histone acetylation, removal of all histone H3 HATs may be required to see any changes in H3K4me3. Unfortunately, gcn5Δ sas3Δ strains are inviable due to an inability of Gcn5 to acetylate Rsc4 (Choi et al. 2008, Howe et al. 2001). However, deletion of ADA2, which encodes a component of all Gcn5-dependent HAT complexes, selectively disrupts Gcn5’s ability to acetylate histones but not Rsc4 (Choi et al. 2008). As a result ada2Δ sas3Δ strains are viable despite having very low detectable levels of histone H3 acetylation (Figure 2.3). It should be noted that there is still residual H3 acetylation in this strain, but Figure 2.3 B and C demonstrate that this is not due to Elp3, a putative H3-specific HAT, or ADA2-independent Gcn5 HAT activity. When we measured the levels of H3K4me3 in an ada2Δ sas3Δ double mutant we found that this strain exhibits levels of H3K4me3 that were less than 60% of wild type (WT) (Figure 2.4 A-B), suggesting that histone acetylation plays a role in establishment or maintenance of H3K4me3 levels. Deletion of ADA2 or SAS3 alone did not affect H3K4me3 levels indicating that these genes play redundant roles in maintaining wild type levels of H3K4me3 (Figure 2.4 A-B).
Figure 2.3: Deletion of ADA2 and SAS3 disrupts acetylation on the histone H3 tail.

Two fold dilutions of whole cell extracts from the indicated strains were subjected to western blot analysis with antibodies against H3, H3K14ac, H3K18ac and H3K23ac. A) Deletion of ADA2 and SAS3 results in elimination of the majority of histone H3 tail acetylation. B) Deletion of ADA2 results in the same loss of acetylation as deletion of GCN5 strain. C) Deletion of the putative HAT ELP3 does not result in further loss of histone H3 acetylation as compared to deletion of ADA2 and SAS3.
Figure 2.4: Loss of H3-specific histone acetyltransferases results in decreased levels of H3K4me3.

A) Quantified western blot of whole cell extracts. Shown is methylation (red) or acetylation (blue) normalized for histone H3, shown relative to WT levels. Error bars indicate mean +/- SEM from three independent experiments. B) Sample western blot image.

To determine the genomic location of the observed loss in H3K4me3 in the H3 HAT mutant, we performed ChIP-on-chip experiments using Affymetrix high-resolution tiling microarrays. Protein-DNA complexes containing H3K4me3 were immunoprecipitated from wild type and ada2Δ sas3Δ strains using the same H3K4me3 specific antibody. Enriched regions were detected as previously described by comparing signal intensities of the ChIP to input DNA (Schulze et al. 2009a). As before, H3K4me3 peaks at the 5’ ends of 4054 genes (Figure 2.5 A) in WT cells. Consistent with our examination of bulk histones, disruption of the H3-specific HATs resulted in loss of H3K4me3, we observed a near complete loss of H3K4me3 at some genes, while others remained unaffected (Figures 2.5 B and 2.6 A), rather than a global 40% loss at all genes.
Figure 2.5: Deletion of ADA2 and SAS3 results in loss of H3K4me3 at several loci.

A) All genes with known TSS were divided into five length classes and the average enrichment for H3K4me3 in a wildtype strain was mapped. As previously shown (Pokholok et al. 2005), H3K4me3 was localized in the 5′end of transcripts and sharply peaked just downstream of the TSS. Transcripts were grouped into five length classes: very short (XS) </= 750 bp, short (S) 750 to 1500 bp, medium (M) 1500 to 2250, long (L) 2250 to 3000, and very long (XL) 3000 to 3750. B) H3K4me3 profiles for WT (red) and ada2Δ sas3Δ (blue) strains generated by ChIP-on-chip. H3K4me3 positioning for the regions surrounding loci that show the greatest loss of H3K4me3 were plotted along the X axis against relative occupancy on the Y axis. Light grey rectangles represent ORFs on the Watson (above axis) or Crick (below axis) strands and dark grey rectangles represent ARS elements. Highlighted in bold font are ORFs tested by ChIP-qPCR. Chromosome number is indicated on the right. C) H3K4me3 levels were measured relative to total histone H3 by ChIP-qPCR in WT (red) or ada2Δ sas3Δ (blue) strains with primers designed to amplify the 5′ region of the indicated gene (see Table 2.3 for details). Error bars indicate the mean +/- SEM from three independent experiments.
To confirm the results of the ChIP-on-chip, we performed ChIP-qPCR experiments at the 5’ ends of select genes whose methylation was found to be dependent on the H3-specific HATs. ChIP-qPCR confirmed the results of ChIP-on-chip at TDH2, ARR1, FUI1 and IPK1 (Figure 2.5 C). Furthermore, we quantitatively measured H3K4me3 levels at the 5’ends of genes that showed only subtle changes in H3K4me3 by ChIP-on-chip, to identify genes whose histone methylation levels are only partially dependent on the state of histone H3 acetylation. Surprisingly, despite showing only modest loss of H3K4me3 by ChIP-on-chip (Figure 2.6 A), YCF1, CDC8, CSE1, YPP1, and PMA1, showed significant loss of H3K4me3 in an ada2Δ sas3Δ strain (Figure 2.6 B). Thus, despite the usefulness of ChIP-on-chip for analyzing genome-wide levels of histone PTMs, the lack of quantitative nature of this assay made it difficult to assess how many genes are truly dependent on H3 acetylation for establishment or maintenance of H3K4me3 levels. Despite the limitations of ChIP-on-chip we were still able to demonstrate that normal H3 acetylation is required for normal levels of H3K4me3.
Figure 2.6: Deletion of ADA2 and SAS3 results in loss of H3K4me3 at loci that only show marginal loss of H3K4me3 by ChIP-on-chip.

A) H3K4me3 profiles for WT (red) and ada2Δ sas3Δ (blue) strains generated by ChIP-on-chip. H3K4me3 positioning for the regions surrounding loci that show marginal or no loss of H3K4me3 were plotted along the X axis against relative occupancy on the Y axis. Light grey rectangles represent ORFs on the Watson (above axis) or Crick (below axis) strands and dark grey rectangles represent ARS elements. Highlighted in bold font are ORFs tested by ChIP-qPCR. Chromosome number is indicated on the right. B) H3K4me3 levels were measured relative to total histone H3 by ChIP-qPCR in WT (red) or ada2Δ sas3Δ (blue) strains with primers designed to amplify the 5' region of the indicated gene (see Table 2.3 for details). Error bars indicate the mean +/- SEM from three independent experiments.
2.3.2 Loss of H3K4me3 is not due to transcriptional defects in \textit{ada2}\textDelta\ sas3\textDelta\ strains

Both histone methylation and acetylation are linked to transcriptional activity; therefore, it is possible that loss of histone acetylation caused by deletion of \textit{ADA2} and \textit{SAS3} results in transcriptional defects that lead to loss of H3K4me3. To rule out this possibility, we performed genome-wide expression profiling on mutant strains. RNA isolated from mid-log phase cells grown in rich media was amplified and hybridized to the Yeast Genome 2.0 array. Profiles were carried out in triplicate and correlation values between replicates were calculated to determine reproducibility (Figure 2.7 A-B). Transcript levels were compared between wild type and mutant profiles and correlation values were again used to determine changes in transcription.

Despite having significant growth defects \textit{ada2}\textDelta\ sas3\textDelta\ strains showed minor changes in transcript levels (Figure 2.8 A) with 206 genes (3.6%) showing greater than two fold increase and 280 (4.9%) showing greater than two fold decrease in transcript levels in the mutant. Thus, although histone H3 acetylation is a generally ubiquitous mark of transcribed genes, it is only important for expression of a subset of genes under these conditions. These results are consistent with a recent study by Lenstra et al. (2011), which demonstrated a similar phenomenon with many histone post-translational modifications.
Figure 2.7: Expression array analysis provides replicable data sets.

XY scatter plot of probe intensities between microarray replicates with array 1 plotted on the X axis against array 2 plotted on the Y axis. $R^2$ is indicated for both A) WT and B) ada2Δ sas3Δ strains.
Figure 2.8: Deletion of ada2Δ sas3Δ does not cause transcriptional defects.

A) XY scatter plot of probe intensities from the expression array with ada2Δ sas3Δ plotted on the X axis against WT plotted on the Y axis. B) The 627 which have the greatest loss of H3K4me3 by ChIP-on-chip were used to create an XY scatter plot of probe intensities from the expression array with ada2Δ sas3Δ plotted on the X axis against WT on the Y axis. C) Venn diagram indicating the overlap of genes which show loss of H3K4me3 in ChIP-on-chip (pink) and transcriptional defects by expression profiling (blue) in ada2Δ sas3Δ strains. The total numbers of genes identified in each assay are indicated below the diagram. D) Western blot analysis of WCE from WT, jhd2Δ, ada2Δ sas3Δ, and ada2Δ sas3Δ jhd2Δ with an HA tagged version of Set1. Histone H3 is used as a control. E) Western blot analysis of WCE from WT or ada2Δ sas3Δ with an HA tagged version of Jhd2 using the same antibodies as in D.
To further rule out the possibility that loss of H3K4me3 in an ada2Δ sas3Δ strain is due to a transcription defect, we identified 15% of genes that showed the greatest loss of H3K4me3 in the mutant by ChIP-on-chip and compared the transcript levels of these genes in WT and ada2Δ sas3Δ strains (Figure 2.8 B). These genes showed the same trend as all genes, with highly transcribed genes representing the smallest class and lesser transcribed genes representing the largest class (Holstege et al. 1998), suggesting there is no particular transcriptional class that is affected by loss of histone acetylation. Additionally, only 175 genes overlap in the 15% of genes exhibiting the greatest H3K4me3 loss with the 15% of genes with the greatest transcript decrease in the ada2Δ sas3Δ mutant (Figure 2.8 C). Finally, we observed no defect in expression of the H3K4 methyltransferase, Set1, or the H3K4 HDM Jhd2, which was confirmed by western blot (Figure 2.8 D-E). Overall, this data suggests that loss of H3K4me3 observed in the H3-HAT mutant is not the result of a transcriptional defect.

2.3.3 Loss of histone H3 acetylation promotes H3K4 demethylation by Jhd2.

The induction of heterochromatin at the HMR locus results in a rapid loss of histone acetylation, followed by loss of H3K4me3 with slightly slower kinetics (Katan-Khaykovich and Struhl 2005). An intriguing hypothesis is that histone acetylation prevents removal of H3K4me3 by a demethylase. If this is true then deletion of JHD2, the major H3K4me3 demethylase, from an ada2Δ sas3Δ strain should result in rescue of H3K4me3 levels. To test this, we used quantitative western blot analysis and found that when JHD2 is deleted from an ada2Δ sas3Δ mutant, H3K4me3
returns to near wild type levels (Figure 2.9 A-B). As expected, H3K14ac remains at background levels. These results suggest that H3 acetylation protects histones from demethylation by Jhd2. To confirm this at a gene specific level, chromatin immunoprecipitation was performed to determine effects of loss of ADA2, SAS3, and JHD2 on H3K4me3 at several loci that were previously shown to lack H3K4me3 upon loss of acetylation. At the 5’ ends of YCF1, CDC8, and CSE1, H3K4me3 levels drop upon deletion of ADA2 and SAS3, but return to wild type levels with subsequent deletion of JHD2 (Figure 2.9 C). Interestingly, PMA1, a gene commonly used to examine levels of H3K4me3, also showed a loss of H3K4me3 in an ada2Δ sas3Δ strain, but this was only slightly rescued by loss of JHD2 (Figure 2.9 C). PMA1 is one of the most highly transcribed loci in yeast (~95 mRNA/hr), a characteristic shared by less than 4% of genes (Holstege et al. 1998, Pokholok et al. 2005) and since extremely high transcription rates are correlated with turnover of H3, the histones present at PMA1 may not be a true reflection of what is occurring at the majority of genes (Dion et al. 2007, Rufiange et al. 2007).
Figure 2.9: Deletion of *JHD2* rescues the H3K4me3 defect in cells lacking H3 acetylation.

A) Quantified western blot of WCEs with methylation (red) or acetylation (blue) normalized for histone H3 and shown relative to wild type levels. Error bars indicate the mean +/- SEM from three independent experiments. B) Sample western blot image. C) H3K4me3 levels were measured relative to total histone H3 by ChIP-qPCR in WT (red), *jhd2Δ* (green), *ada2Δ sas3Δ* (blue) or *ada2Δ sas3Δ jhd2Δ* (purple) strains with primers designed to amplify the 5' region of the indicated gene (see Table 2.3 for details). Error bars indicate the mean +/- SEM from three independent experiments. D) XY scatter plot on of probe intensities from the expression array with *ada2Δ sas3Δ* plotted on the X axis against *ada2Δ sas3Δ jhd2Δ* plotted on the Y axis.
To rule out the possibility that the restoration of H3K4me3 levels in an \( \text{ada2}\Delta \sas3\Delta \) mutant via loss of \( \text{JHD2} \) is an indirect effect of suppression of transcription-related defects we used genetic analysis demonstrating that deletion of \( \text{JHD2} \) does not rescue \( \text{ada2}\Delta \sas3\Delta \) phenotypes such as slow growth, temperature sensitivity, or sensitivity to 6-azauracil (Figure 2.10). Additionally we carried out genome-wide expression profiling as before and compared \( \text{ada2}\Delta \sas3\Delta \) to \( \text{ada2}\Delta \sas3\Delta \jhd2\Delta \) strains and found deletion of \( \text{JHD2} \) does not change the transcription profile of \( \text{ada2}\Delta \sas3\Delta \) strains (Figure 2.9 D). Similarly, the \( \jhd2\Delta \) strain shows very minor changes in expression compared to wild type (Figure 2.11 C), which is consistent with the fact that there are no changes in H3K4me3 in this strain. Taken together, the fact that deletion of \( \text{JHD2} \) results in rescue of wild type levels of H3K4me3 in both bulk histones and at the 5’ end of several loci, without rescuing transcriptional defects, confirms that acetylation of histone H3 protects against demethylation by Jhd2.
Figure 2.10: Deletion of JHD2 does not rescue ada2Δ sas3Δ phenotypes.

Ten fold serial dilutions of wild type, ada2Δ sas3Δ or ada2Δ sas3Δ jhd2Δ strains. Cells were plated on rich media (YPD) for 7 days A) at 30, 37, and 25°C or B) on synthetic drop out media without (–URA) and with (6-AU) 6-azauracil. The results show that deletion of JHD2 does not rescue the temperature sensitive, cold sensitive or 6-AU sensitive phenotypes of ada2Δ sas3Δ, indicating that rescue of methylation is not due to rescue of ada2Δ sas3Δ defects.
Figure 2.11: Correlation between replicates of full genome expression profiles.

A-B) XY scatter plots of probe intensities between microarray replicates with array 1 or mutant strain plotted on the X axis against array 2 or WT plotted on the Y axis. $R^2$ is indicated.  

C-D) XY scatter plots of probe intensities between WT and jhd2Δ expressions arrays or WT and ada2Δ sas3Δ jhd2Δ expression arrays with WT plotted on the Y axis and mutant strain plotted on the X axis. $R^2$ is indicated.
2.3.4 Histone H3 acetylation negatively regulates demethylation by Jhd2 in vitro.

The above data suggest that Jhd2 is unable to demethylate H3K4 when the H3 tail is acetylated. There are two explanations for these observations. First, Jhd2 may be unable to bind to nucleosomes that contain hyperacetylated histone H3. We consider this unlikely because previous work has shown that the histone H3 tail is dispensable for the interaction of Jhd2 with chromatin (Huang et al. 2010). Additionally, we are unable to detect Jhd2 bound to peptides representing the first 23 amino acids of the H3 tail regardless of modification state (Figure 2.12 A). We also performed a chromatin association assay to test if deletion of ADA2 and SAS3 affected the interaction of Jhd2 with chromatin. Proteins from yeast strains expressing HA-tagged Jhd2 were fractionated into a soluble “non-chromatin” fraction and a pellet containing the bulk of the chromatin in the cell. Jhd2 levels in each fraction were monitored by western blot. Loss of ADA2 and SAS3 did not affect the relative levels of Jhd2 bound to chromatin (Figure 2.12 B).
Figure 2.12: Histone H3 acetylation negatively regulates demethylation but not binding by Jhd2 in vitro.

A) Peptide binding assays were performed using acetylated (H3K9, 14, 18a) and unacetylated (H3K4me3) peptides representing the first 23 amino acids of H3K4 tri-methylated histone H3. B) Chromatin fractionation assay using an HA tagged version of Jhd2. Pellet; chromatin fraction, sup; non-chromatin associated supernatant. C) Purified Jhd2 was subjected to histone demethylase assays using the same peptides as in (A). Error bars indicate the mean +/- SEM from at least three independent experiments. * = p<0.05.
An alternate explanation for our observations is that although Jhd2 can bind acetylated histone H3, it is unable to efficiently demethylate it. To test this, Jhd2, purified from yeast, was subjected to demethylase assays using synthetic peptides corresponding to the H3 tail (residues 1 to 23) that were tri-methylated at K4, with and without acetylation at lysines 9, 14, and 18. Our findings demonstrate that in this assay, Jhd2 cannot demethylate an acetylated peptide to the same extent as an unacetylated one (Figure 2.12 C), indicating that the ability of Jhd2 to demethylate H3K4me3 is negatively regulated by histone H3 acetylation.

2.4 CONCLUSIONS

Our results explain several inconsistencies observed in numerous previous studies. First, despite being the major H3K4 demethylase in yeast, Jhd2 seems unable to demethylate the majority of histones (Ingvarsdottir et al. 2007, Seward et al. 2007). However, given that H3 acetylation and H3K4me3 co-localize to specific regions of the genome, H3K4me3 will often be found on acetylated histone H3, and as a result be protected from the activity of Jhd2. Second, although H3K4me3 is generally confined to the 5’ region of transcriptionally active genes, multiple labs have observed Set1 bound to the 3’ ends of genes (Ingvarsdottir et al. 2007, Zhang et al. 2005). Because these regions are normally hypoacetylated, thus susceptible to demethylation by Jhd2, this may partially explain why the 3’ regions of genes do not show high levels of H3K4me3, despite the presence of Set1. Finally, induction of heterochromatin by regulated expression of the silencing protein, Sir3, results in loss of both histone H3 acetylation and H3K4me3 from the HMR locus (Katan-
Khaykovich and Struhl 2005). However, removal of H3K4me3 occurs with noticeably slower kinetics than histone deacetylation, which is consistent with a requirement for deacetylation prior to demethylation.

From this study we can propose a mechanism for the loss of H3K4me3 that is observed upon transcriptional repression. During active transcription, histone H3 acetylation prevents Jhd2 from demethylating H3K4me3 (Figure 2.13 A). Transcriptional repression is initiated by binding of a transcriptional repressor to the promoter which mediates recruitment of one or more histone deacetylase complexes and acetylation is lost. This would allow Jhd2 to demethylate H3K4me3 (Figure 2.13 B).
**Figure 2.13: Model of control of histone acetylation and methylation on the H3 tail.**

Schematic representation of the H3 tail.

A) In the presence of an activating stimulus, transcriptional activators bind DNA sequences and recruit HAT complexes. At the same time, Set1 methylates all three methylation states of H3K4 while Gcn5 and Sas3 dependent HAT complexes acetylate histone H3. This acetylation prevents Jhd2 demethylase activity on H3K4me3. B) After loss of an activating stimulus or in the presence of a repressive stimulus a transcriptional repressor element binds to its DNA sequence, which recruits HDAC complexes. HDACs deacetylate the H3 tail allowing Jhd2 to demethylate H3K4me3.
The fact that H3K4me3 is dependent on histone H3 acetylation and not vice versa is surprising when one considers that both Gcn5 and Sas3 are components of complexes that contain H3K4me3-binding motifs (Martin et al. 2006a, Pray-Grant et al. 2005, Shi et al. 2006, Vermeulen et al. 2010). In this study however, we only examined the impact of loss of SET1 on H3K14ac levels and not other acetylation marks; thus, it is possible that acetylation at other sites is dependent on H3K4 methylation. Moreover, we have been unable to determine which sites of acetylation on the H3 tail are required to prevent demethylation by Jhd2. Although mutation of individual H3 tail lysines to alanines, glutamines, or arginines does result in loss of H3K4me3 as seen by others (Nakanishi et al. 2008), it is not rescued by deletion of JHD2 indicating that these mutations affect H3K4me3 levels through independent means. However if H3 acetylation and H3K4me3 are mutually dependent on each other, this would potentially result in a positive feedback loop that would cause a continuous increase in both H3K4me3 and H3 acetylation levels during the period when a gene is transcriptionally active. While it is still a matter of debate whether histone PTMs are heritable, an intriguing possibility is that any inheritance that does exist may depend more on the “intensity” of a specific PTM rather than the actual modification per se.
3.1 INTRODUCTION

Histone post-translational modifications play a role in regulation of chromatin structure by acting as docking sites for chromatin remodelling complexes. Chromatin remodelers have the ability to disrupt DNA-histone contacts, resulting in altered nucleosome positioning and changes in nucleosome structure. Numerous protein domains contained within remodelling complexes have been identified and shown to selectively interact with specific histone modifications. In particular, lysine methylation has emerged as an important modification in chromatin regulation. Several protein domain families have been identified as methyl-lysine binding domains that include, but are not limited to: chromodomains, tudor domains, MBT (malignant brain tumour) domains, WD40 domains, PWWP (proline, tryptophan, tryptophan, proline) domains, and PHD (plant homeodomain) fingers. With the exception of the PHD finger, all of these domains are members of the Tudor domain “Royal Family” (Maurer-Stroh et al. 2003). These domains are characterized by hydrophobic cavities, made up of 2-4 aromatic residues, which are capable of interacting with chromatin either through methylated histones or methylated DNA (Maurer-Stroh et al. 2003).

1 A version of Chapter 3 has been submitted to Molecular and Cellular Biology for publication (control number MCB06341-11). Maltby, VE, Schulze, J., Johnson, I., Kobor, MS., Howe, LJ. *Author formerly known as MacDonald, VE.
The PWWP domain is a poorly conserved domain. The DNA methyltransferase, Dmnt3a, was originally predicted to bind methylated DNA via its PWWP domain (Qiu et al. 2002). This PWWP domain has recently been demonstrated to bind H3K36me3, an interaction thought to be important for proper placement of de novo DNA methylation; thus, this domain may be capable of interaction with both DNA and histone methylation (Dhayalan et al. 2010). Another study demonstrated that the PWWP domain of the Schizosaccharomyces pombe protein Pdp1 (PWWP domain protein-1) binds to H4K20me (Wang et al. 2009c). More recently, BRPF1, a subunit of the mammalian HAT complex, MOZ, was found to interact with methylated lysines via its PWWP domain (Laue et al. 2008, Vezzoli et al. 2010). Vezzoli and colleagues solved the NMR and crystal structures of the PWWP domain of BRPF1, and demonstrated that the PWWP domain binds H3K36me3. They also identified residues required for binding not only within the PWWP domain, but also residues on the H3 tail surrounding K36 that are suspected to provide specificity for H3K36me3 (Vezzoli et al. 2010). Taken together it is likely that methyl-lysine binding, particularly H3K36me3, represents a function of the PWWP domain.

Isw1 is an ATP-dependent chromatin remodelling protein found in S. cerevisiae. It has roles in both transcriptional activation and repression, where it is responsible for altering nucleosome positioning over promoter sequences (Erkina et al. 2010). Isw1 can be isolated as a monomer, or as a member of two different complexes in vivo (Vary et al. 2003). Independent Isw1 is associated with the rDNA
locus, where it regulates the maintenance and silencing of RNA polymerase II transcribed genes in the rDNA (Mueller and Bryk 2007). Isw1a is a complex of Isw1 and Ioc3 (Isw1 One Complex 3) and is involved in gene repression by positioning nucleosomes at promoters, thus, preventing transcription (Morillon et al. 2003). Isw1b is a complex of Isw1, Ioc2 and Ioc4 that is involved in facilitating transcription elongation and termination by interacting with the coding regions of genes (Morillon et al. 2003). Previous studies show that Isw1b is required for normal distribution of RNAPII across active genes and that Ioc2 in particular prevents 3’ end accumulation of RNAPII (Morillon et al. 2003). Ioc2 and Ioc4 have also been demonstrated to have a role in regulating CTD phosphorylation and histone H3 methylation (Morillon et al. 2003). *IOC2* deletion strains show lower than normal levels of serine 5 phosphorylation on the RNAPII CTD and *IOC4* deletion strains show lower than normal levels of serine 2 phosphorylation on the RNAPII CTD (Morillon et al. 2003). The same study showed that deletion of *IOC2* marginally reduces the levels of H3K4me3 at the 5’ end of genes, and that deletion of *IOC4* drastically reduces levels of H3K4me3 and H3K36me2 at the promoter and throughout the ORF (Morillon et al. 2003).

While Isw1 complex function is partially understood, how each complex is targeted to specific regions of the genome is unknown. Isw1 contains a SANT and a SLIDE domain, which have been proposed to interact with histone tails (Boyer et al. 2002, Boyer et al. 2004); thus, they could recruit independent Isw1 to the rDNA loci. Ioc3 has no known chromatin binding domains; thus, Isw1a may rely on the SANT or
SLIDE domain for targeting, or interaction with another complex. Ioc2 and Ioc4 both contain a single potential methyl-lysine binding domain that could be responsible for targeting Isw1b to the coding regions of genes. Ioc2 contains a non-canonical PHD finger and Ioc4 contains a PWWP domain. While both of these domains have been shown to interact with histones in a methylation-dependent manner in other proteins, they have not been directly demonstrated to bind methylated histones in the context of Isw1b (Dhayalan et al. 2010, Martin et al. 2006a, Shi et al. 2006, Taverna et al. 2006, Vezzoli et al. 2010, Wang et al. 2009c).

The PWWP domain is a particularly good candidate for methyl-lysine binding in Isw1b for several reasons. First, previous studies have shown that Isw1b is localized to the 3’ end of actively transcribed genes, which correlates with the position of H3K36me3 which is also localized to the coding region and 3’ ends of genes (Morillon et al. 2003, Pokholok et al. 2005). Additionally, another study used Illumina high throughput sequencing to map nucleosome positioning and found that Isw1 preferentially influences nucleosome positioning in the coding regions of genes which is consistent with Isw1b’s role in transcription elongation and termination by RNA Polymerase II (Tirosh et al. 2010).

Previous studies show that cryptic promoters are active in mutants which fail to reassemble chromatin after passage of RNA polymerase II (Kaplan et al. 2003). Cryptic promoters result from an internal TATA consensus sequence present in some ORFs that, in WT cells, is repressed presumably due to nucleosome positioning.
(Kaplan et al. 2003). Several studies have revealed that there are a variety of mutants involved in transcription elongation, including deletion of the H3K36 HMT Set2, that allow initiation of cryptic transcription to occur (Carrozza et al. 2005, Joshi and Struhl 2005, Keogh et al. 2005). Isw1 has also been implicated as having a role in cryptic transcription. One study found that loss of ISW1 resulted in cryptic initiation at FLO8, while a second study found that upon deletion of ISW1, nucleosomes in the mid-coding region were shifted towards the 5' end and this shift was particularly prominent at genes with cryptic promoters (Cheung et al. 2008, Tirosh et al. 2010). Given that lone Isw1 and Isw1a are thought to have repressive roles and Isw1b is known to facilitate transcription elongation, Isw1b is the most likely candidate involved in Isw1 repression of cryptic initiation (Morillon et al. 2003, Mueller and Bryk 2007). Despite the evidence showing a correlation between Isw1b and H3K36me3 how Isw1b is actually targeted to specific regions of the genome is still unknown.

In this study we present evidence that the PWWP domain of Ioc4 is capable of interaction with chromatin in an H3K36me3-dependent manner. Using chromatin immunoprecipitation and co-immunoprecipitation assays we show that Ioc4 requires both its PWWP domain and H3K36me3 to interact with histone proteins. Our genome-wide study shows that Ioc4 is present at various target genes, not just a subset of certain genes. Finally, we also show that Ioc2 is required for Isw1b complex integrity. Overall, we have elucidated the targeting mechanism for Isw1b to specific areas of the genome.
3.2 METHODS AND MATERIALS

3.2.1 Yeast strains and plasmids

All strains used in this study are isogenic to S288C and are listed in Table 3.1. Yeast culture and genetic manipulations were performed using standard protocols (Ausubel et al. 1987). Genomic deletions were verified by PCR analysis and whole-cell extracts were generated as previously described (Ausubel et al. 1987, Kushnirov 2000).
<table>
<thead>
<tr>
<th>Yeast Strain</th>
<th>Mating type</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>YLH101</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63</td>
</tr>
<tr>
<td>Yi75</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 ioc4::KANMX6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ioc2::HISMX6 IOC4-6HA.405 pRS.406 ISWTAP::TRP</td>
</tr>
<tr>
<td>Yi73</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 ioc4::KANMX6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ioc2::HISMX6 IOC4.405 IOC2.406 ISWTAP::TRP</td>
</tr>
<tr>
<td>Yi81</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 ioc4::KANMX6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ioc2::HISMX6 pRS.405 IOC2.406 ISWTAP::TRP</td>
</tr>
<tr>
<td>Yi80</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 ioc4::KANMX6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ioc2::HISMX6 IOC4.405 IOC2-6HA.406 ISWTAP::TRP</td>
</tr>
<tr>
<td>Yi74</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 ioc4::KANMX6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ioc2::HISMX6 IOC4-6HA.405 IOC2.406 ISWTAP::TRP</td>
</tr>
<tr>
<td>YVM268</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 ioc4::KANMX6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IOC4-6HA.405</td>
</tr>
<tr>
<td>YVM266</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 ioc4::KANMX6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IOC4-405</td>
</tr>
<tr>
<td>Yi86</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 IOC4-6HA.405</td>
</tr>
<tr>
<td>Yi87</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 set1::HISMX6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IOC4-6HA.405</td>
</tr>
<tr>
<td>Yi88</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 set2::KANMX6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IOC4-6HA.405</td>
</tr>
<tr>
<td>YVM243</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 (hht1-hhf1)::LEU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(hht2-hht2)::KAN IOC4-6HA::HIS pHHF2.hht2K36A.314</td>
</tr>
<tr>
<td>YVM244</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 (hht1-hhf1)::LEU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(hht2-hht2)::KAN IOC4-6HA::HIS pHHF2.hht2K36R.314</td>
</tr>
<tr>
<td>YVM245</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 (hht1-hhf1)::LEU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(hht2-hht2)::KAN IOC4-6HA::HIS pHHF2.HHT2.314</td>
</tr>
<tr>
<td>YVM268</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 ioc4::KANMX6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IOC4-6HA.405</td>
</tr>
<tr>
<td>YVM273</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 ISW1TAP::TRP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ioc4::KANMX6 IOC4-6HA.405</td>
</tr>
<tr>
<td>YVM275</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 ISW1TAP::TRP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ioc4::KANMX6 ioc4F18A-6HA.405</td>
</tr>
<tr>
<td>YVM276</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 ISW1TAP::TRP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ioc4::KANMX6 ioc4W21A-6HA.405</td>
</tr>
<tr>
<td>YVM277</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 ISW1TAP::TRP</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ioc4::KANMX6 ioc4F114A-6HA.405</td>
</tr>
<tr>
<td>YVM272</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 IOC4TAP::TRP</td>
</tr>
<tr>
<td>L1106</td>
<td>Mat a</td>
<td>his3Δ200 ura3Δ0 KANMX-GAL1pr-FLO8-HIS3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rco1Δ0::kanMX</td>
</tr>
<tr>
<td>YLH511</td>
<td>Mat a</td>
<td>his3Δ200 ura3Δ0 NATMX-GAL1pr-FLO8-HIS3</td>
</tr>
<tr>
<td>YLH523</td>
<td>Mat a</td>
<td>his3Δ200 ura3Δ0 NATMX-GAL1pr-FLO8-HIS3</td>
</tr>
</tbody>
</table>

Table 3.1: Yeast strains used in this study
3.2.2 Chromatin immunoprecipitation and ChIP-on-chip

Chromatin immunoprecipitation (ChIP) and genome wide location analyses were performed as previously described (Schulze et al. 2009a). Cells for both assays were processed similarly and as previously described (Schulze et al. 2009a) with the exception that cells for ChIP-qPCR were grown in smaller volumes, in synthetic complete lacking methionine and cells for ChIP-on chip were grown in YPD. Antibodies used for immunoprecipitations are listed in Table 3.2.

In ChIP-qPCR assays, after reversal of crosslinking and DNA purification, immunoprecipitated and input DNA were amplified using an ABI StepOne thermocycler using the primer pair 781 (MET16 s+661 CATTCCACACAAACCGTCAA) and 782 (MET16 a+760 CGAATCGGCTGGCTTCAT). Each PCR reaction consisted of 13.5 μl ddH₂O, PCR buffer (20 mM Tris-Cl, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄ and 0.1% TX-100), 0.2 mM dNTPs, 1.6 mM of each primer, and 1 μl of a 1:1000 dilution of SYBR green. PCRs went through a program of 95°C for 2 min followed by 40 cycles of 95°C 30 s, 60°C 20s, 72°C 1 min. Percent IP values were calculated and are shown relative to WT.

For ChIP-on-chip, immunoprecipitated and input DNA was amplified to 1μg of aRNA using T7 RNA polymerase in two rounds. Samples were labelled with biotin and input and immunoprecipitated samples were hybridized to two affymetrix 1.0R S. cerevisiae microarrays. Data was analyzed as previously described (Schulze et al.
2009a). All ORFs (derived from the SGD database) were aligned according to the location of their translational initiation and termination sites. Each ORF was divided into 40 bins of equal length and average enrichment values were calculated for each bin. The 500 bp upstream and downstream of the coding start and end sites, respectively, were assigned to 20 bins and the average enrichment value for each bin was plotted as the 5’ and 3’ intergenic region (IGR).

Table 3.2: Antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Cat. number/notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit Immunoglobulin G (IgG)</td>
<td>Chemicon</td>
<td>PP64</td>
</tr>
<tr>
<td>αHA</td>
<td>Roche Diagnostics</td>
<td>High affinity 3F10 clone 11867423001 (antigen YPYDVPDYA)</td>
</tr>
<tr>
<td>αH3</td>
<td>GenScript</td>
<td>Rabbit polyclonal - raised to yeast specific antigen CKDIKLARRLRGERS</td>
</tr>
<tr>
<td>αH3K4me3</td>
<td>Abcam</td>
<td>ab1012</td>
</tr>
<tr>
<td>Goat anti-mouse (800)</td>
<td>Licor</td>
<td>926-32221</td>
</tr>
<tr>
<td>Goat anti-rabbit (680)</td>
<td>Licor</td>
<td>926-32210</td>
</tr>
<tr>
<td>Goat anti-rat (800)</td>
<td>Licor</td>
<td>926-32219</td>
</tr>
<tr>
<td>αH3</td>
<td>GenScript</td>
<td>Mouse monoclonal clone 5F4A5—raised to antigen CKDIKLARRLRGERS</td>
</tr>
<tr>
<td>αH3K36me3</td>
<td>Upstate</td>
<td>07-549</td>
</tr>
<tr>
<td>αH3K79me3</td>
<td>Abcam</td>
<td>ab2621 (cross reacts with H3K79me2)</td>
</tr>
</tbody>
</table>

3.2.3 Modified TAP tag immunoprecipitation (IP) and enrichment assays

The modified TAP tag IP was performed as previously described (Lambert et al. 2009) with the exception that after extract preparation, extracts were clarified by centrifugation at 12 000 rpm (Eppendorf microfuge, model 5415D) for 5 min at 4°C. Following incubation for 2 hours at 4°C, beads were washed and boiled for 10 min with SDS loading buffer. The resulting proteins were analyzed by western blotting.
For enrichment assays, total histone H3 signal in the IP fraction was normalized to input H3 signal using the Licor Odyssey system. Enrichment was determined by the relative intensity of the modification signal in the bound fraction as compared to the input fraction. An increased modification signal relative to WT indicates enrichment for that modification.

### 3.2.4 In vitro binding assay

To isolate lsyw1b we used the modified TAP protocol described above in loc4TAP strains with the following modifications. Following cell lysis, whole cell extracts were not sonicated, but were immediately clarified by centrifugation at 12,000 rpm (Eppendorf microfuge, model 5415D) for 5 min at 4°C. After incubation and washes, the remaining bound fraction was washed an additional three times (1 x 10min, 1 x 5min, 1x 1 min) with wash buffer (100mM HEPES pH 8.0, 20mM Mg acetate, 10% glycerol, 10mM EGTA, 0.1mM EDTA, 300mM Na acetate, 0.5% NP-40) plus 600mM NaCl to remove all bound histones. Whole cell lysates were prepared from WT, set2Δ, and hht2K36R strains following the modified TAP protocol and were normalized for total protein using a Bradford assay. The resulting lysates were incubated with washed beads carrying immobilized loc for 3 hours at 4°C and histone binding was determined by western blot.
3.3 RESULTS AND DISCUSSION

3.3.1 loc4 is bound throughout the coding region of genes

Isw1b contains two auxiliary subunits, loc2 and loc4. First, we wanted to determine if either of these two proteins is required for the integrity of the Isw1b complex. To do this, we fused a TAP tag to the carboxyl terminus of Isw1 in an ioc2Δ strain harbouring an HA tagged version of loc4, or an ioc4Δ strain harbouring an HA tagged version of loc2. We then used co-immunoprecipitation to purify Isw1TAP and analysed complex stability by western blot with antibodies against the HA tag. Our findings indicate that, in an ioc2Δ strain, loc4 is no longer a stable component of the Isw1b complex (Figure 3.1A). On the other hand, there were no changes of loc2 association with Isw1b, following deletion of IOC4. This suggests that loc2 is required for overall complex stability.

Isw1, loc2 and loc4 were shown to localize to the 3’ end of a small set of transcribed candidate genes, including MET16, PMA1, IMD2 and PKA1 (Morillon et al. 2003). The same study found that loc3 does not associate with the 3’ region of MET16 during induction, suggesting that it is the Isw1b complex (and not the Isw1a complex) involved in transcription elongation of MET16 (Morillon et al. 2003). With this in mind, we wondered if 3’ ORF localization and transcription elongation is a general function Isw1b. To investigate this, we performed ChIP-on-chip experiments using Affymetrix high resolution tiling microarrays. Protein-DNA complexes containing a 6xHA tagged version of loc4 were immunoprecipitated from a wild type strain to ensure that only the Isw1b complex was analyzed. Enriched regions were
detected as previously described by comparing signal intensities of ChIP to input DNA (Schulze et al. 2009a).

Figure 3.1: Deletion of IOC2 results in destabilization of the Isw1b complex, whereas deletion of IOC4 has no effect on complex integrity.

A) Co-immunoprecipitation of Isw1TAP with loc2 or loc4. Isw1TAP was purified from WCEs from the indicated strains. Results were analysed by western blot with antibodies against IgG (Isw1TAP) or the HA tag (loc2 or loc4).

We used stringent cut-off criteria to identify a set of genes most enriched for loc4 and found that loc4 localizes to 372 actively transcribed genes in WT cells. loc4 is absent from promoter and 5' ends of genes, peaks at the mid-coding region and tapers off in the 3' intergenic region (IGR) (Figure 3.2). An untagged strain was used as a control and, as seen in Figure 3.2, does not show a similar binding pattern, indicating that this pattern is specific to loc4. The pattern of loc4 localization is similar to that of H3K36me3, which previous studies also found to be largely absent from promoters and 5' ends, at a peak in the mid-coding sequence (Pokholok et al. 2005). loc4 may be localized to more than the genes found in this study as the cells were grown in YPD; thus, genes inducible during certain stress conditions
(such as the absence of methionine in the media) were omitted from this protocol. Altogether, this result indicates that Isw1b is localized to the mid-coding and 3’ ends of genes, consistent with previous findings demonstrating Isw1b localization at the 3’ ends of a small subset of genes (Morillon et al. 2003).

![Figure 3.2](image.png)

**Figure 3.2: loc4 is found within the coding region and the 3’ end of ORFs**

A and B) ORFs were aligned according to their transcription start and end sites and were given an enrichment score for loc4HA. The average enrichment for loc4HA was mapped in a tagged and an untagged strain. As shown the untagged strain shows no enrichment for loc4HA at any region, whereas the tagged strain shows enrichment within the coding region and the 3’ end of the ORFs.

### 3.3.2 Histone H3 lysine 36 methylation is required for loc4 association with chromatin

Histone H3K36me3 is found within the ORFs and the 3’ ends of actively transcribed genes (Pokholok et al. 2005). Using ChIP-on-chip we have shown that loc4 is localized throughout the coding regions in a similar pattern to H3K36me3; therefore, we wondered if H3K36me3 was required for targeting of Isw1b to
chromatin (Figure 3.2). To test this, we performed ChIP-qPCR on a strain expressing an HA tagged version of loc4, with primers specific to the 3’ end of MET16, where loc4 is known to be localized during active transcription (Morillon et al. 2003). To induce MET16, cells were grown on media lacking methionine. Association of loc4 with chromatin appears to be partially dependent on the H3K36 HMT, Set2, but not on the H3K4 HMT, Set1 (Figure 3.3A). This observation was not due to changes in loc4 levels in either the set1Δ or set2Δ strains (Figure 3.3B). Surprisingly we found that deletion of SET1 actually increases the amount of loc4 localized to MET16. A possible explanation for this is that in the set1Δ strain, there are increased levels of H3K36me3. Jhd1 is an H3K36me3 demethylase that interacts with H3K4me3 in vitro (Kim and Buratowski 2007, Shi et al. 2007, Tu et al. 2007). Deletion of SET1, therefore, may result in less Jhd1 bound to histone H3, resulting in less H3K36me3 demethylase activity, which would then lead to increased loc4 binding.
Figure 3.3: The HMT Set2 and H3K36 are required for loc4 association with chromatin.

A and C) Levels of loc4HA at the 3' region of induced MET16 were measured relative to input by ChIP in the indicated strains. The primer pair used amplifies the region of +661 to +760 relative to the transcription start site (+1). Results are shown relative to WT strains which were set to 1. Error bars indicate the mean +/- SEM from three independent experiments. B and D) Western blot analysis of WCE from the indicated strains with an HA tagged version of loc4. Histone H3 is used as a control.
To confirm that loc4HA binding is dependent on H3K36me, loc4HA was tagged in strains which carried deletions of both the HHT1 and HHT2 loci and expressed histone H3 from a URA3 plasmid. A TRP-based plasmid carrying HHT2 or HHT2 with lysine 36 mutated to either an alanine or arginine was introduced into these strains. Plating cells on 5-fluoroorotic acid (5-FOA) was used to screen for cells containing the mutant HHT2 as the only copy of histone H3. These transformants were then subjected to ChIP-qPCR, in the same manner as the HMT mutants. Interestingly, mutation of lysine 36 results in near complete loss of loc4 bound to the 3’ end of MET16, without any effect to the protein expression of loc4 (Figure 3.3 C and D). The requirement of both Set2 and lysine 36 for loc4 association with chromatin by ChIP supports our hypothesis that Isw1b interaction with chromatin throughout the coding region is dependent on lysine 36 methylation.

### 3.3.3 The PWWP domain is required for Isw1b association with chromatin

Recent work investigating the binding properties of the human PWWP containing protein BRPF1 to histone tail peptides determined that BRPF1 interacts with histone H3 peptides methylated at K36, but not with peptides methylated at other known methylation sites (Vezzoli et al. 2010). Additionally, they identified three residues, Tyr1091, Tyr1099 and Phe1147, which form a binding pocket for the trimethyl ammonium group (Vezzoli et al. 2010). When any of these residues were mutated, binding of the PWWP domain to the H3K36me3 peptides was abolished (Vezzoli et al. 2010). To investigate whether the PWWP domain of loc4 could serve a similar function, we performed a CLUSTAL alignment of BRPF1 and loc4 as well
as two other PWWP domain containing proteins: Pdp1 recently found to interact with histone H4 methylated at lysine 20, and Nto2 (Ylr455w) an uncharacterized PWWP domain-containing protein in *S. cerevisiae* (Wang et al. 2009c).

For simplicity, we performed this analysis on regions containing the amino acids surrounding those that form the binding pocket in BRPF1. In BRPF1, Tyr1091 and Tyr1099 are found in the domain’s best conserved region (the “PWWP” motif), whereas Phe1147 is 23 amino acids downstream of this region (Figure 3.4 A). In Ioc4, Phe18 and Trp21 correspond to Tyr1091 and Tyr 1099 and although the exact amino acid identity is not conserved, there are conserved aromatic amino acids present in Ioc4 as well as most other PWWP domains (Figure 3.4 A and (Vezzoli et al. 2010)). The amino acid corresponding to Phe1147 in the different PWWP domain–containing proteins is found at varying distances downstream of the PWWP region, depending on the protein (Figure 3.4 A and (Vezzoli et al. 2010)). Despite this variation between the last residue of the binding pocket and the PWWP motif, the region surrounding the residue is quite well conserved between species (Figure 3.4 A). In Ioc4, Phe114 is most likely the final amino acid that forms the binding pocket. Having conserved aromatic residues that form a methyl-lysine binding pocket is a common architecture of the Tudor superfamily methyl-lysine binding domains as well as in PHD fingers; thus, we hypothesized that these three residues will form an aromatic cage, similar to BRPF1, that binds H3K36me3 (Taverna et al. 2006).
Figure 3.4: The PWWP domain of loc4 is required for loc4’s interaction with chromatin.

A) Alignment of part of the PWWP domain of Brpf1, Ioc4, Nto2 and Pdp1. Residues predicted to form the aromatic cage are highlighted in dark grey. An * (asterisk) indicates positions which have a single, fully conserved residue. A : (colon) indicates conservation between groups of strongly similar properties - scoring > 0.5 in the Gonnet PAM 250 matrix. A . (period) indicates conservation between groups of weakly similar properties - scoring ≤< 0.5 in the Gonnet PAM 250 matrix. B) Western blot analysis of co-immunoprecipitation experiments. Isw1TAP was isolated from strains carrying an HA tagged copy of wild type or mutant loc4 by immunoprecipitation. The resulting co-immunoprecipitation fractions were analyzed by western blot analysis with antibodies against the TAP tag (IgG) or the HA tag (αHA). C) Western blot analysis of WCE from the indicated strains with an HA tagged version of loc4. Histone H3 is used as a control. D) Levels of Ioc4HA at induced MET16 were measured relative to input by ChIP in the indicated strains. The primer pair used amplifies the region of +661 to +760 relative to the transcription start site (+1). Results are shown relative to WT strains which were set to 1. Error bars indicate the mean +/- SEM from three independent experiments.
With this in mind, we mutated the conserved residues in loc4 to alanines and examined mutants to determine whether loc4 was still able to associate with Isw1. To do this, we isolated Isw1TAP from a strain with an HA tagged version of either wild type or mutant loc4. The resulting co-immunoprecipitation was then analyzed by western blot with antibodies against the HA tag to determine the presence of loc4. All three mutants associate with Isw1 at similar levels to wild type, indicating that the loc4 PWWP domain mutants are still stably incorporated into the Isw1b complex (Figure 3.4 B). We next wanted to determine if mutation of the PWWP domain results in loss of interaction with chromatin. To do this, we performed ChIP-qPCR and found that loc4 levels are significantly reduced at the 3' end of MET16 in the PWWP domain mutants, which is not due to changes in protein expression of loc4 (Figure 3.4 C and D). Taken together with our previous data, this result supports our hypothesis that Isw1b is localized to the coding region of genes and that this localization is dependent on both H3K36me3 and the PWWP domain of loc4.

3.3.4 loc4 preferentially interacts with histones which are methylated at lysine 36.

To further confirm the interaction between loc4 and H3K36me3, we used histone-modification enrichment assays. Isw1b was purified from an loc4TAP strain using a modified TAP protocol (Lambert et al. 2009). The co-purifying histones were then analyzed by western blot using antibodies specific to histone H3 H3K4me3, H3K36me3 and H3K79me3. Since these antibodies are generated in different
animals were able to simultaneously analyze the signals from the two antibodies on the same blot and overlay the signals using the Licor Odyssey system. Histone H3K4me3 is not enriched in loc4 bound histones, with equal signal strength observed for the histone H3 (red) and H3K4me3 (green) antibodies in both the input and the IP fraction (indicated by a yellow signal) (Figure 3.5 A). H3K36me3 on the other hand shows a distinct enrichment in the loc4 bound fraction, with more H3K36me3 (red) than histone H3 (green) present in the bound fraction compared to the input (indicated by a predominately red signal). Interestingly, H3K79me3 is also slightly enriched in the loc4 bound fraction; however, this is likely because H3K79me and H3K36me3 are found to be co-localized throughout the open reading frames of genes (Pokholok et al. 2005).

To strengthen the argument that loc4 interacts with H3K36me3 histones, we developed an in vitro binding assay. loc4TAP was isolated from WT strains using the same method as the enrichment assays. After incubation and washing, the histones were washed away with a high salt (600mM NaCl) wash. In this assay, bands corresponding to all three Isw1b components remained bound to the resin, however all histones were washed away (Figure 3.6). Next, whole cell extracts, prepared by the modified TAP protocol, from WT, set2Δ, or HHT2.hht2K36R strains were incubated with the purified Isw1b complex. While there is some residual binding to histones from the set2Δ and HHT2.hht2K36R extracts, this interaction is very weak compared to that of wild type (Figure 3.6B), suggesting that loc4 and the Isw1b complex preferentially interact with H3K36me3 histones.
Figure 3.5: loc4 preferentially binds histones that are tri-methylated at H3K36

**Histone modification enrichment assays.** loc4TAP was used in a modified TAP protocol to co-immunoprecipitate histones with loc4. Results were analyzed by western blot analysis. A stronger signal is present in the bound fraction for both H3K36me3 and H3K79me3, suggesting that histones bound to loc4 are enriched for these two modifications.
Figure 3.6: Isw1b preferentially interacts with H3K36 methylated histones

A) Isw1b was purified from an loc4TAP strain using a modified TAP protocol followed by removal of histones with high salt wash. B) WCE’s from WT, set2Δ and HHF2.hht2K36R strains were incubated with the bound Isw1b complex and interaction of histones was detected by western blot analysis with antibodies against histone H3 and IgG (loc4).
3.4 CONCLUSIONS

Isw1 is present as three separate complexes in yeast, each of which is targeted to different regions of the genome. How the different complexes are targeted to these specific areas is mostly unknown. This study demonstrates, for the first time, a mechanism for how Isw1b is targeted to the coding regions of genes. First, we have provided insight into the architecture of the Isw1b complex. Deletion of \textit{IOC2} clearly results in loss of interaction between Isw1 and Ioc4; therefore, a possible function for Ioc2 in the Isw1b complex is to act as a molecular scaffold, facilitating the interaction between Isw1 and Ioc4 (Figure 3.7).

Secondly, we revealed that Ioc4 is capable of interaction with histones methylated at H3K36 both \textit{in vivo} and \textit{in vitro}. Previous studies investigating the targeting of Isw1 to chromatin found that association of Isw1 with histones is dependent on the SLIDE domain of Isw1 \textit{in vivo}; however, this study found that the SLIDE domain was also required for proper interaction of Ioc2 and Ioc4 with Isw1 (Pinskaya et al. 2009). Our data suggests that the dependence of Isw1 on its SLIDE domain for interaction for chromatin is likely due to the dependence on the SLIDE domain for interaction with Ioc4. Ioc4 in turn binds with H3K36me3 thus, targeting Isw1b to chromatin. Similarly, the SLIDE domain of Isw1 is required for interaction of Ioc2 (Pinskaya et al. 2009). Deletion of \textit{IOC2} causes loss of Ioc4 interaction with Isw1, further explaining why loss of the SLIDE domain results in loss of Isw1 interaction with chromatin.
In this study we determine a possible targeting mechanism for Isw1b to the mid-coding and 3’ regions of genes during transcription elongation. It is possible that the interaction between H3K36me3 and loc4 at these regions helps promote repression of cryptic transcription from internal TATA elements by moving nucleosomes back into place over the TATA element.

Figure 3.7: Schematic diagram of the architecture of Isw1b

Shown is a schematic representation of the Isw1b complex. The loc2 complex acts as a molecular scaffold, bridging the catalytic subunit Isw1 with the chromatin interaction subunit loc4. loc4 is targeted to H3K36me3, resulting in Isw1b targeting to the 3’ coding regions of genes and allowing transcription elongation.
CHAPTER 4. THE PWWP DOMAIN OF NTO2 IS A PUTATIVE H3K36ME3 INTERACTION DOMAIN

4.1 INTRODUCTION

The MYST (MOZ-YBF2(SAS3)-SAS2-TIP60) family of HATs is conserved in eukaryotes. The characteristic feature of MYST HATs is the MYST domain, named for the four founding members of this family, which is comprised of an acetyl Co-A interaction domain along with a zinc finger. MYST HATs are found as multiprotein complexes, which all include an inhibitor of growth (ING) family protein, Eaf6, and at least one other auxiliary subunit. In humans, there are at least four MYST family members, MOZ/MORF, TIP60, HBO1, and MOF. In yeast, there are two MYST family proteins, Sas3 and Esa1, which are part of the NuA3 and NuA4 complexes respectively.

NuA3 (Nucleosome Acetyltransferase of histone H3) is a multiprotein HAT complex containing the catalytic subunit, Sas3 and at least five other subunits (Yng1, Eaf6, Taf14, Nto1 and Ylr455w), each of which contains a potential histone-modification interaction domain (Howe et al. 2002, John et al. 2000, Krogan et al. 2006, Taverna et al. 2006). NuA3 HAT activity is specific to histone H3, particularly lysines 14 and 23 (Howe et al. 2001). Interestingly, lysine 14 and 23 acetylation is found at elevated levels, not only at the promoter, but also throughout the transcribed region of active genes (Liu et al. 2005, Pokholok et al. 2005, Wang et al. 2008). Consistent with this, ChIP-on-chip studies with Yng1 found that Yng1 is
enriched throughout the transcribed regions of active genes (Taverna et al. 2006). This genome-wide study found a definite peak at the 5’ region of genes and lower levels of Yng1 throughout the rest of the ORF (Taverna et al. 2006). However, how NuA3 is targeted to these areas is not fully understood. MOZ and MORF are structurally and functionally related to each other and both form complexes similar to NuA3. In addition to MOZ or MORF as the catalytic subunit, these complexes contain ING5, hEaf6, and BRPF1/2/3, which are homologous to Yng1, Eaf6 and Nto1 respectively. MOZ/MORF complexes are also responsible for acetylating lysine 14 of the H3 tail and function as coactivators of transcription factors.

One mechanism regulating targeting of NuA3 may be post-translational modification of lysines 4 and 36 on histone H3. Lysines 4 and 36 are methylated by the HMTs Set1 and Set2 respectively and are enriched at active loci (Barski et al. 2007, Bernstein et al. 2005, Pokholok et al. 2005, Schubeler et al. 2004). Both of these HMTs are associated with RNAPII in a manner dependent on the phosphorylation of the largest subunit, Rbp1 and the PAF1 complex (Krogan et al. 2003a, Krogan et al. 2003b, Ng et al. 2003). Recruitment of Set1, and H3K4me3, to the promoter and 5’ regions of genes, is dependent on phosphorylation of serine 5 of the CTD and the Paf1 complex (Krogan et al. 2003a, Ng et al. 2003). During transcription initiation, serine 5 is phosphorylated by Kin28, a TFIIDH-associated kinase (Rodriguez et al. 2000). Serine 5 phosphorylation decreases during early transcription elongation stages and is replaced by serine 2 phosphorylation, which is mediated primarily by the cyclin-dependent kinase Ctk1 (Komarnitsky et al. 2000).
Serine 2 and the PAF1 complex then mediate the interaction of Set2 with RNAPII, resulting in H3K36me3 throughout the transcribed regions of genes (Cho et al. 2001, Krogan et al. 2003b). As a consequence of these interactions, H3K4me3 is found primarily at the promoter and the early transcribed region of genes, whereas H3K36me3 is largely absent from these regions but is found spread throughout the open reading frame (Barski et al. 2007, Pokholok et al. 2005). NuA3 interaction with the H3 tail is dependent on both lysine 4 and 36 methylation, thus, this could represent a possible targeting mechanism for NuA3 throughout the open reading frame (Martin et al. 2006b).

Previous studies found that NuA3 interaction with the H3 tail is dependent not only on lysines 4 and 36 but also on the Yng1 PHD finger (Martin et al. 2006a, Martin et al. 2006b, Taverna et al. 2006). Studies demonstrated that the Yng1 PHD finger was capable of interaction with H3K4me3, providing a possible mechanism for NuA3 targeting to the 5' regions of genes (Martin et al. 2006a, Taverna et al. 2006). Surprisingly, in a yng1ΔPHD strain and a set1Δ strain NuA3 was still able to interact with the histone H3 tail (Martin et al. 2006a). In addition to this, Taverna and colleagues demonstrated that NuA3 is present throughout all regions of genes, whereas H3K4me3 is primarily found towards the 5' region (Barski et al. 2007, Bernstein et al. 2005, Pokholok et al. 2005, Schubeler et al. 2004, Taverna et al. 2006).
In an effort to explain this observation, we proposed that NuA3 interacted with the H3 tail through another histone modification (Martin et al. 2006b). To test this hypothesis, we used strains that lacked a number of post translational modifications and found that deletion of the H3K36 HMT SET2, in combination with truncation of the Yng1 PHD finger or deletion of SET1, resulted in loss of NuA3 interaction with the H3 tail (Martin et al. 2006b). This led us to propose that NuA3 has another subunit that is able to interact with H3K36me3 that, in combination with the Yng1 PHD finger and H3K4me3, helps recruit NuA3 to the histone H3 tail, possibly through interaction with H3K36me3.

Each of the subunits of NuA3 is a potential candidate involved in interaction with H3K36me3. Nto1 contains tandem PHD finger domains, one of which has been shown to interact with H3K36me in vitro; however, whether this occurs in vivo is unknown (Shi et al. 2007). Taf14 contains a YEATS domain, which is found in many other chromatin modification and transcription complexes both in yeast as well as higher eukaryotes (Schulze et al. 2009b). Another YEATS domain protein in yeast, Yaf9, has been demonstrated to interact with histones, however, little is known about the function of the Taf14 YEATS domain (Wang et al. 2009a). Eaf6 is a small protein, which is a member of both the NuA3 and NuA4 (histone H4 HAT) complexes (Doyon and Cote 2004, Taverna et al. 2006). Although Eaf6 does not have any protein domains known to interact with chromatin, it is conserved in humans (hEaf6) and is found in complexes homologous to NuA3, such as MOZ and MOF (Doyon et al. 2006). Finally, Ylr455w is an uncharacterized protein, which contains a PWWP
domain. As demonstrated in Chapter 3 of this thesis, as well as several other studies, the PWWP domain is a H3K36me3 binding domain in the context of other proteins (Dhayalan et al. 2010, Vezzoli et al. 2010, Wu et al. 2011). Ylr455w contains the residues required for formation of the H3K36me3 binding pocket; thus, Ylr455w represents another potential candidate for interaction of NuA3 with chromatin.

In this study, we use a genetic approach as a primary method of determining which subunits of NuA3 function as H3K36me3 binding proteins. While preliminary evidence suggested that TAF14, EAF6, and YLR455W interact with the YNG1PHD finger, investigation of the architecture of the NuA3 suggested Ylr455w as the most likely candidate, leading us to hypothesize that Ylr455w serves as the H3K36me3-binding subunit. This study demonstrates that Ylr455w is a stable component of the NuA3 complex and that it represents the best candidate for interaction of NuA3 with H3K36me3. For this reason, we propose the name Nto2 (for NuA Three Open reading frame 2) for this protein and will use this terminology for the remainder of this chapter. In addition, we demonstrate that SET1 and SET2 are required for interaction of NuA3 with chromatin in a non-redundant fashion, meaning that the methyl-lysine interaction domains contained within the NuA3 complex could represent multivalent binding domains.
4.2 MATERIALS AND METHODS

4.2.1 Yeast strains and plasmids

All strains used in this study are isogenic to S288C and are listed in Table 4.1. Yeast culture and genetic manipulations were performed using standard protocols (Ausubel et al. 1987). Genomic deletions were verified by PCR analysis and whole-cell extracts were generated as previously described (Ausubel et al. 1987, Kushnirov 2000).
### Table 4.1 Yeast strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mating type</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>YLH101</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63</td>
</tr>
<tr>
<td>YVM103</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 gcN5::TRP ylr455w::HIS3 pGCN5.416</td>
</tr>
<tr>
<td>YVM106</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 gcN5::HIS3 nto1::HISMX6 yng1ΔPHD::KAN pNTO1.415</td>
</tr>
<tr>
<td>YVM107</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 gcN5::TRP yng1ΔPHD::HISMX6 pGCN5.416</td>
</tr>
<tr>
<td>YVM108</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 gcN5::TRP yng1ΔPHD::HISMX6 eaf6::KANMX6 pGCN5.416</td>
</tr>
<tr>
<td>YVM109</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 gcN5::TRP eaf6::KANMX6 pGCN5.416</td>
</tr>
<tr>
<td>YLH314</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 gcN5::TRP pGCN5.416</td>
</tr>
<tr>
<td>YLH367</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 gcN5::HIS3 sas3::KANMX6 pGCN5.416</td>
</tr>
<tr>
<td>YVM111</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 eaf6::KANMX6 pGCN5.416</td>
</tr>
<tr>
<td>YLH370</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 taf14::KANMX6 pTAF14Flg.416</td>
</tr>
<tr>
<td>YLH427</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 taf14::KANMX6 gcN5::TRP pTAF14Flg.416</td>
</tr>
<tr>
<td>YLH428</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 taf14::KANMX6 gcN5::TRP yng1ΔPHD::HIS3 pTAF14Flg.416</td>
</tr>
<tr>
<td>YVM193</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 gcN5::TRP ylr455w::HIS3 yng1ΔPHD::KAN pGCN5.416</td>
</tr>
<tr>
<td>YVM128</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 gcN5::HIS3 nto1::HIS3 yng1ΔPHD::KANMX6 pGCN5.416 pNTO1.415</td>
</tr>
<tr>
<td>YVM129</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 gcN5::HIS3 nto1::HIS3 yng1ΔPHD::KANMX6 pGCN5.416 pNTO1PHDFQ5T/A6Y.415</td>
</tr>
<tr>
<td>YVM130</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 gcN5::HIS3 nto1::HIS3 yng1ΔPHD::KANMX6 pGCN5.416 pNTO1PHDF23G.415</td>
</tr>
<tr>
<td>YVM131</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 gcN5::HIS3 nto1::HIS3 yng1ΔPHD::KANMX6 pGCN5.416 pNTO1PHD46A.415</td>
</tr>
<tr>
<td>YVM132</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 gcN5::HIS3 nto1::HIS3 yng1ΔPHD::KANMX6 pGCN5.416 pNTO1PHDI29E/A30W.415</td>
</tr>
<tr>
<td>Strain</td>
<td>Mating type</td>
<td>Genotype</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>YVM133</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 gcn5::HIS3 nto1::HIS3 yngΔPHD::KANMX6 pGCN5.416 pnto1PHDΔ28A.415</td>
</tr>
<tr>
<td>YVM146</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 SAS3-6HA::TRP</td>
</tr>
<tr>
<td>YVM142</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 SAS3-6HA::HIS set1Δ::KANMX6 set2Δ::TRP</td>
</tr>
<tr>
<td>YVM147</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 SAS3-6HA::TRP yng1ΔPHD::KANMX6</td>
</tr>
<tr>
<td>YVM148</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 SAS3-6HA::TRP eaf6Δ::KANMX6</td>
</tr>
<tr>
<td>YVM157</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 SAS3-6HA::TRP set1Δ::HISMX6</td>
</tr>
<tr>
<td>YVM158</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 SAS3-6HA::TRP set2Δ::HISMX6</td>
</tr>
<tr>
<td>YVM155</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 SAS3-6HA::TRP yng1ΔPHD::KANMX6 eaf6Δ::HISMX6</td>
</tr>
<tr>
<td>YVM211</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 SAS3-6HA::TRP ylr455w::HIS pEAF6FLAG.416</td>
</tr>
<tr>
<td>YVM212</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 SAS3-6HA::TRP ylr455w::HIS pTAF14FLAG.416</td>
</tr>
<tr>
<td>YVM213</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 SAS3-6HA::TRP ylr455w::HIS pYNG1FLAG.416</td>
</tr>
<tr>
<td>YVM241</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 SAS3TAP::TRP YLR455w-6HA::HIS</td>
</tr>
<tr>
<td>YVM242</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 YLR455w-6HA::HIS</td>
</tr>
<tr>
<td>YVM214</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 SAS3TAP::TRP eaf6::KAN YLR455w-6HA::HIS</td>
</tr>
<tr>
<td>YVM112</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 SAS3TAP pTAF14FLAG.416</td>
</tr>
<tr>
<td>YVM113</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 SAS3TAP yng1ΔPHD pTAF14FLAG.416</td>
</tr>
<tr>
<td>YVM114</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 SAS3TAP eaf6 pTAF14FLAG.416</td>
</tr>
<tr>
<td>YVM115</td>
<td>Mat a</td>
<td>his3Δ200 leu2Δ1 lys2-128d ura3-52 trp1Δ63 SAS3TAP yng1ΔPHD eaf6 pTAF14FLAG.416</td>
</tr>
</tbody>
</table>
4.2.2 Co-immunoprecipitations

Cells were grown in rich media (YPD) to an OD$_{600}$ of 1.0. Cells were harvested by centrifugation (4000 rpm, 3 min, Sorvall Legend RT), followed by two washes in PBS. Harvested cells were re-suspended in 600 µl of ex150 buffer (150 mM NaCl, 20 mM HEPES pH 8.0, 10% v/v glycerol, 0.5% v/v Tween plus protease inhibitor cocktail (Roche)) and an equivalent volume of glass beads was added. Samples were vortexed 3 x 5 min using the Vortex Genie (Scientific Industries). Lysates were clarified by centrifugation at 13,200 rpm for 5 min at 4°C in an Eppendorf Centrifuge (model 5415D). Whole cell extracts were then incubated with αFLAG M2 resin (Sigma Aldrich) or IgG coupled magnetic beads (Invitrogen, prepared as per manufacturer instructions) for 3 hours at 4°C. Following incubation, the beads were washed 5 times with ex350 (350 mM NaCl, 20 mM HEPES pH 8.0, 10% glycerol, 0.5% Tween). The resulting proteins were run on SDS PAGE gel and analyzed by western blot analysis using the antibodies listed in Table 4.2.

Table 4.2: Antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Cat. no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit Immunoglobulin G (IgG)</td>
<td>Chemicon</td>
<td>PP64</td>
</tr>
<tr>
<td>αHA (rat 3F10 clone)</td>
<td>Roche</td>
<td>11 867 423 001 (antigen YPYDVPDYA)</td>
</tr>
<tr>
<td>αHA-HRP (rat 3F10 clone)</td>
<td>Roche</td>
<td>12 013 819 001 (antigen YPYDVPDYA)</td>
</tr>
<tr>
<td>Goat anti-rabbit (680)</td>
<td>Licor</td>
<td>926-32210</td>
</tr>
<tr>
<td>αFLAG</td>
<td>SIGMA</td>
<td>F7425</td>
</tr>
</tbody>
</table>
4.2.3 Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as previously described, with a few modifications (Nelson et al. 2006). Briefly, cells were grown in 50 mL of YPD to mid-log phase and cross-linked with 1% (v/v) formaldehyde for 30 min at room temperature. The reaction was stopped with 125 mM glycine for 15 min and cells were washed twice with PBS. Samples were sonicated (Biorupter, Diagenode, Sparta NJ, high output for 5 x 30 on/off) to obtain an average fragment length of 500 bp. Antibodies used for ChIP are listed in Table 4.2. Antibodies were coupled to appropriate Dynabeads overnight at 4°C (Protein A or G, Invitrogen). After reversal of crosslinking and DNA purification, immunoprecipitated and input DNA were amplified using an MJ Research Opticon Monitor 3 Thermal Cycler using a primer pair designed to the 5’ end of COX10 (primers 636 (sense 5’ CGG AAT CAT GGC GGG AAA C 3’) and 637 (antisense 5’ GGA AGT TGT GTG CTT GCA TCG 3’)). Each PCR reaction consisted of 13.5 μl ddH₂O, PCR buffer (20 mM Tris-Cl, 10 mM KCl, 10 mM (NH₄)₂SO₄, 2 mM MgSO₄ and 0.1% TX-100), 0.2 mM dNTPs, 1.6 mM of each primer, and 1 μl of a 1:1000 dilution of SYBR green. PCRs went through a program of 94°C for 2 min followed by 40 cycles of 94°C 30 s, 55°C 1 min, 72°C 1 min using the Opticon Monitor 3 (MJ Research). Average IP values for each sample were normalized to average input values (%IP) and compared to a mock (untagged) control.
4.2.4 Modified TAP tag immunoprecipitation

The modified TAP tag immunoprecipitation (IP) was performed as previously described (Lambert et al. 2009), with the following modifications. After extract preparation, extracts were clarified by centrifugation at 12 000 rpm (Eppendorf microfuge, model 5415D) for 5 min at 4°C. Following incubation for 2 hours at 4°C, beads were washed and boiled for 10 min with SDS loading buffer. The co-immunoprecipitating proteins were analyzed by Coomassie B-250 staining.

4.3 RESULTS AND DISCUSSION

4.3.1 Deletion of TAF14, EAF, and YLR455w show a sas3Δ phenotype in a yng1ΔPHD background.

Deletion of SAS3 alone does not have an effect on cell viability; however, deletion of the loci coding for the two primary histone H3-specific HATs, GCN5 and SAS3 results in a synthetic lethal phenotype due to Gcn5’s inability to acetylate Rsc4 in combination with loss of H3 acetylation (Choi et al. 2008, Howe et al. 2001). A strain harbouring deletions of both GCN5 and the YNG1 PHD finger did not have a synthetic lethal phenotype, suggesting that Sas3 is capable of interacting with the H3 tail and acetylating H3K14 (Figure 4.1A and (Martin et al. 2006a)). This suggests that there is a redundant subunit in the NuA3 complex mediating the interaction of NuA3 with the histone H3 tail and loss of both subunits would be required to see a synthetic lethal phenotype with gcn5Δ.
Figure 4.1: A genetic screen to identify NuA3 complex components as potential H3K36me3 interactors shows that Nto1 shows a sas3Δ phenotype due to loss of Sas3 from the cell.

A) Ten fold serial dilutions of the indicated strains were plated on synthetic drop out (-URA) media or synthetic complete media with 5-FOA for 2 days at 30°C. B) Western blot of Sas3HA from WCEs of either an NTO1 or nto1Δ strain with antibodies against the HA tag.
We reasoned that if NuA3 is unable to interact with chromatin, then cells will display a sas3Δ phenotype, which is synthetically lethality following deletion of GCN5. To investigate this, we developed a genetic screen to identify the redundant subunit. For this screen, we created a strain that has a truncation of the Yng1 PHD finger and deletion of GCN5. GCN5 was then added back on a URA3 based vector, which can be selected against using media containing 5-fluoroorotic acid (5-FOA). The uracil biosynthetic pathway analog 5-FOA is converted to a toxic product in the presence of the URA3 enzyme, so that cells which have lost the wild type GCN5 plasmid can grow on media containing 5-FOA (Boeke et al. 1984). In this strain, we individually deleted each of the genes encoding subunits of NuA3 and plated them on media lacking uracil, as a control, or 5-FOA to score for the sas3Δ phenotype by loss of viability.

Nto1 contains two PHD fingers, the first of which (PHD1) has been shown to specifically interact with H3K36me3 peptides in vitro (Shi et al. 2007). Deletion of NTO1 in combination with deletion of GCN5 results in loss of cell viability on 5-FOA (Figure 4.1 A). This could be due to significantly reduced levels of Sas3 in the cell following deletion of NTO1 (Figure 4.1 B). The study which first demonstrated that Nto1 PHD1 interacts with H3K36me3 also identified residues that abolished interaction of PHD1 with H3K36me3 peptides (Shi et al. 2007). To determine whether Nto1 PHD1 has a redundant function with the Yng1PHD finger in binding chromatin, we mutated amino acids required for the interaction of this motif with H3K36me3
peptides *in vitro* (Shi et al. 2007). All the point mutations rescued growth on 5-FOA, suggesting that Nto1 is not the redundant subunit interacting with the H3 tail (Figure 4.1 A). The possibility remains that the second PHD finger (PHD2) of Nto1 is capable of interaction with H3K36me3. PHD2 interacts weakly with H3K4me1/2/3, H3K36me1/2/3 and unmodified histones *in vitro*, suggesting that PHD2 does not have specific affinity for lysine 36. Based on these findings, we suggest that it is unlikely that Nto1 is the redundant subunit interacting with H3K36me3.

Taf14 contains an uncharacterized YEATS domain. YEATS domain proteins are found in several chromatin remodelling complexes, and the YEATS domain of another yeast protein, Yaf9, has been shown to interact with histones, making Taf14 a reasonable candidate for histone-binding (Schulze et al. 2009b, Wang et al. 2009a). Consistent with previous data, *TAF14* deletion strains exhibit slow growth (Zhang et al. 2004), which is further reduced in a *gcn5Δ yng1ΔPHD* background (Figure 4.2A). However, upon further analysis we found that similar to *nto1Δ* strains, *taf14Δ* strains show reduced levels of Sas3 in the whole cell extracts, providing a likely explanation for this phenotype (Figure 4.2B). Another study being conducted in our lab has characterized point mutations of the Taf14 YEATS domain and demonstrated that these do not affect levels of Sas3 or the stability of the NuA3 complex (Kristina McBurney, unpublished data). We used these point mutations in our genetic screen, but all of them fail to show a *sas3Δ* phenotype (Kristina McBurney, unpublished data). Additionally, mass spectrometry analysis revealed
that the Taf14 YEATS domain is likely interacting with other complexes and not with histones (Kristina McBurney, unpublished data). Overall, our data suggests that Taf14 is also not mediating the interaction of NuA3 complex with histones.

**Figure 4.2:** A genetic screen to identify NuA3 complex components as potential H3K36me3 interactors shows that disruption of *TAF14* also results in loss of Sas3 from the cell.

A) Ten fold serial dilutions of the indicated were plated on synthetic drop out (-URA) media or synthetic complete media with 5-FOA for 2 days at 30°C. B) Western blot of Sas3HA from WCEs of either a *TAF14* or *taf14Δ* strain with antibodies against the HA tag.
Unlike the other members of the NuA3 complex, there are no known histone-interaction domains in Eaf6 and its function within this complex is unknown. However, Eaf6 is a member of both the NuA3 and NuA4 complexes and is conserved in human NuA3-like complexes such as MOZ and MORF (Doyon and Cote 2004, Doyon et al. 2006, Taverna et al. 2006). In our genetic screen, eaf6Δ does not display any loss of cell viability on its own or in combination with gcn5Δ (Figure 4.3 A). Deletion of EAF6 in a gcn5Δ, yng1ΔPHD background results in loss of cell viability comparable to a gcn5Δ sas3Δ strain (Figure 4.3A compare top row to bottom row). In addition to this (as discussed later) eaf6Δ does not have an effect on the levels of Sas3 in the cell. This data suggests that Eaf6 may serve as the subunit required for NuA3 interaction with chromatin.

Nto2 is an uncharacterized ORF which contains a PWWP domain. PWWP domains have been shown by us and others to interact with H3K36me3 (Chapter 3 and (Dhayalan et al. 2010, Vezzoli et al. 2010, Wu et al. 2011)). A previous study determined the amino acid residues required for interaction of another PWWP containing domain protein, BRPF1, H3K36me3 (Vezzoli et al. 2010). Nto2 contains the same residues, which form an H3K36me3 binding pocket; therefore, it is a reasonable candidate protein for histone binding. In our genetic screen, deletion of NTO2 shows minimal loss of viability on its own or in combination with gcn5Δ. In the gcn5Δ yng1ΔPHD background, deletion of NTO2 results in loss of cell viability (Figure 4.3 B). In addition to this, the data in section 4.3.2 suggests that this is not
due to instability of Sas3. Taken together, Nto2 is also a reasonable candidate for interaction of NuA3 with H3K36me3.

Figure 4.3: A genetic screen to identify NuA3 complex components as potential H3K36me3 interactors indicates that deletion of either EAF6 or NTO2 shows a sas3Δ phenotype.

A) Ten fold serial dilutions of the indicated were plated on synthetic drop out (-URA) media or synthetic complete media with 5-FOA for 2 days at 30°C.
4.3.2 Nto2 is not required for stable association of Eaf6 with Sas3

Because deletion of both NTO2 and EAF6 resulted in a sas3Δ phenotype in our screen, we postulated that one of these subunits may be required for stable association of the other with the NuA3 complex. To test this, we used co-precipitation followed by western blot analysis. Briefly, Eaf6 was tagged with a FLAG tag and affinity purified from whole cell extracts in wild type or an NTO2 deletion strain. The ability of Eaf6 to interact with the catalytic subunit, Sas3, was determined by western blot analysis. In the nto2Δ strain there is an equal amount of Sas3 in the bound fraction as compared to wild type strains (Figure 4.4 A). This suggests that deletion of NTO2 does not affect the ability of Eaf6 to interact with Sas3. As a control, we repeated this experiment using strains with a FLAG tagged version of Taf14 or Yng1 (Figure 4.4 B and C). In these experiments the levels of Sas3 in the bound fraction are similar to those of wild type, suggesting that Nto2 is also not required for stable association of Taf14 or Yng1 with Sas3 (Figure 4.4 B and C).
Figure 4.4: Deletion of NTO2 does not disrupt NuA3 complex stability.

Yng1 (A), Taf14 (B) or Eaf6 (C) were FLAG tagged in wild type (WT) or nto2Δ strains and immunoprecipitated using αFLAG resin. Co-immunoprecipitating Sas3 was detected by western blot with antibodies against the HA tag. αFLAG antibodies were used to show equal precipitation of the FLAG tagged protein. (-) not present (+) present.
4.3.3 Eaf6 is required for stable association of Nto2 with Sas3

Nto2 was not required for association of Eaf6 with Sas3, but deletion of either NTO2 or EAF6 results in a sas3Δ phenotype. Next, we determined whether Eaf6 is required for stable incorporation of Nto2. Using a TAP tagged version of the catalytic subunit Sas3, we immunoprecipitated NuA3 from wild type and eaf6Δ strains. The ability of Nto2 to interact with the catalytic subunit, Sas3, was determined by western blot analysis. In a wild type strain, Nto2HA is clearly visible in the bound fraction, indicating that Nto2 is capable of interaction with Sas3 (Figure 4.5 A). However, in the eaf6Δ strain the amount of Nto2 in the bound fraction drops to nearly undetectable levels, suggesting that Eaf6 affects the ability of Nto2 to interact with Sas3 (Figure 4.5 A). Conversely, antibodies against Taf14 were used as a control and, as demonstrated in Figure 4.5 A, deletion of EAF6 does not affect the interaction between Taf14 and Sas3. As an additional control, we affinity purified Taf14 FLAG from WT, eaf6Δ, yng1ΔPHD and eaf6Δ yng1ΔPHD strains and analyzed for the presence of Sas3HA by western blot. The levels of Sas3 in the bound fraction remains constant between the wild type and all three mutant strains, suggesting that neither Eaf6 nor the Yng1 PHD finger are required for the interaction between Taf14 and Sas3 (Figure 4.5 B). Taken together, our data demonstrates that Eaf6 mediates the interaction between Nto2 and Sas3, which could explain why deletion of either EAF6 or NTO2 results in a sas3Δ phenotype.
Figure 4.5: Deletion of *EAF6* results in loss of Nto2 from the NuA3 complex but does not affect Taf14 incorporation.

A) Taf14 was FLAG tagged in WT, *eaf6Δ*, *yng1ΔPHD* or *eaf6Δ yng1ΔPHD* strains and immunoprecipitated using αFLAG resin. Co-immunoprecipitating Sas3 was detected by western blot with antibodies against the HA tag. αFLAG antibodies were used to show equal precipitation of the FLAG tagged protein. (-) not present (+) present.

B) Sas3 was TAP tagged in WT or *eaf6Δ* strain and immunoprecipitated using IgG coupled to magnetic beads. Co-immunoprecipitating Nto2 and Taf14 were detected by western blot with antibodies against the HA tag (Nto2) or Taf14. (-) not present (+) present.
4.3.4 Set1, Set2, and Eaf6 are required for Sas3 interaction with chromatin

Our lab previously demonstrated, using chromatin co-pull down assays, that NuA3 interaction with chromatin is only disrupted in strains harbouring deletions of both Set1 and Set2 (Martin et al. 2006b). We were also able to demonstrate that interaction of NuA3 with chromatin was dependent on H3K4 and H3K36, which suggested that methylation of these two residues mediates the interaction of NuA3 with chromatin (Martin et al. 2006b). However, chromatin co-pull down assays are problematic in that they are only able to detect interaction of Sas3 with chromatin in bulk histones and not at specific loci as chromatin immunoprecipitation (ChIP) does. Unfortunately at the time of the study by Martin et al. (2006), we were unable to use ChIP to localize Sas3 to any loci (Martin et al. 2006b). However, a new protocol (Nelson et al. 2006) and antibodies for ChIP, along with results from a genome wide study of Sas3 interaction with chromatin (Rosaleny et al. 2007), allowed us to successfully ChIP Sas3 to specific loci in the current study.

Using ChIP-qPCR, we looked for interaction of Sas3 with chromatin at the 5' region of the COX10 and CWC25 loci. We chose to look at the 5' region because this is where H3K4me3 peaks; therefore, the Yng1PHD finger should be able to recruit NuA3 to chromatin in this region, and we chose COX10 and CWC25 due to the strong interaction of Sas3 at these loci in genome wide studies (Martin et al. 2006a, Pokholok et al. 2005, Rosaleny et al. 2007, Taverna et al. 2006). Unlike our previous studies, we were able to successfully detect Sas3HA at both the COX10
(Figure 4.6 A) and CWC25 loci (data not shown) by ChIP. Sas3HA is significantly enriched at the 5’ end of COX10, when compared to an untagged control (Figure 4.6 A).

Our previous studies suggested that both H3K4me and H3K36me are required to maintain this interaction between Sas3 and chromatin, so we aimed to replicate these results (Martin et al. 2006a). To do this, we performed ChIP of Sas3 in strains with deletions of SET1 and/or SET2. Surprisingly, deletion of either SET1 or SET2 showed a significant loss of Sas3 interaction with chromatin and deletion of both did not increase this loss (Figure 4.6 A), suggesting that methylation of both K4 and K36 is required for NuA3 interaction with chromatin and not one or the other as previously described (Martin et al. 2006a). The same result was observed at the CWC25 locus (data not shown). Similarly, we deleted both the YNG1 PHD finger and EAF6 and found that deletion of either of these subunits abolishes most of the Sas3 interaction with chromatin and that this interaction was not further affected by the double mutation (Figure 4.6 B). Our results suggest that Set1, Set2, Eaf6 and the Yng1 PHD finger are all required for normal levels of Sas3 interaction with chromatin.
Figure 4.6: SET1, SET2, Yng1PHD finger and EAF6 are all required for Sas3 interaction with chromatin at the 5’ end of COX10.

Levels of Sas3HA at the 5’ end of COX10 were measured by ChIP-qPCR in the strains indicated. The amount of Sas3 co-precipitating was measured as a percent of input (%IP) and error bars indicate standard error of the mean (SEM) from at least three independent experiments.
4.3.5 Nto2 interacts with histones

Through genetic and biochemical assays we have determined that Nto2 represents the most likely candidate for interaction with H3K36me3 in the NuA3 complex. To determine if Nto2 interacts with histones we used a modified TAP tag immunoprecipitation protocol (Lambert et al. 2009). TAP tagged Nto2 was immunoprecipitated from wild type cells. The resulting co-immunoprecipitating proteins were run on a 12% SDS PAGE gel and stained with Coomassie G-250 stain. As seen in Figure 4.7, Nto2 co-immunoprecipitates with all four histones at near stoichiometric levels. Conversely, the same protocol performed using Sas3TAP yields undetectable levels of histones by Coomassie staining (data not shown). Further studies will be required to confirm the specificity of this interaction, however, the near stoichiometric ratio of histones to Nto2 suggests that Nto2 binds to histones with high affinity and provides more support for our hypothesis that Nto2 functions as the other histone interaction domain in NuA3 (in addition to Yng1 PHD finger).
Figure 4.7: Nto2 co-immunoprecipitates with histones.

Nto2 was immunoprecipitated from WCEs and the co-immunoprecipitated fraction was run on a 12% SDS PAGE gel and stained with Coomassie G-250 to determine which proteins associate with Nto2TAP. Numbers on the side indicate molecular weight of the standard in kDa.
4.4 CONCLUSIONS

We have previously shown that the interaction of NuA3 with chromatin requires either H3K4me or H3K36me (Martin et al. 2006a). Subsequent studies demonstrated that the Yng1 PHD finger was capable of interaction with H3K4me3 but not H3K36me3, suggesting that some other subunit of NuA3 may be responsible for the latter interaction (Martin et al. 2006a, Taverna et al. 2006). The results of the current study suggest that Nto2 is the most likely candidate for this interaction. A genetic screen identified both Eaf6 and Nto2 as reasonable candidates for interaction with H3K36me3. Subsequent biochemical assays suggest that Eaf6 mediates the interaction between Nto2 and Sas3; thus, it is probable that the identification of Eaf6 in our genetic screen is due to loss of Nto2. Furthermore, immunoprecipitation studies demonstrate that Nto2 has a strong affinity for histones.

The work in Chapter 3 of this thesis, and by others (Dhayalan et al. 2010, Vezzoli et al. 2010, Wu et al. 2011) demonstrates that the PWWP domain is capable of specific interaction with H3K36me3. Nto2 has a conserved PWWP domain that contains all the amino acid residues required for the formation of the H3K36me3-binding pocket (Figure 3.4). In addition to this, co-immunoprecipitation studies found that Nto2 has a strong affinity for histones. Based on this study, and previous studies of the PWWP domain, we predict that Nto2 interacts with H3K36me3 via its PWWP domain. Determining an interaction between Nto2 and H3K36me3 and the dependence of this interaction on the PWWP domain remains a topic for further study.
Previous data suggests that either lysine 4 or 36 is required for interaction of NuA3 with chromatin (Martin et al. 2006b). Data from our lab and others demonstrated that the Yng1 PHD finger interacts with H3K4me3, but that truncation of the PHD finger does not result in loss of Sas3 interaction with chromatin (Martin et al. 2006a, Shi et al. 2007, Taverna et al. 2006). This led us to predict that another subunit of NuA3 interacts with H3K36me3 in a manner that is redundant with the Yng1 PHD finger. Our genetic screen supports this hypothesis demonstrating that the \textit{yng1ΔPHD} strain does not show a \textit{sas3Δ} phenotype on its own, but a \textit{sas3Δ} phenotype results with loss of \textit{yng1ΔPHD} in combination with loss of putative H3K36me3 binding domains.

Conversely our ChIP-qPCR data suggests that the interaction of Sas3 with chromatin is dependent not on \textit{either} Set1 \textit{or} Set2 but is dependent on \textit{both} Set1 \textit{and} Set2. The most likely reason for this discrepancy is the relative sensitivity of each of these assays. The \textit{gcn5Δ sas3Δ} phenotype can be rescued with very minimal amounts of either Gcn5 or Sas3. We are able to rescue this phenotype by adding \textit{SAS3} back into the strain under the control of a \textit{GAL} promoter and plating on dextrose (this should not promote transcription of \textit{SAS3})(data not shown). This “leaky expression” from the \textit{GAL} promoter is enough to fully restore growth of a \textit{gcn5Δ sas3Δ} strain; therefore, it is possible that the residual binding of Sas3 to chromatin in a \textit{yng1ΔPHD} or \textit{eaf6Δ} strain is enough rescue the synthetic lethality. ChIP-qPCR is a much more sensitive assay which allows us to quantify the amount
of Sas3 bound to chromatin; therefore, this could account for the differences seen between these two assays.

This study, in combination with other work, allows us to predict the architecture of the NuA3 complex (Figure 4.8). We have demonstrated that both Taf14 and Nto1 are required for Sas3 stability. In addition to this, we now know that while Nto2 is not required for incorporation of Eaf6, Taf14 or Yng1, Eaf6 is required for Nto2 association with NuA3. Likewise, unpublished work in our lab shows that Yng1 is required for Eaf6 association with Sas3; thus, it is also required for Nto2 association. In mammals, the MOZ and MORF complexes are closely related to NuA3 (Avvakumov and Cote 2007). Each of these complexes contain a MYST family HAT (Sas3 homolog), an ING protein (Yng1 homolog), hEaf6, and BRPF1/2/3 which contains tandem PHD fingers (like Nto1) and a PWWP domain (like Nto2) (Doyon et al. 2006).

One study, investigating the molecular architecture of MOZ and MORF, found that ING5, hEAF6 and BRPF1 form a tri-meric core where BRPF1 was found to have a role as a molecular scaffold by bridging the association of ING5 and EAF6 with MOZ or MORF (Ullah et al. 2008). This is consistent with our finding that Nto1 is required for Sas3 stability and we predict that Nto1 may have the same molecular scaffold function in NuA3. In addition to this, the PWWP domain of BRPF1, which is conserved with that of Nto2, has been demonstrated to interact with H3K36me3 (Laue et al. 2008, Vezzoli et al. 2010). If the PWWP domain of Nto2 interacts with
H3K36me3, then there is a possibility that Nto1 and Nto2 combined make up the function of BRPF1. Lastly, the function of Eaf6 is unknown in all the complexes with which it is associated. We have provided evidence here that Eaf6 helps stabilize Nto2 in the NuA3 complex; therefore, it may have a similar role in other complexes.

Overall, our study provides a possible targeting mechanism for the NuA3 complex and therefore also provides a targeting mechanism for global acetylation. The Yng1PHD finger is able to interact with H3K4me3, and the PWWP domain of Nto2 may interact with H3K36me3. Together these two proteins tether NuA3 to the transcribed regions of genes where it acetylates lysines 14 and 23.

Figure 4.8: Schematic representation of the NuA3 complex interacting with the histone H3 tail.
A visual representation of the proposed architecture of the NuA3 complex, based on the literature and novel findings reported in the current study. (?) indicates a proposed interaction that has not yet been confirmed.
CHAPTER 5. CONCLUSIONS AND DISCUSSION

My research focused on the regulation and function of histone lysine methylation in *S. cerevisiae*, particularly on methylation of lysines 4 and 36 of the histone H3 tail. Originally it was thought that methylation was a stable histone modification; however, the recent discovery of HDMs has led to several studies aimed at determining the specificity of demethylases (reviewed in (Klose et al. 2006)). In my thesis, I have presented data demonstrating that crosstalk between histone H3 acetylation and methylation is one mechanism regulating H3K4me3 demethylation (Chapter 2). We find that histone H3 acetylation is negatively regulating the demethylase activity of Jhd2, thus, preventing demethylation. My results answer several questions raised by previous studies. For example, Jhd2 is the major demethylase in yeast, yet it is unable to demethylate the majority of histones (Ingvarsdottir et al. 2007, Seward et al. 2007). Histone H3 acetylation and methylation co-localize throughout the genome, therefore, at the majority of locations throughout the genome lysine methylation is protected from demethylation by Jhd2 (Pokholok et al. 2005). This study helps further our understanding of how histone demethylation is regulated at the molecular level.

A great deal of work has also focussed on the function of lysine methylation as a binding target for other histone modifying proteins. As a result, many methyl-lysine binding domains have been identified. My studies demonstrate a function for H3K36me3 as a target for PWWP domain-containing proteins; thus, it functions to recruit the Isw1b remodelling complex and possibly the NuA3 HAT complex (Chapter
3 and 4). Taken together, my findings improve our understanding of the function and regulation of methylation on the histone H3 tail.

5.1 CHAPTER FINDINGS

In Chapter 2, we demonstrated that histone H3 lysine acetylation prevents demethylation of H3K4me3 by the HDM Jhd2. These findings support our hypothesis that crosstalk exists between lysine acetylation and methylation and that this crosstalk results in co-regulation of these two modification marks. Data from previous studies suggested that histone H3 lysine acetylation would be dependent on H3K4me3, because the two major HAT complexes specific to the histone H3 tail contain subunits which interact with H3K4me3 (Martin et al. 2006b, Pray-Grant et al. 2005, Shi et al. 2007, Taverna et al. 2006, Vermeulen et al. 2010). Chapter 2 provides evidence that the opposite is true and that H3K4me3 is in fact dependent on histone H3 acetylation. We did rule out the possibility that H3K14ac was dependent on H3K4me3, however, the histone H3 tail is acetylated at several other residues. The possibility remains that some of these other residues are dependent on H3K4me3. This study provides an explanation not only for the correlation between these two modification marks, but also for the fact that H3K4me is primarily vulnerable to demethylation following gene inactivation.

The data in Chapter 3 supports our hypothesis that the PWWP domain of loc4 is a methyl-lysine binding domain that specifically recognizes H3K36me3. Most studies of Isw1 to date have focussed on elucidating the function of this chromatin
remodeler and identifying separate roles for each of the three Isw1 complexes (Morillon et al. 2003, Mueller and Bryk 2007, Tirosh et al. 2010, Vary et al. 2003). These studies revealed that Isw1b is involved in transcription elongation and that it is localized to the 3’ ends of actively transcribed genes; however, the exact targeting mechanism of Isw1b to these regions was unknown (Morillon et al. 2003). We confirm that Ioc4 is found localized to the mid and 3’ regions of genes and that this localization is dependent not only on H3K36me3 but also the PWWP domain of Ioc4. Taken together our results suggest that Ioc4 interaction with H3K36me3 through its PWWP domain serves as a targeting mechanism for Isw1b to the mid to 3’ regions of genes.

In Chapter 4 we identified Nto2 as a reasonable candidate for interaction with H3K36me3 and elucidated some of the molecular architecture of the NuA3 complex. Previous studies suggested that the interaction of NuA3 with chromatin is dependent on both lysine 4 and 36 methylation on the histone H3 tail, leading to the hypothesis that there are redundant methyl-lysine binding proteins within the NuA3 complex (Martin et al. 2006b). The Yng1 PHD finger was already identified as an H3K4me3 interaction domain; therefore, we attempted to identify the methyl-lysine domain that interacts with H3K36me3 (Martin et al. 2006a, Shi et al. 2007, Taverna et al. 2006). Chapter 4 supports the model that the NuA3 complex requires lysines 4 and 36 for interaction with chromatin; however, unlike previous studies we find that these interactions are not redundant. Our findings suggest that the interaction of NuA3 with chromatin is a multivalent interaction requiring both H3K4 and H3K36 methylation.
Genetic and biochemical assays suggest that Nto2 is the best candidate for interaction with H3K36me and provide support for a model where Eaf6 is required for the stable association of Nto2 with the NuA3 complex.

5.2 GENERAL DISCUSSION

In Chapter 2 we demonstrate that disruption of one histone modification can lead to changes in other modifications and support our hypothesis that there is crosstalk between H3K4me3 and H3 lysine acetylation. The importance of this histone crosstalk is particularly evident in relation to observations of links between placement, reading and removing modifications in the context of human diseases, such as cancer (reviewed in (Chi et al. 2010)). In some forms of cancer, histone modifiers work together at different modification sites, regulating gene transcription states. For example, the HDM JHDM1B is overexpressed in various human leukemias leading to repression of the Ink4b-Arf-Ink4a tumour suppressor locus normally responsible for promoting cell senescence (He et al. 2008, He et al. 2011, Tzatsos et al. 2009). During oncogenesis JHDM1B is upregulated, resulting in demethylation of the active marks, H3K36me2 and H3K4me3 (He et al. 2008, He et al. 2011, Tzatsos et al. 2009). At the same time JHDM1B promotes binding of the repressor protein, Bmi1, and upregulation of EZH2 (Tzatsos et al. 2009). EZH2 tri-methylates H3K27me3, which is a repressive mark that leads to chromatin condensation and gene inactivation (Cao et al. 2002). H3K27me3 and recruitment of Bmi1 alone were not sufficient to fully silence this locus suggesting that these
modifications (H3K36me2, K4me3 and K27me3) require co-operative regulation to repress the Ink4b-Arf-Ink4a locus and promote cancerous growth (Tzatsos et al. 2009).

HDMs have been linked to human cancers as both repressors and stimulators of cancerous transformation. For example, JARID1B, a histone H3K4me2/3 demethylase, is overexpressed in both prostate and breast cancer cells and this expression is even higher in cells which have undergone metastasis (Lu et al. 1999, Xiang et al. 2007, Yamane et al. 2007). This overexpression results in demethylation and subsequent repression of tumour suppressor genes, leading to cancerous transformation (Yamane et al. 2007). Conversely, LSD1 is an H3K4me1/2 demethylase, which inhibits the invasive properties of breast cancer cells and suppresses their metastatic potential (Wang et al. 2009d). LSD1 removes H3K4me1/2 from genes required for cell growth, migration and invasion and is downregulated in cancerous mammary cells (Wang et al. 2009d).

Our study also contributes to the understanding of the function of the PWWP domain. The importance of understanding the specificity of histone PTM recognition domains is made apparent in light of two recent studies, which identified novel potential therapeutic compounds that mimic acetylated histones (Filippakopoulos et al. 2010, Nicodeme et al. 2010). Both studies identified small-molecule inhibitors of the BET family of proteins. This family consists of BRD2, BRD3, BRD4 and BRDT and is characterized by the presence of two amino-terminal bromodomains, a well
characterized acetyl-lysine binding domain (Mujtaba et al. 2007, Wu and Chiang 2007). In an aggressive form of carcinoma, BRD4 forms a fusion with the NUT protein (nuclear protein in testis) as a result of a t(15;19) translocation (French et al. 2001, French et al. 2003). This fusion results in a distinct proliferation advantage and blockage of epithelial differentiation (French et al. 2008).

Two compounds were identified that compete with acetyl-lysines for interaction with the binding pocket of the bromodomain of BRD4 (Filippakopoulos et al. 2010, Nicodeme et al. 2010). This competition resulted in displacement of BRD4 from chromatin and results in phenotypes similar to wild type cells (Filippakopoulos et al. 2010, Nicodeme et al. 2010). Studies like these that are focussed on developing inhibitors of epigenetic targets, have become a more common occurrence in biomedical studies and the potential for development of drugs, such I-BET and JQ1, demonstrates the importance of understanding the specificity of histone interaction domains (reviewed in (Cole 2008)).

Similar to histone acetylation, histone methylation is also linked to human diseases (Chi et al. 2010). Nucleoporin 98 (NUP98) is a nuclear pore complex component that has been reported to undergo fusions with a wide variety of partners in various leukemia's (reviewed in (Xu and Powers 2009)). In these leukemias the amino-terminal region of NUP98 fuses with the PHD finger of either PHF23 or JARID1A (Wang et al. 2009b). The ability of these fusion proteins to cause disease stems from the ability of the PHD finger to interact with H3K4me2/3; thus, it is
possible that future studies could be directed at finding small molecule inhibitors for this interaction (Wang et al. 2009b). As the pool of knowledge surrounding PWWP domains increases, this domain may also be found to be involved in human disease. The NuA3 homolog, MOZ, is found as a fusion with CBP (CREB-binding protein) (Borrow et al. 1996), TIF2 (Carapeti et al. 1998, Liang et al. 1998) and p300 (Chaffanet et al. 2000, Kitabayashi et al. 2001) in acute monocytic leukemia (AML). MOZ fusions result in transformation of haematopoietic progenitor cells leukemic cells and induce changes in acetylation resulting in overexpression of various genes. Chapter 3 of this study adds to the current understanding of the PWWP domain as a methyl-lysine binding domain and could assist future studies in finding small molecule inhibitors of this domain.

Eleven years ago, the histone code hypothesis was proposed. This hypothesis proposed that “distinct histone modifications, on one or more tails, act sequentially or in combination to form a ‘histone code’ that is, read by other proteins to bring about distinct downstream events” (Strahl and Allis 2000). Since the proposal of the histone code, much more information about the number, type and function of histone modifications has become available. This has resulted in a dispute over whether or not an actual code exists. Some critics suggest that the histone code is contradictory given that multiple binding partners, with diverse downstream effects, exist for a single histone PTM (Becker 2006). This however, is a simplistic view of the code where a single protein motif recognizes a single PTM.
Another primary critique of the histone code is that it states that combinatorial readout of histone modifications results in downstream events (Strahl and Allis 2000). This statement has received a great deal of criticism because, until very recently, no complex or protein that interacts simultaneously with two or more modifications had been identified. Thus, while the concept of multivalency in terms of chromatin biology had been described in theory, there was little evidence for this in practice (Ruthenburg et al. 2007). One study has been published recently that provides evidence for the multivalent hypothesis. BPTF codes for a protein that contains both a PHD finger and bromodomain. The PHD finger, which has been demonstrated to interaction with H3K4me2/3, (wysocka 2006) is followed by a bromodomain. The authors of this study found that the bromodomain is capable of interaction with histone H4 at lysines 12, 16, and 20. Furthermore, the authors were able to demonstrate that the affinity of BPTF for nucleosomes is enhanced for doubly modified nucleosomes as compared to the affinity of either the PHD finger or the bromodomain alone. In addition to this, the double interaction appears to enhance the specificity of the bromodomain for H4K16ac compared to the peptide studies alone which suggested that it had no preference for H4K16ac over H4K12 or H4K20. Taken together, this study by Ruthenburg and colleagues provides evidence for multivalent interacts and provides support for the histone code hypothesis.

In Chapter 4 we provide some evidence that NuA3 may also contain bivalent interaction domains. We find that NuA3 interaction with chromatin is enhanced when both lysine 4 and 36 are methylated as compared to either methylation mark
alone. Additionally, we find that the Yng1 PHD finger and Eaf6 (through recruitment of Nto2) together enhance the interaction capability of NuA3 with chromatin. According to the histone code, a single protein or a complex could function in combinatorial readout of histone modifications. The recent study by Ruthenburg and colleagues demonstrates the combinatorial readout at the single protein level, whereas in this study, we have demonstrated combinatorial readout at the level of a whole complex. This field of study is still in its infancy and further testing will be required to determine if NuA3 is a bona fide multivalent chromatin interaction complex.

5.3 FUTURE DIRECTIONS

One interesting observation from our studies is that hyperacetylation of the histone H3 tail prevents Jhd2 from demethylating H3K4me3. Previous studies show that mutation of lysine 14 to an alanine, arginine, or glutamine results in loss of H3K4me3 but not H3K4me1/2 (Nakanishi et al. 2008). In addition to this, lysine 14 is the preferred substrate for both Gcn5 and Sas3 dependent HAT complexes (Grant et al. 1997, Howe et al. 2001). Together with our data, it is likely that lysine 14, and not just a hyperacetylated tail, is blocking the demethylase activity of Jhd2; thus, future investigation into which specific lysine residue is required for this interaction should be considered.
Repeating the demethylase assay experiments with peptides that are acetylated at lysine 14 and methylated at lysine 4 will help determine if lysine 14 responsible for regulating Jhd2 activity. Also of interest for future investigation will be determining if lysine acetylation is regulating demethylation of other methylation marks. Currently there is no known H3K79 demethylase and very little known about demethylation of H3K36me3. Jhd1 is capable of demethylating H3K36me3 in vitro and in vivo; however, most studies find minimal, if any, changes of global H3K36me3 upon deletion or overexpression of Jhd1 (Fang et al. 2007, Kim and Buratowski 2007, Kwon and Ahn 2011, Tsukada et al. 2006). Two recent studies found that deletion of RPH1 results in marginal increases in H3K36me using ChIP at actively transcribed genes, suggesting that Rph1 may also play a role in demethylating this residue (Kim and Buratowski 2007, Kwon and Ahn 2011).

Although the study that found H3K14 was required for H3K4me3 did not find the same to be true for H3K36me (Nakanishi et al. 2008), it is possible that another modification on histone H3 or another histone is regulating H3K36me. The histone H3 tail is littered with numerous modifications and crosstalk between modifications on other tails is well documented (Bhaumik et al. 2007). It is also of interest to determine if this mechanism of action is conserved in higher eukaryotes or if this is a mechanism specific to our model system.
Our study has identified a role for the PWWP domain in *S. cerevisiae* as an H3K36me3 interaction domain in the Isw1b complex and possibly the NuA3 complex. However, both of these complexes have other protein domains with uncharacterized function. Further studies directed at the function of these protein domains will supplement our understanding of how these complexes are targeted to specific regions in the genome and their molecular architecture. The other non-catalytic subunit of the Isw1b complex, loc2, contains a PHD finger domain. Most PHD finger domains in yeast were found to interact with H3K4me; however, in my study I omitted loc2 from my investigations. loc2 is a non-canonical PHD finger which is missing two of the 7 cysteines that are required for co-ordination of the zinc ions as well as one of the tyrosines required for formation of the binding pocket (Ian Johnson, unpublished data). With this in mind it is possible that the PHD finger of loc2 is required for some other function, possibly maintaining the molecular architecture of the Isw1b complex, and is worth further investigation.

Determining if the Nto2 PWWP domain is a bona fide methyl-lysine binding domain will be of immediate interest. Future studies will first focus on the Nto2 PWWP domain, to determine if there is a direct interaction between Nto2 and H3K36me3. First, we will generate strains with point mutations in binding pocket of the PWWP domain mutant and use them in our genetic screen. If the Nto2 PWWP domain is required for NuA3 interaction with chromatin then the point mutations should not rescue the sas3Δ phenotype of an NTO2 deletion strain. Next, we will use these mutants along with a SET2 and K36R mutant to determine if Nto2 is
capable of interaction with chromatin in any of these strains. *In vitro* binding assays will help determine the preference of Nto2 for H3K36me3 over differentially modified substrates and show a direct interaction between the PWWP domain and the H3K36me3. Finally, we will also perform ChIP-qPCR experiments in an NTO2 deletion strain to establish whether or not Sas3 interaction with chromatin is dependent on Nto2.
REFERENCES


Biol 17: 1272-1278.


