A Characterization of the Role of Post-Translational Modifications in Transcriptional Regulation by the Histone Variant H2A.Z

by

Ryan Raymond Draker

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Medical Biophysics
University of Toronto

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Abstract

H2A.Z is an essential histone variant that has multiple chromosomal functions. One such role is transcriptional regulation. However, its role in this process is complex since it has been reported to function both as a repressor and activator. Earlier work in our lab showed that H2A.Z can be post-translationally modified with monoubiquitin (H2A.Zub1) and this form of H2A.Z is linked to transcriptional silencing. We further predicted that changes in the H2A.Z ubiquitylation status directly modulated its function in transcription. Furthermore, H2A.Z-containing nucleosomes possess a unique set of post-translational modifications (PTMs), compared to H2A nucleosomes, many of which are linked to transcriptional activation. The central aim of this thesis was to characterize the role of PTMs on H2A.Z nucleosomes in transcriptional regulation. To this end, I have provided the first evidence linking H2A.Z deubiquitylation to transcriptional activation. I demonstrated that ubiquitin specific protease 10 (USP10) is a deubiquitylase that targets H2A.Z in vitro and in vivo. Moreover, I found that both H2A.Z and USP10 are required for activation of androgen-receptor (AR)-regulated genes, and that USP10 regulates the levels of H2A.Zub1 at these genes. To understand how H2A.Z engages downstream effector proteins, in the nucleosome context, we used a mass spectrometry
approach to identify H2A.Z-nucleosome-interacting proteins. Many of the identified proteins contained conserved structural motifs that bind post-translationally modified histones. For example, we found that Brd2 contains tandem bromodomains that engage H2A.Z nucleosomes through acetylated H4 residues. To investigate the biological relevance of this interaction, I present evidence that Brd2 is recruited to AR-regulated genes in a manner dependent on H2A.Z and the bromodomains of Brd2. Consistent with this observation, chemical inhibition of Brd2 recruitment greatly inhibited AR-regulated gene expression. Collectively, these studies have defined how H2A.Z mediates transcriptional regulation through multiple mechanisms and pathways.
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Table of Contents

Abstract ................................................................................................................................................. ii

Acknowledgments....................................................................................................................................... iv

Table of Contents....................................................................................................................................... vi

Chapter 1 .................................................................................................................................................... 1

1 GENERAL INTRODUCTION................................................................................................................. 2

1.1 CHROMATIN STRUCTURE AND FUNCTION ................................................................................ 2

1.2 HISTONE MODIFICATIONS AND HISTONE VARIANTS ............................................................. 6

1.2.1 Histone post-translational modifications ..................................................................................... 6

1.2.1.1 Intrinsic and extrinsic mechanisms of chromatin regulation .................................................... 6

1.2.1.2 Effector-mediated mechanisms of chromatin regulation .......................................................... 10

1.2.2 The Histone Code Hypothesis: translating combinations of histone PTMs .................................. 13

1.2.3 Histone modifying enzymes ......................................................................................................... 15

1.2.4 Histone PTMs and disease ............................................................................................................ 15

1.2.5 Histone variants ............................................................................................................................. 17

1.2.5.1 Intrinsic, extrinsic, and effector-mediated mechanisms of regulation ..................................... 17

1.2.6 Histone variant H2A.Z ................................................................................................................ 21

1.2.6.1 Localization of H2A.Z within the genome ................................................................................. 24

1.2.6.1.1 H2A.Z in euchromatin, heterochromatin, and centromeric regions .................................... 24

1.2.6.1.2 Localization of H2A.Z at the 5’ ends of genes ................................................................... 28

1.2.6.2 Role of H2A.Z in the regulation of gene expression ................................................................. 29

1.2.6.3 Links between H2A.Z and epigenetic regulatory mechanisms ............................................... 31

1.2.6.3.1 Post-translational modifications of H2A.Z further define its functions ................................ 35

1.3 THESIS RATIONALE ...................................................................................................................... 38

Chapter 2 .................................................................................................................................................. 39

2 IDENTIFICATION AND CHARACTERIZATION OF USP10 AS AN H2A.Z DEUBIQUITYLASE AND A
TRANSSCRIPTIONAL CO-ACTIVATOR .................................................................................................... 40

2.1 INTRODUCTION ............................................................................................................................. 40

2.2 MATERIALS AND METHODS ........................................................................................................ 42

2.2.1 Cell culture, transfection, plasmids and antibodies ....................................................................... 42

2.2.2 Immunoprecipitation of HA-ubiquitin-conjugated proteins ........................................................ 43
2.2.3 In vitro deubiquitylation assay ..............................................................................44
2.2.4 Immunofluorescence analysis .............................................................................44
2.2.5 Small-scale biochemical fractionation ..................................................................44
2.2.6 Stable shRNA and protein expression via retroviral transduction of LNCaP cells...44
2.2.7 Luciferase assays ..................................................................................................45
2.2.8 RT-qPCR analysis ...............................................................................................45
2.2.9 Chromatin immunoprecipitation (ChIP) and sequential ChIP assays ..............46

2.3 RESULTS ..................................................................................................................48
2.3.1 Detection of monoubiquitylated histones ............................................................48
2.3.2 USP10 specifically deubiquitylates H2A.Zub1 and H2Aub1 .............................49
2.3.3 USP10 is localized to both the cytoplasm and nucleus .....................................54
2.3.4 USP10 and H2A.Z regulate AR-mediated transcription .....................................57

2.4 DISCUSSION .............................................................................................................68

Chapter 3 .......................................................................................................................73

3 CHARACTERIZATION OF BRD2 AS A TRANSCRIPTIONAL CO-ACTIVATOR THAT ENGAGES H2A.Z NUCLEOSOMES .................................................................74

3.1 INTRODUCTION ......................................................................................................74

3.2 MATERIALS AND METHODS ..................................................................................77
3.2.1 Cell culture, reagents, plasmids, and antibodies ..................................................77
3.2.2 Mononucleosome immunoprecipitation ..............................................................78
3.2.3 Mass spectrometry analysis and proteins identification .....................................79
3.2.4 Peptide competition assays ................................................................................80
3.2.5 Stable knockdown of H2A.Z in LNCaP cells ......................................................80
3.2.6 RT-qPCR analysis and Chromatin immunoprecipitation (ChIP) assays ...........80

3.3 RESULTS ..................................................................................................................81
3.3.1 Identifying proteins that interact with H2A.Z nucleosomes .................................81
3.3.2 Brd2 engages H2A.Z nucleosomes through the recognition of acetylated H4 residues by its bromodomains ..........................................................83
3.3.3 Brd2 is recruited to AR-regulated genes in a manner dependent on H2A.Z and the bromodomains of Brd2 .........................................................................92

3.4 DISCUSSION .............................................................................................................103

Chapter 4 .......................................................................................................................111
4  **CONCLUSIONS AND FUTURE DIRECTIONS**................................................................................. 112

4.1 **THESIS SUMMARY** .................................................................................................................. 112

4.2 **GENERAL DISCUSSION AND FUTURE DIRECTIONS** ............................................................. 114

  4.2.1 Characterizing a role of H2A.Zub1 in transcriptional regulation .................................................... 114

  4.2.2 Utility of an H2A.Zub1-specific antibody ...................................................................................... 116

  4.2.3 Identifying novel E3 ligases and DUBs ......................................................................................... 118

  4.2.4 Defining an H2A.Z ‘code’ ........................................................................................................... 119

  4.2.5 Mechanisms of transcriptional regulation by H2A.Z at androgen-responsive genes . 122

4.3 **CLOSING REMARKS** ............................................................................................................... 123

Appendix I ........................................................................................................................................ 126

References ........................................................................................................................................ 130
List of Figures

Figure 1-1. Basic structure of the nucleosome ................................................................. 3
Figure 1-2. Common histone post-translational modifications (PTMs)............................... 9
Figure 1-3. Amino acid alignment between H2A and H2A.Z............................................. 23
Figure 1-4. Amino acid sequence alignment of H2A.Z from different organisms............... 26
Figure 1-5. Functional states of H2A.Z are associated with distinct PTMs.......................... 37
Figure 2-1. Detection of mono-ubiquitylated H2A.Z and H2A by Western blot.................. 50
Figure 2-2. Expression of USP10 causes a reduction in H2A.Zub1 and H2Aub1 levels......... 51
Figure 2-3. USP10 deubiquitylates H2A.Zub1 and H2Aub1 in vitro .................................... 53
Figure 2-4. USP10 localizes to the cytoplasm and nucleus............................................... 56
Figure 2-5. USP10 acts as a co-activator of AR in luciferase-based assays....................... 59
Figure 2-6. USP10 and H2A.Z are required for full expression of androgen-responsive genes 61
Figure 2-7. H2A.Z localizes to androgen response elements at AR-regulated genes .......... 63
Figure 3-1. Nucleosome IP/Mass spectrometry approach and analysis of H2A.Z-nucleosome interacting proteins .................................................................................. 82
Figure 3-2. Validation of H2A.Z-nucleosome-interacting proteins ................................... 84
Figure 3-3. Brd2 binds to H2A.Z nucleosomes through the recognition of acetylated H4...... 88
Figure 3-4. H2A.Z nucleosomes are enriched with H3 and H4 acetylation, compared to H2A nucleosomes .................................................................................................... 90
Figure 3-5. Brd2 is recruited to the PSA gene in androgen-treated cells ............................ 94
Figure 3-6. H2A.Z influences H4 acetylation and Brd2 recruitment at AR-regulated genes ... 97
Figure 3-7. JQ1 interferes with the interaction between Brd2 and H2A.Z nucleosomes, and inhibits expression of AR-regulated genes................................................................. 99

Figure 3-8. JQ1 treatment of LNCaP cells reduces androgen-stimulated recruitment of Brd2 and H4 acetylation at AR-regulated genes................................................................. 101

Figure 3-9. Brd4 is recruited to AR-regulated genes in an H2A.Z-dependent manner......... 102

Figure 3-10. A model of Brd2 recruitment to AR-regulated genes........................................ 109

Figure 4-1. Model of H2A.Z’s regulation of androgen-responsive genes.......................... 124
List of Appendices

Appendix I.................................................................................................................................................. 134

Identification of Ring1b as an H2A.Z E3 Ubiquitin Ligase........................................................................ 134
Chapter 1

General Introduction
1 GENERAL INTRODUCTION

1.1 CHROMATIN STRUCTURE AND FUNCTION

All cells within the human body, with few exceptions, contain a full complement of genetic material, which is roughly $3.0 \times 10^9$ base pairs (bp) of DNA and contains approximately 20,000 – 25,000 protein-coding genes. If stretched end to end this molecule would measure approximately 2 meters, and yet is able to fit all within a nucleus that is 1 million times smaller in diameter. The cell achieves this feat of engineering by packaging the DNA into chromatin through the intimate association of DNA with histone proteins. The basic structure of chromatin consists of approximately 150bp of DNA wrapped around a histone octamer in a left-handed supercoil making roughly 1.7 turns around the octamer. The histone octamer consists of two copies each of the four core histones (H2A, H2B, H3 and H4) in the form of an $(H3-H4)_2$ tetramer flanked by two H2A-H2B dimers. A linker histone, H1, can also bind the nucleosome at the DNA entry/exit point. This fundamental unit of chromatin is referred to as the nucleosome, and is depicted in Figure 1-1. By packaging the DNA into chromatin, the cell can achieve various levels of compaction to balance its structural needs with functional requirements.

It was not until the discovery of its basic structure by Watson and Crick in 1964, that DNA was recognized, and then studied, as the medium for the cell’s genetic information and heritability. However, even before this discovery, it was not until the middle of the twentieth century that biologists recognized the commonality of genetics and developmental biology. One such biologist was Conrad Waddington, who, in 1942 coined the term ‘epigenetics’ (Waddington, 1942). The term ‘epigenetics’ was used by Waddington to describe the causal mechanisms of development. This included classical genetics, as well as the general process that occurs during
Figure 1-1. **Basic structure of the nucleosome.** A cartoon version of the basic structure of the nucleosome, showing the two H2A-H2B dimers in blue, and the \((\text{H3-H4})_2\) tetramer in orange, which together comprise the histone octamer. Approximately 150bp of DNA wrap around the histone octamer in a left-handed helix of roughly 1.7 turns. Also shown are the N-terminal tails of the histones, which are unstructured and protrude from the surface of the nucleosome. Histone H1 binds the nucleosome at the DNA entry/exit point.
the course of a developmental program. This latter process, which culminates in a mature organism, was also known as epigenesis (Choudhuri, 2011). Following the work by Waddington, scientists continued to use the term epigenetics; however, the term evolved to describe observations that were not easily explained by genetics, but nevertheless had a heritable component (Holliday, 2006). But, a major question in developmental biology remained: How does a single-cell embryo develop into a multi-cellular animal, such as a human being, which has over 200 different cell types? It is now clear that the execution of specific genetic programs dictate a cell’s morphology and function, and hence its specific identity. Indeed, a cell’s identity is determined by its unique pattern of gene expression: For example, despite having the same complement of genes, a heart cell expresses a unique combination of genes compared to a skin cell. Therein, the cell faces another dilemma with respect to its genome: How does each cell type maintain a unique pattern of gene expression and gene silencing? Research over the last half century has revealed that the cell organizes its chromatin into distinct physiological structures, or states, to dictate and maintain patterns of cell-type-specific gene expression and silencing. These chromatin states are heritable, and, therefore, once a cell has been committed to a particular developmental fate, that unique pattern of gene expression will be stably maintained. Insofar as the physiological structure of chromatin carries important phenotypic information not encoded in the DNA sequence, chromatin structure fits the criteria of epigenetic phenomenon (Ruthenburg et al., 2007).

As a basic mechanism of transcriptional regulation, it is generally accepted that the overall structure of chromatin can affect the accessibility of DNA-binding proteins that participate in the transcriptional activation process (Campos and Reinberg, 2009). Through the modulation of inter-nucleosomal contacts and higher-order folding, chromatin can either assume a highly condensed structure known as heterochromatin, which is inaccessible to factors that bind DNA,
or relatively uncondensed structure referred to as *euchromatin*, which is more accessible to nuclear factors. Heterochromatin can be further subdivided into *facultative heterochromatin* and *constitutive heterochromatin*. Facultative heterochromatin can interconvert between euchromatic and heterochromatic states, and often comprises genes that are expressed during development or differentiation and then become silenced. In contrast, constitutive heterochromatin maintains a condensed form, comprising permanently silenced genes and repetitive DNA elements, and is localized to centromeres and telomeres (Trojer and Reinberg, 2007).

Interestingly, studies reported as early as 1925 identified a link between the transcriptional activity of a gene and its position within the chromosome (Sturtevant, 1925). However, it was the pioneering work by H. J. Muller on radiation-induced chromosomal translocations that formally described the phenomenon of position effect variegation (PEV) (Muller, 1930; Muller, 1932). In these studies of the fruit fly *Drosophila melanogaster*, Muller observed that mutant flies exhibited a “mottled” eye pigmentation phenotype. This was in contrast to the wild type eyes, which are solid red in colour. Importantly, Muller also observed that the appearance of the variegated eye colour correlated with specific chromosomal rearrangements of the *Drosophila* eye pigment gene, *white*. It is now known that the PEV phenotypic effects observed in those early studies results from a chromosomal break that places the affected gene in close proximity to heterochromatin, leading to silencing of the gene. Indeed, it has since been shown that the cause of PEV is the relocation of a euchromatic gene to a region adjacent to heterochromatin. The variegated phenotype results from the variability in the spreading of the heterochromatic structure into the euchromatic gene, causing the gene to be silenced in some cells, but expressed in others (Girton and Johansen, 2008). PEV therefore provides an elegant example of how chromatin structure contributes to epigenetic regulation of gene expression.
1.2 Histone Modifications and Histone Variants

Although originally believed to be a static physical structure, it is now clear that chromatin is highly dynamic in nature, which is of key importance in its regulation of DNA-templated processes. Given the importance of chromatin structure in the epigenetic regulation of transcription, the mechanisms governing this process have been the focus of intense research within the field of chromatin biology. Chromatin structure and function can be regulated through three general mechanistic pathways: intrinsic, extrinsic, and effector-mediated (Campos and Reinberg, 2009; Ruthenburg et al., 2007). Intrinsic mechanisms directly alter the physical properties of the nucleosome, including DNA contacts, mobility, conformation, and stability. Extrinsic mechanisms act through the alteration of inter-nucleosome contacts and chromatin structure. Finally, effector-mediated mechanisms act via the recruitment of chromatin-binding proteins, termed effectors, which can directly or indirectly alter the physical properties of the chromatin fibre. Regulation of chromatin through these pathways can be mediated through the addition of post-translation modifications (PTMs) to histone proteins, and/or the incorporation of histone variants, which will be discussed in turn.

1.2.1 Histone post-translational modifications

1.2.1.1 Intrinsic and extrinsic mechanisms of chromatin regulation

The crystal structure of the nucleosome was a significant achievement in understanding the interactions that hold the nucleosome core particle together, and how these interactions influence the formation of higher order structures (Luger et al., 1997). The histone octamer is held together and stabilized by various protein-protein interactions, mainly through contacts between H3 and H3 within the (H3-H4)$_2$ tetramer, and H3-H2B contacts between the tetramer and the H2A-H2B dimers. Given the highly basic nature of histone proteins, the octamer interacts with the negatively charged phosphate backbone of DNA through electrostatic forces
and hydrogen bonding, as well as through non-polar contacts with the sugar groups. Interestingly, the amino-terminal tails of histone proteins, which contain many highly basic amino acids, are unstructured and extend out past the DNA double helix (see Figure 1-1). The histone tails are thought to also contribute to the formation of higher order structures since they can make contacts with neighbouring nucleosomes. For example, residues 16-25 of H4 make extensive contact with the H2A-H2B dimer of the adjacent nucleosome (Luger et al., 1997). Indeed, as the eukaryotic genome became larger and more complex throughout evolution, it is believed that the addition of tails to the histone proteins (archaeal histones lack tails) was an important development in allowing for greater compaction, and improving regulation by impeding access to the underlying DNA sequences (Malik and Henikoff, 2003). Thus, the ability to modulate the various protein-protein and protein-DNA interactions is a crucial aspect of chromatin regulation and metabolism.

It is now a generally accepted concept in chromatin biology that histone post-translation modifications participate in the regulation of chromatin structure and function. In fact, we now know that histone PTMs can participate in the regulation of chromatin through all three pathways described above: intrinsic, extrinsic, and effector-mediated (Campos and Reinberg, 2009; Ruthenburg et al., 2007). For almost 50 years it has been known that histone proteins possess PTMs. Furthermore, the initial pioneering studies also made the crucial observation that certain modifications correlated with increased transcription, suggesting a causal relationship between histone PTMs and the functional state of chromatin (Allfrey, Faulkner, and Mirsky, 1964; Allfrey and Mirsky, 1964). Although the original studies by Allfrey et al. described the presence of methy and acetyl groups, histones are also commonly modified by phosphorylation, ubiquitylation, sumoylation, and ADP-ribosylation. The majority of the modification sites are concentrated in the extended N-terminal tails; see Figure 1-2 for some common examples. For
instance, of first 30 amino acids of H3, 16 residues are known sites of modification. In addition, some modifications also occur on the C-terminal tails as well as within the central globular domains.

So how do these PTMs regulate chromatin structure and function? Fundamentally, the addition of PTMs can directly perturb chromatin structure, acting through the intrinsic and extrinsic pathways, affecting histone-DNA interactions, and/or altering inter-nucleosome interactions, respectively (Bannister and Kouzarides, 2011; Campos and Reinberg, 2009; Ruthenburg et al., 2007). These changes can occur by the PTM directly altering the net charge of histones, thereby contributing to the stability of the nucleosome and higher-order chromatin structures. For example, both acetylation and phosphorylation can effectively reduce the net positive charge of histones, resulting in a disruption of histone-histone and/or histone-DNA interactions, which ultimately can lead to a less compact chromatin structure. For example, it has long been know that hyper-acetylated chromatin is more accessible to endonuclease digestion, suggesting it has a more relaxed conformation (Simpson, 1978). In particular, lysine 16 of H4 (H4K16), which is part of a short basic patch of H4 involved in inter-nucleosomal interaction via binding to H2A in the neighbouring nucleosome, has been shown to be of critical importance in chromatin compaction: Acetylation of H4K16 (H4K16ac) directly prevented compaction of nucleosomal arrays in vitro (Shogren-Knaak et al., 2006). Similar to phosphorylation, ADP-ribosylation may also affect chromatin structure through its negative charge, and has accordingly been associated with relaxed chromatin states (Hassa et al., 2006). In contrast, ubiquitin, unlike other PTMs that are small chemical modifications, is a 76-amino acid polypeptide and is a relatively large addition to the histone proteins (similarly true for sumoylation). Although monoubiquitylation (ub1) has been observed on all four of the core histones as well as H1, H2Aub1 and H2Bub1 are the most abundant in higher eukaryotes (Jason et al., 2002). Given its relative size, it was
Figure 1-2. **Common histone post-translational modifications (PTMs).** Shown are the N-terminal tails of the four core histones with sites of known common modifications marked with representative symbols. Lysine residues that can be either acetylated or methylated are shown with both symbols to indicate this fact. Residues numbers are shown below each sequence.
commonly believed that the addition of ubiquitin to histones would impact overall chromatin structure. However, this point is moot. Whereas several studies have noted decreased levels of ubiquitylated histones in the highly compacted metaphase chromosomes, suggesting that this PTM inhibits higher-order chromatin compaction, the effect of histone ubiquitylation in *in vitro* assays of chromatin assembly suggest it does not impact higher-order folding or assembly (Jason et al., 2005; Jason et al., 2002). Although, histone ubiquitylation may destabilize individual nucleosomes, it has been reported that H2Aub1 and H2Bub1 can destabilize the H2A-H2B dimer, weakening its association with the H3-H4 tetramer (Lan et al., 2012; Li et al., 1993). Therefore, the contribution of the ubiquitin moiety to overall chromatin structure may be complex in nature. Interestingly, histone ubiquitylation is also associated with opposing functional states of chromatin. Although both H2A and H2B can be monoubiquitylated, H2Aub1 is a well-characterized mark of transcriptional repression, mediated by polycomb group proteins (de Napoles et al., 2004; Fang et al., 2004; Wang et al., 2004), whereas H2Bub1 is a well-established PTM known to be required for transcriptional activation and elongation (Berger, 2007; Shilatifard, 2006; Zhang, 2003). Although the addition of ubiquitin to histones may affect impact chromatin structure, a more important aspect dictating its function in chromatin metabolism may involve the effector-mediated pathway, participating in the recruitment of chromatin-binding proteins (Jason et al., 2002).

### 1.2.1.2 Effector-mediated mechanisms of chromatin regulation

Aside from directly altering the structure of chromatin, the unique chemical and physical properties of PTMs impart the nucleosome with a unique surface, which can be specifically recognized by protein domains. Moreover, the presence of the PTMs on the extended N-terminal tails (or C-terminal tail in the case of ubiquitylation) make ideal binding platforms by
which proteins can engage chromatin. The binding domains that recognize histone acetylation, methylation and phosphorylation have been best characterized to date. Methylated lysines and arginines can be recognized by proteins containing domains from the Royal family (chromo, MBT, tudor, and PWWP) and the non-related PHD domain, acetylated lysines can be recognized by bromodomains, and phosphorylated serine residues can be recognized by a domain within 14-3-3 proteins and the BRCT domain of MDC1 (Kouzarides, 2007; Yun et al., 2011). Given the large size of the ubiquitin moiety, and the fact that most ubiquitin binding domains recognize the surface of ubiquitin or the C-terminal region where ubiquitin is attached to the target protein, it has been more difficult to clearly identify proteins that specifically recognize ubiquitylated histones (Yun et al., 2011). However, a recent study identified the ZRF1 protein in a screen for H2Aub1-binding proteins and reported that ZRF1 recognizes H2Aub1 through a novel ubiquitin-binding domain located in the zoutin domain of the protein (Richly et al., 2010). Furthermore, ubiquitylated histones can influence the recruitment of chromatin-binding proteins. For example, H2Aub1 is known to be required for the recruitment of the poly-ubiquitin E3 ligase RNF168 in the DNA damage response pathway (Doil et al., 2009; Stewart et al., 2009), and H2Bub1 is required for incorporation of the Cps35 subunit of the COMPASS methyltransferase complex (Lee et al., 2007; Zheng, Wyrick, and Reese, 2010).

The effector-mediated pathway is thus named due to the fact that chromatin-binding proteins, or effectors, mediate downstream effects on the chromatin template. These proteins are referred to as ‘readers’ since their recruitment to chromatin often depends on the recognition of a specific PTM or combination of PTMs. There are four basic types of effectors that are recruited by histone PTMs: chromatin architectural proteins, chromatin remodeling proteins/complexes, chromatin-modifying enzymes, and finally, additional protein co-factors involved in chromatin metabolism (Yun et al., 2011). Recruitment of architectural proteins can directly affect
chromatin structure. For example, heterochromatin protein 1 (HP1), which recognizes, or ‘reads’, tri-methylated H3K9 (H3K9me3), assists in the formation of higher-order chromatin structures by cross-linking adjacent nucleosomes (Bannister et al., 2001; Lachner et al., 2001). Chromatin remodelers can also have a direct impact on local chromatin structure by using ATP hydrolysis to mobilize nucleosomes or make DNA more accessible through nucleosome eviction (Clapier and Cairns, 2009). These complexes are often targeted to specific regions of chromatin—gene promoters, for example—through the recognition of distinct PTMs. The remodeling complex NURF, for example, contains a subunit, BPTF, which binds to H3K4me3 and H4K16ac through its PHD and bromodomains, respectively (Ruthenburg et al., 2011). Certain histone PTMs are also known to influence the deposition, or removal, of other PTMs through the recruitment of enzymes with histone PTM activity. The enzymatic activity of the enzyme can occur in cis, where the reaction takes place on the same tail to which the enzyme was recruited, or it can occur in trans, acting on a different histone tail. For example, the histone demethylase, JMJD2A, which demethylates H3K9me3 and H3K36me3, is targeted to chromatin by its recognition of H3K4me3 via its tudor domain (Huang et al., 2006b; Klose et al., 2006; Whetstine et al., 2006). Recruitment of additional protein co-factors involves general factors often required for the various DNA-templated processes of transcription, DNA damage repair, recombination, RNA processing, and replication, and may or may not have inherent enzymatic activity (Yun et al., 2011). The general transcription factor TFIID, for example, binds to gene promoters through the recognition of acetylated lysines and H3K4me3, which then facilitates the recruitment of RNA polymerase II (Lee and Young, 2000; Vermeulen et al., 2010; Vermeulen et al., 2007). Through the recruitment of these four different types of effectors, histone PTMs can contribute to the regulation of various DNA-templated processes.
1.2.2 The Histone Code Hypothesis: translating combinations of histone PTMs

Although many histone modifications have been studied in isolation, and linked to specific functional outcomes, research over the last decade has revealed that biology is much more complex. For instance, certain modifications can influence one another, in either a positive or negative manner. This crosstalk between histone PTMs was first described for H3 phosphorylation of serine 10 (H3S10p) and acetylation of H3K14 (Cheung et al., 2000; Lo et al., 2000). Not only do these marks increase at gene promoters upon transcriptional activation, the enzyme that adds the acetyl mark to H3K14 (Gcn5) prefers H3 tails that contain the S10p modification. Therefore, in addition to histone PTMs specifically recruiting histone modifying enzymes, certain histone PTMs can affect the histone tail, as a substrate, in a more general way. Many additional combinations of histone PTMs have since been identified and have been linked to specific functional outcomes. An important conceptual development arising from these observations was the idea that histone modifications are endpoints of cellular signaling pathways that begin on the surface of the cell and ultimately converge on chromatin through the addition and/or removal of histone PTMs. Together, combinations of PTMs lead to specific functional outcomes, e.g. transcription (Cheung, Allis, and Sassone-Corsi, 2000). By integrating the chromatin template into cellular signaling pathways, it is now appreciated that the dynamic regulation of chromatin is a crucial step in the cell’s ability to respond to external stimuli. Integrating chromatin into cellular signaling pathways also provided a mechanistic link between histone PTMs and the epigenetic regulation of gene expression (Turner, 2000). These concepts led to the formal proposal of the Histone Code Hypothesis, which predicted that combinations of histone PTMs—acting in direct combination or in sequential fashion—specify distinct chromatin structures, and hence resulted in specific functional outcomes and epigenetic states (Jenuwein and Allis, 2001; Strahl and Allis, 2000). Since its publication, there has been
debate within the field as to whether combinations of histone PTMs actually specify a ‘code’ (Turner, 2007). Despite debate rooted in semantics, the predictions of the Histone Code Hypothesis provided testable explanations as to why certain histone PTMs are associated with different functional outcomes. Furthermore, the large number of possible combinations of different histone PTMs would add an exquisite level of information beyond that encoded in the DNA sequence alone (Jenuwein and Allis, 2001).

In support of the hypothesis, a recent study used genome-wide occupancy data of 38 different histone methylation and acetylation marks in human CD4 T-cells to define 51 distinct chromatin states based on combinations of the various marks (Ernst and Kellis, 2010). Each of the states showed specific enrichments in functional annotations and sequence motifs, suggesting that the different states correspond to distinct biological roles. Characterizing unique combinations of histone PTMs—and their cognate effectors—may prove to be a powerful tool for classifying genes. Analogous to how cell surface marker expression is used by immunologists to classify subpopulations of lymphocytes, combinations of PTMs could be used to similarly classify genes based on cell type and stage of differentiation (Tarakhovsky, 2010). Moreover, it has been observed that many chromatin-binding proteins contain multiple PTM recognition domains in tandem, suggesting that effector proteins that engage chromatin can do so in a multivalent manner (Ruthenburg et al., 2007). An elegant example of this concept was demonstrated for BPTF, which contains a PHD finger domain and a bromodomain. Although it was previously known that BPTF could bind H3K4me2/3 through its PHD finger, Ruthenburg et al. showed that the bromodomain can also recognize acetylated lysine residues on H4, and more importantly, the two domains are used simultaneously to engage mononucleosome substrates (Ruthenburg et al., 2011). Moreover, the specificity of the bromodomain increases on the mononucleosome substrate. Whereas the bromodomain can recognize several different acetylated residues on H4
peptides, it is specific for H4K16ac on mononucleosomal substrates. Therefore, BPTF is able to recognize a trans-histone modification pattern through the use of tandem domains. Many other examples of multivalent engagement have since been reported (Agricola et al., 2011; Eustermann et al., 2011; Nady et al., 2011), highlighting the prevalence of this phenomenon, and providing further support for the predictions of the Histone Code Hypothesis.

1.2.3 Histone modifying enzymes

With the popularity of the Histone Code Hypothesis, enzymes that catalyze the addition and removal of histone PTMs are now appreciated as key regulators of chromatin function. Indeed, the functionally opposing enzymes are often referred to as ‘writers’ and ‘erasers’, which dynamically regulate the PTM levels on the chromatin fibre. For example, histone acetylation is regulated by the opposing actions of histone acetyltransferases (HATs) and histone deacetylases (HDACs); phosphorylation is regulated by the activity of kinases and phosphatases; methylation by methyltransferases and demethylases; and ubiquitylation by E3 ligases and deubiquitylases. The precise regulation of these enzymes can dictate whether histone PTMs will contribute to short-term outcomes (ongoing functions) or long-term outcomes (heritable). In cellular signaling events, rapid responses to environmental stimuli would necessitate a high turnover rate of histone PTMs, whereas defining and maintaining chromatin structures, such as constitutive heterochromatin, throughout the cell cycle or from one cell generation to the next, would not require the PTMs to remain dynamic (Turner, 2007).

1.2.4 Histone PTMs and disease

The importance of epigenetics in normal development and cellular homeostasis is highlighted by the observation that alterations in the levels of histone PTMs and the expression or activity levels of histone modifying enzymes are often altered in human diseases, including: cancer,
neurological disorders, and autoimmune diseases (Portela and Esteller, 2010). For instance, spatial or temporal alterations of epigenetic marks can lead to the development of disease. Genetic mutations, or non-genetic causes such as exposure to certain environmental factors, including exposure to asbestos, tobacco, and heavy metals, can contribute to these epigenetic alterations. Indeed, interest in characterizing the histone modifications across the entire genome from several different cell types, including cancer cells, led to the formation of an international task force, Alliance for the Human Epigenome and Disease (AHEAD) (American Association for Cancer Research Human Epigenome Task Force; European Union, 2008).

The links between epigenetics and disease are best exemplified by cancer oncogenes. Although it is unclear if alterations of histone PTM levels are causative or upstream of cancer initiation, it is clear that the disease state results in perturbations of these marks. Therefore, comparison of PTMs in diseased versus normal cells may be of significant clinical value. For instance, the clinical outcome for most cancers is based on tumor stage and degree of spread from the site of the primary tumor; however, additional predictive information can be gained by tumor grade, histological type, and patient demographics (Kurdistani, 2011). Recent work studying changes in histone PTM levels has provided additional prognostic information beyond what is known using the current predictive factors. For example, examination of the global levels of H3K4me2, H3K9ac, H3K18ac, H4R3me2, and H4K12ac in primary prostate tumors defined two groups of patients with significantly different clinical outcomes: Patients with lower global levels of the histone PTMs had a poorer prognosis and increased risk of tumor recurrence after removal of the primary tumor compared to patients with higher relative PTM levels (Seligson et al., 2005). The prognostic utility of global histone modifications has since been validated in cancers of the breast (Elsheikh et al., 2009), pancreas (Wei et al., 2008), prostate (Ellinger et al., 2010; Seligson et al., 2009), ovary (Wei et al., 2008), lung (Barlesi et al., 2007; Seligson et al.,...
2009; Van Den Broeck et al., 2008), and squamous cell carcinoma of the esophagus (Tzao et al., 2009). While a global reduction in histone modifications is generally associated with a poorer clinical outcome, there is also evidence that individual marks may contribute differentially to the disease state. For instance, levels of H3K18ac was identified as an independent prognosticator that correlated with survival time, and H4K16ac, being low in the majority of tumors, was hypothesized to be an early event in the pathogenesis of invasive breast cancer (Elsheikh et al., 2009). Changes in H4K16ac levels were also hypothesized to be an early event in a mouse model of skin cancer (Fraga et al., 2005).

Since the levels of various histone PTMs are altered in cancer cells, it is not surprising that histone-modifying enzymes are affected as well. For example, HDACs are overexpressed or mutated in different cancer types and the H3K27 methyltransferase EZH2 is also frequently overexpressed in various cancers (Chase and Cross, 2011; Portela and Esteller, 2010). In other instances, histone-modifying enzymes are mutated through chromosomal translocation events. Translocation of the H3K4 methyltransferase MLL is found within 10% of human leukemias, involving 50 different fusion partners (Krivtsov and Armstrong, 2007). The resulting fusion product lacks the methyltransferase activity, but is also often aberrantly linked to other chromatin modifying enzymes such as HATs. Ultimately, the altered activity of the fusion product can lead to modified expression patterns of MLL target genes, and likely contributes to disease initiation and progression.

1.2.5 **Histone variants**

1.2.5.1 **Intrinsic, extrinsic, and effector-mediated mechanisms of regulation**

In addition to histone PTMs, the intrinsic and extrinsic properties of the nucleosome can be modified through the replacement of canonical histones with histone variants. Histone variants
differ in their primary amino acid sequence from their canonical counterparts, thereby imparting different structural properties to the nucleosome, and ultimately, different functional outcomes. In general, the canonical, or ‘core’ histones H2A, H2B, H3, and H4 serve the basic functions of DNA packaging and general chromatin-mediated regulation of the underlying DNA. In contrast, histone variants have evolved specialized roles in a range of processes such as DNA repair, meiotic recombination, chromosome segregation, transcription initiation and termination, sex chromosome condensation, and sperm chromatin packaging (Talbert and Henikoff, 2010). The difference in function between the core histones and histone variants is even apparent in the organization and expression of their respective genes: In humans and other animals, each core histone protein is encoded by multiple gene copies, which are clustered into repeat arrays and their expression is tightly coupled to DNA replication during S phase of the cell cycle. In addition, core histone genes lack introns, and their mRNAs are devoid of poly-A tails. In contrast, histone variant genes are usually found singly within the genome and are often constitutively expressed, reflecting their dynamic involvement in the various processes occurring outside of DNA replication and re-packaging of chromatin. Furthermore, histone variant genes contain introns and their mRNAs are poly-adenylated.

Amongst the four core histones, a significant amount of heterogeneity is evident within H3 and H2A. The diversity of histone variants is reflective of their specialized roles that have evolved beyond the functions of the canonical histones. Some variants are considered ‘universal’, being found in nearly all eukaryotic species, likely participating in an evolutionarily conserved cellular function. Conversely, some variants are lineage-specific, representing a need for a distinct function that evolved due to the expanding size and complexity of the eukaryotic genome (Talbert and Henikoff, 2010).
In replacement of the canonical H3, variants H3.3 and CENP-A can be incorporated into the nucleosomes, each with its own unique effect on chromatin structure and function. H3.3 is most similar to canonical H3, differing in only four amino acids. Despite the small number of differences in the primary amino acid sequence, incorporation of H3.3 significantly alters the stability of the nucleosome. H3.3-containing nucleosomes are reported to be less stable than those containing canonical H3 (Jin and Felsenfeld, 2007; Jin et al., 2009) and the variant protein itself is rapidly turned over (Schwartz and Ahmad, 2005). These properties likely dictate H3.3’s function in transcription. H3.3 is located in actively transcribing genes, located through the gene body, promoter, and other regulatory elements (Henikoff, 2008). Not surprisingly, H3.3 is enriched with PTMs associated with active transcription, such as H3K4me2/3, H3K9ac, and K3K14ac (Wirbelauer, Bell, and Schubeler, 2005).

A critical event during the cell cycle is the equal division of newly replicated DNA into two new daughter cells. This event occurs through the action of microtubules, which pull sister chromatids apart, the end result being a full genome complement in each of the new daughter cells. Attachment of the microtubules to the sister chromatids occurs through the kinetochore, a specialized protein complex which assembles at the centromere of each sister chromatid. The centromere itself is a specialized heterochromatic structure and contains the H3 variant, CENP-A. CENP-A differs from H3 mainly in its N-terminus, the result of which reduces its structural flexibility compared to H3. The incorporation of CENP-A, and the resulting unique structure imparted to the chromatin, is critical for proper assembly and formation of the kinetochore (Black and Bassett, 2008).

Several variants of H2A exist in eukaryotes, most notably H2A.X, H2A.Bbd, macroH2A, and H2A.Z. H2A.X is present in nearly all species, and, in fact, it is the main H2A variant in yeast.
The near ubiquitous presence of H2A.X is perhaps reflective of its crucial role in the DNA damage response pathway. H2A.X is randomly incorporated throughout the genome and does not appear to result in any major structural changes. However, although the globular domain of H2A.X is similar to that of H2A, it is distinguished by a sequence motif in its C-terminal tail (Ser-Gln-Glu/Asp-Φ), where Φ represents a hydrophobic residue. The critical function of this motif lies in the ability of the serine residue to be targeted for phosphorylation on nucleosomes that reside near the sites of double-strand breaks. The phosphorylated form of H2A.X is known as γH2A.X. Phosphorylation promotes the recruitment and retention of DNA repair proteins, histone modifying enzymes, and chromatin remodeling complexes required for repair of the double-strand break (Talbert and Henikoff, 2010).

H2A.Bbd is unique to mammals and its incorporation results in unique intrinsic and extrinsic effects on the chromatin fibre. The primary amino acid sequence of H2A.Bbd differs substantially from H2A, with only 48% identity between the two proteins. H2A.Bbd lacks most of the C-terminal tail region, which contains the site of ubiquitylation in H2A, it is missing 13 of the 14 lysine residues found in H2A, and has a much smaller acidic patch (a conserved region of H2A containing acidic protein residues). These differences translate into the H2A.Bbd-containing nucleosome being less stable than nucleosomes containing the canonical H2A. First, the H2A.Bbd-H2B dimer loosely docks onto the (H3-H4)$_2$ tetramer and only organizes approximately 118bp of DNA around the octamer. Second, due to the reduction in size of the acidic patch, which is critical for interaction with neighbouring H4 tails, the formation of higher-order chromatin structures are inhibited (Bao et al., 2004). Given these properties, H2A.Bbd is generally associated with more accessible chromatin. It is, in fact, excluded from the highly condensed heterochromatin of the inactivated copy of the X chromosome in female
cells (the bar body), an observation that led to the appellation, bar body deficient (Bbd). However, its precise function in chromatin is not completely understood.

Like H2A.Bbd, macroH2A (mH2A) is a lineage-specific variant, present mainly in vertebrate species (Talbert and Henikoff, 2010). Although its N-terminal one third is roughly 65% identical to canonical H2A, its main difference from H2A is in the large C-terminal tail of approximately 200 residues, making it nearly three-times the size of H2A. Structurally, incorporation of mH2A leads to a stabilization of the mH2A-H2B dimer with the (H3-H4)$_2$ tetramer (Chakravarthy et al., 2005). This difference causes the mH2A-containing nucleosome to be more refractory to chromatin remodeling complexes. For example, the human SWI/SNF remodeling complex has a nine-fold preference for canonical nucleosomes as substrates, over those containing mH2A (Chang et al., 2008). Additionally, the extended C-terminal tail was shown to interact with HDACs (Chakravarthy et al., 2005) and mH2A nucleosomes have been reported to inhibit the binding of transcription factors as well as RNA pol II (Angelov et al., 2003; Doyen et al., 2006). Consistent with these properties, mH2A is mostly located within heterochromatic structures throughout the genome.

1.2.6 Histone variant H2A.Z

H2A.Z is a highly conserved universal variant of the canonical histone H2A. At the primary amino acid level, H2A.Z is approximately 60% identical to H2A (see Figure 1-3 for an alignment); the main areas of difference reside in the L1 loop region within the globular domain, and in the C-terminal tail. Based on the crystal structure of the H2A.Z nucleosome, these differences were predicted to affect nucleosome structure and stability (Suto et al., 2000). In particular, the structure predicted that differences in the L1 loop, which mediates H2A-H2A interactions within the nucleosome, would preclude the existence of heterotypic nucleosomes
containing both H2A and H2A.Z. This prediction has since been disproven, since heterotypic nucleosomes have been assembled in vitro (Chakravarthy et al., 2004; Ishibashi et al., 2009), and endogenous heterotypic nucleosomes have been found in vivo (Luk et al., 2010; Viens et al., 2006; Weber, Henikoff, and Henikoff, 2010). Differences in the docking domain in the C-terminal tail of H2A.Z were predicted to cause a destabilization between the H2A.Z-H2B dimer and the (H3-H4)$_2$ tetramer. Analysis in CD4+ T-cells found that H2A.Z nucleosomes only protect approximately 120bp of DNA, suggesting an altered nucleosome-DNA interaction compared to that of H2A-containing nucleosomes (Tolstorukov et al., 2009). However, the overall effect of H2A.Z on nucleosome stability and chromatin structure has been a topic of considerable debate due to conflicting reports (reviewed in: Ausio, 2006; Zlatanova and Thakar, 2008). The nature of how H2A.Z affects nucleosome stability is further complicated by the fact that additional PTMs and/or incorporation of other histone variants have been reported to alter the stability of H2A.Z nucleosomes and chromatin structure. For example, both acetylation (Ishibashi et al., 2009) and the presence of the H3.3 variant (Henikoff, 2009; Jin and Felsenfeld, 2007; Thakar et al., 2009) have both been reported to promote the destabilization of H2A.Z nucleosomes. Therefore, the effect of H2A.Z incorporation on nucleosome stability may be context specific.

The importance of H2A.Z in eukaryotic cells is highlighted by the fact that it is essential for viability in organisms such as *Tetrahymena thermophila* (Liu, Li, and GorovskyMa, 1996), *Drosophila melanogaster* (Clarkson et al., 1999), *Xenopus laevis* (Ridgway et al., 2004), and mice (Faast et al., 2001). In contrast, loss of H2A.Z is tolerated in *Saccharomyces cerevisiae*, suggesting that this variant may have distinct functions in different organisms. Although the role of H2A.Z in regulating gene expression is generally conserved amongst different organisms, there are also distinct functional differences between H2A.Z in yeast and higher
Figure 1-3. Amino acid alignment between H2A and H2A.Z. Identical residues are highlighted in yellow. The L1-loop and docking domain regions are indicated, based on the crystal structure of the H2A.Z nucleosome (Suto et al., 2000). Alignment was generated using the Geneious alignment program (Drummond A.J. et al., 2011) using the ClustalW alignment method.
eukaryotes. In agreement with this concept, yeast encode one version of H2A.Z from a single gene \((htz1)\), whereas it has recently been discovered that two isoforms of H2A.Z exist in both mammals and birds, H2A.Z-1 and H2A.Z-2, that are expressed from separate genes and differ by only three amino acids (Coon et al., 2005; Dryhurst et al., 2009; Eirin-Lopez et al., 2009; Matsuda et al., 2010). It is not clear how the role of the newly discovered H2A.Z-2 differs from that of H2A.Z-1, but it does appear to have non-overlapping functions based on the following observations: 1) Deletion of H2A.Z-1 in mice is embryonic lethal (Faast et al., 2001); 2) H2A.Z-1 and H2A.Z-2 show unique distributions within chromatin and slight differences in the associated H3 PTMs (Dryhurst et al., 2009); 3) Deletion of the individual isoforms display unique alterations in the patterns of cellular growth and gene expression (Matsuda et al., 2010). However, dissecting the functional differences between the two isoforms has been hindered by the lack of antibodies that can distinguish between H2A.Z-1 and H2A.Z-2. Nonetheless, discovery of the second isoform of H2A.Z (H2A.Z-2) has added a new layer of complexity to H2A.Z’s role within chromatin. Since little is currently known regarding this new isoform, discussion of mammalian H2A.Z throughout the remainder of this thesis will focus on H2A.Z-1 (unless explicitly stated otherwise), which will simply be referred to as H2A.Z.

1.2.6.1 Localization of H2A.Z within the genome

1.2.6.1.1 H2A.Z in euchromatin, heterochromatin, and centromeric regions

Since chromatin is compartmentalized both structurally and functionally within the nucleus, examination of the distribution of H2A.Z within the genome has yielded useful insights into its functions. In *Tetrahymena*, the H2A.Z equivalent, hv1 (see Figure 1-4 for sequence alignment and nomenclature of H2A.Z in different species), is found exclusively in the transcriptionally active macronucleus, but is absent in the transcriptionally inert micronucleus, suggesting that this variant has a function associated with transcription (Allis et al., 1980). The *Drosophila*
H2A.Z variant, H2AvD, has a non-random distribution on polytene chromosomes, and antibodies against H2AvD stain both euchromatic and heterochromatic regions of the genome (Leach et al., 2000). Immunofluorescence staining of H2AvD also showed a much wider distribution compared to staining against RNA polymerase II (pol II), suggesting that localization of this variant does not strictly correlate with transcription activity. It is of interest to note that the *Drosophila* H2AvD variant is unique in that it is a functional fusion of the H2A.Z and H2A.X variants, and, therefore, the distinctive distribution of the H2AvD in flies may have been adopted to accommodate the dual functions of H2A.Z and H2A.X in this organism. More recent studies have shown that H2A.Z is also non-randomly distributed in other organisms, and specific genome localization is associated with distinct functions.

Similar to the *Tetrahymena* studies, immunofluorescence examination of differentiated mouse fibroblasts showed that H2A.Z is distributed across the entire interphase nucleus, but is excluded from the transcriptionally silent and HP1α-enriched pericentric heterochromatin (Sarcinella et al., 2007). Staining of mouse metaphase chromosomes also showed that the majority of H2A.Z is enriched on chromosome arms, and depleted at the constitutive heterochromatin that surrounds the centromere. Complementing these observations, Bulynko et al. found a mutually exclusive distribution between H2A.Z and tri-methylated H3K9 (a hallmark of constitutive pericentric heterochromatin) in mouse embryonic fibroblasts (Bulynko et al., 2006).

However, in trophoblast cells of the developing mouse embryo, H2A.Z appears to have an opposite localization pattern by being concentrated at pericentric heterochromatin and co-localizes with HP1α (Rangasamy et al., 2003). It is unclear why the genomic distribution of H2A.Z differs between early versus late developmental stages, but points to the possibility that
Figure 1-4. Amino acid sequence alignment of H2A.Z from different organisms. The primary amino acid sequences from human, mouse (H2A.Z in both), Drosophila melanogaster (H2AvD), Tetrahymena thermophilus (hv1), and Sachromyces cerivisiae (Htz1) were used for alignment. Identical residues are highlighted in yellow. Alignment was generated using the Geneious alignment program (Drummond A.J. et al., 2011) using the ClustalW alignment method.
H2A.Z’s functional roles switch at different stages of development or differentiation. The discovery of the second isoform of H2A.Z also suggests that the two isoforms may differ functionally at different stages of development and it will be important to distinguish between the two. Interestingly, the Rangasamy et al. study also showed, through *in vitro* biochemical analyses, that H2A.Z physically associates with the centromere-interacting passenger protein INCENP (Rangasamy et al., 2003). The same authors also subsequently found that in differentiated cells, a small fraction of H2A.Z is specifically localized to centromeric regions of the genome within the pericentric heterochromatin (Greaves et al., 2007). Using 2D and 3D immunofluorescence analyses to examine the inactive X chromosome in female mouse cells, they found that H2A.Z forms a single domain that surrounds only one side of the centromere of each sister chromatid. Additional studies further support the idea that a distinct fraction of H2A.Z may have centromere-related functions. First, biochemical purification of nucleosomes containing CENP-A (the H3 variant specific to centromeres) showed that H2A.Z is often found within these centromere-specific nucleosomes (Foltz et al., 2006). Second, RNAi-mediated depletion of H2A.Z in mouse L929 and monkey Cos-7 cells resulted in chromosome segregation defects characterized by formation of chromatin bridges between separating nuclei (Rangasamy, Greaves, and Tremethick, 2004). Finally, in yeast, colony-sectoring studies showed that the H2A.Z-deletion strain (*htz1Δ*) has increased rates of chromosome loss, suggesting that a chromosome segregation function of H2A.Z is conserved between yeast and human cells (Krogan et al., 2004). All together, these studies suggest that, depending on the localization of H2A.Z to different chromosomal regions, this variant may have both transcription- and non-transcription-related functions in the cell.
1.2.6.1.2 Localization of H2A.Z at the 5’ ends of genes

Chromatin immunoprecipitation (ChIP) and tiling microarray technologies have provided high-resolution information regarding the genome-wide localization of H2A.Z. A number of these analyses in *S. cerevisiae* showed that H2A.Z preferentially maps to the 5’ ends of genes within euchromatic regions (Guillemette et al., 2005; Li et al., 2005; Raisner et al., 2005; Zhang, Roberts, and Cairns, 2005). For example, the study by Guillemette et al. estimated that 63% of all promoters within the yeast genome contain H2A.Z, and Zhang et al. reported a preference of H2A.Z for TATA-less promoters (Guillemette et al., 2005; Zhang, Roberts, and Cairns, 2005). Yeast promoters are commonly deficient in nucleosomes (Bernstein et al., 2004; Lee et al., 2004). In fact, a common feature of yeast RNA pol II promoters is the presence of a nucleosome-free region (NFR) of approximately 150 base pairs (bp), located about 200 bp upstream of the initiation codon (Yuan et al., 2005). The NFR is frequently flanked by well-positioned nucleosomes, and H2A.Z ChIP-microarray analyses showed that H2A.Z is specifically found in the nucleosomes that flank one or both sides of the NFR (Raisner et al., 2005). Finally, some of these yeast studies further found that H2A.Z preferentially associates with inducible genes under repressed or basal-expression conditions (Guillemette et al., 2005; Li et al., 2005; Millar et al., 2006). Interestingly, deletion of the yeast H2A.Z gene does not lead to de-repression of these genes, but results in reduced transcription under inducing conditions. Therefore, deposition of H2A.Z at the 5’ ends of genes does not function to repress gene expression, but is required to set up a chromatin architecture that is compatible with gene regulation at promoters.

Similar to the yeast studies, genome-wide ChIP-sequencing or ChIP-microarray analyses of mammalian cells showed that H2A.Z preferentially localizes to gene promoters (Barski et al., 2007; John et al., 2008). However, there are several distinctive features of the distribution of
mammalian H2A.Z compared to the yeast system. First, H2A.Z deposition is not restricted to the nucleosomes flanking the NFR; instead, it is spread over several nucleosomes upstream and downstream of the transcription start site (TSS) (Barski et al., 2007; Schones et al., 2008). Second, in human T cells, promoter enrichment of H2A.Z correlates with gene activity since H2A.Z is often found associated with actively transcribing genes (Barski et al., 2007). Additional studies showed that H2A.Z could also be found at promoters of inducible genes. For example, genome-wide analyses of chromatin at binding sites of a nuclear hormone receptor, glucocorticoid receptor (GR), revealed an enrichment of H2A.Z at constitutive and hormone-induced GR-binding sites (John et al., 2008). Similarly, H2A.Z was enriched in the promoter of the p53-regulated p21 gene in U2OS osteosarcoma cells, specifically at p53 binding sites (Gevry et al., 2007). It is likely that the additional complexities of the mammalian genome account for differences in the characteristics of H2A.Z in the respective organisms.

1.2.6.2 Role of H2A.Z in the regulation of gene expression

The localization of H2A.Z to the 5′ end of genes close to the transcription start site concurs with early studies that suggest H2A.Z has a function in regulating gene expression. Indeed, proper expression of a variety of genes has now been shown to be dependent on H2A.Z. For example, in yeast, this variant is important for the induction of GAL1-10 and PHO5 genes (Adam et al., 2001; Santisteban, Kalashnikova, and Smith, 2000). Transcription of these genes is induced in response to changing environmental conditions: Growth on media containing galactose as the sole carbon source induces the GAL genes, whereas PHO5 is induced under low phosphate conditions. In \(htz1\Delta\) cells, GAL1-10 and PHO5 display impaired transcriptional induction upon activating conditions. More recent work has provided evidence that H2A.Z may also contribute to proper repression of GAL1 under non-inducing conditions (growth in glucose). In \(htz1\Delta\) cells, Zhao et al (2011) found that two populations of cells exist: One group contains an H2A
nucleosome at the GAL1 promoter and the gene is repressed, and a second group has a nucleosome-free promoter and the gene is de-repressed. Both of these chromatin states were stably inherited through multiple cell divisions. Therefore, H2A.Z contributes to the deposition of a nucleosome at the GAL1 promoter that is responsive to changes in environmental conditions. Another group of highly inducible genes that require H2A.Z for proper induction are the heat shock genes. In wildtype cells, genes that are normally activated by heat shock contain a high level of H2A.Z at their promoters, and the \( \text{htz1}\Delta \) cells display an attenuated activation of these genes upon heat shock (Zhang, Roberts, and Cairns, 2005).

Additional gene expression profiling in yeast using microarray analyses found that H2A.Z has a role in regulating expression of genes proximal to telomeres. Deletion of H2A.Z in \( S. \text{cerevisiae} \) resulted in the down-regulation of 214 genes, the majority of which clustered at regions adjacent to telomeres (Meneghini, Wu, and Madhani, 2003). Since telomeric DNA resides in a highly compacted heterochromatin state, the authors surmised that down-regulation of these genes in \( \text{htz1}\Delta \) cells was a consequence of heterochromatin spreading into adjacent euchromatin. Indeed, ChIP assays demonstrated the redistribution of the heterochromatin-associated proteins Sir2 and Sir3 into telomere-proximal regions. More importantly, deletion of Sir2 in the \( \text{htz1}\Delta \) background restored expression of many of the genes down-regulated in \( \text{htz1}\Delta \) cells. This study therefore defined a role for H2A.Z in regulating expression of genes residing proximal to heterochromatin-euchromatin boundaries, and further suggested that H2A.Z functions to antagonize the physical spreading of heterochromatin. A similar function is likely conserved in higher eukaryotes since H2A.Z is distributed throughout the euchromatic regions of the mouse cell nucleus, but is excluded from the pericentric heterochromatin (Sarcinella et al., 2007). Moreover, ChIP analyses of H2A.Z distribution in chicken cells as well as human T cells
showed that this variant is enriched at insulator regions of the respective genomes (Barski et al., 2007; Bruce et al., 2005). Insofar as insulators are specialized structures that define heterochromatin-euchromatin boundaries, the presence of H2A.Z at these specialized structures further supports a role for H2A.Z in mediating a boundary function.

While the H2A.Z deletion yeast strains are viable, genetic deletion of H2A.Z genes in other organisms is not tolerated. Therefore, gene expression studies similar to the ones done using H2A.Z null yeast cells cannot be done in higher eukaryotes. However, it is possible to use RNAi-mediated methods to knockdown expression of H2A.Z in human cells, and one such study found that reduction of H2A.Z results in de-repression of the p53-regulated p21 gene in the non-induced state (Gevry et al., 2007). This suggested that H2A.Z normally functions to repress this inducible gene. Whether knockdown of H2A.Z expression affects induction of the p21 gene under activating conditions (such as upon DNA damage) is not known. Therefore, additional studies using the RNAi-mediated knockdown of H2A.Z will be useful to fully characterize the requirement for H2A.Z in gene induction in mammalian cells. Moreover, use of RNAi-based approaches will be useful in dissecting functional differences between H2A.Z-1 and H2A.Z-2.

1.2.6.3 Links between H2A.Z and epigenetic regulatory mechanisms

While ample evidence indicates that H2A.Z is required for proper transcriptional regulation, the exact mechanistic links between this variant and the transcription process remain poorly understood. In yeast, H2A.Z is mostly associated with inducible but inactive genes; however, in most cases, loss of H2A.Z in these cells does not de-repress these inactive genes, suggesting that this variant does not directly function in transcription silencing (Adam et al., 2001; Guillemette et al., 2005; Li et al., 2005; Raisner et al., 2005; Santisteban, Kalashnikova, and Smith, 2000;
In human cells, H2A.Z is associated with both active and inactive genes. Knockdown of H2A.Z in human fibroblasts resulted in de-repression of the p21 gene, indicating that H2A.Z has an active role in repressing its transcription (Gevry et al., 2007). On the other hand, knockdown of the SRCAP protein, one of the human orthologues of the yeast SWR protein that mediates deposition of H2A.Z into chromatin, resulted in reduced expression of genes such as SP-1 and G3BP (Wong, Cox, and Chrivia, 2007), suggesting that some genes do require H2A.Z for active transcription. Taken together, these studies argue that, for different sets of genes, H2A.Z can perform distinct and sometimes contrasting functions in their transcriptional regulation. So, is there a common feature of H2A.Z that could be compatible with these diverse functions? One possibility is that the presence of H2A.Z at promoters maintains the chromatin structure of the promoters in an open or poised state that is amenable to either transcriptional activation or repression. Gene promoters contain important regulatory DNA sequences that act as docking sites for both transcriptional activators and repressors. The ability of a cell to alter its transcriptional output in response to environmental cues depends on rapid recruitment, and possibly exchange, of various factors at the promoter. Consequently, the chromatin architecture at the promoter plays a crucial role in the dynamics of promoter accessibility to these factors (Kornberg and Lorch, 1999). For example, this appears to be the case at the GAL1 promoter in yeast. As discussed in the previous section, H2A.Z contributes to both the inducible activation, as well as the repression of the GAL1 gene. Based on the work of Zhao et al (2011), the key to H2A.Z’s function at the GAL1 gene may be in its ability to promote an architecture at the promoter that allows for suitable, rapid transcriptional responses to environmental changes. In the absence of H2A.Z, the promoter is either repressed by an H2A nucleosome, or is de-repressed due to the absence of a nucleosome. In this manner, H2A.Z can contribute to both the repression and activation by promoting the deposition of a
nucleosome at the promoter that can be targeted for remodeling and ejection upon transcriptional activation.

In recent years, methylation of histones at distinct sites has been shown to be functional marks that determine epigenetic states (Martin and Zhang, 2005). For example, for most organisms, H3 methylated at K4 is often associated with open chromatin/euchromatic states, and such methylated H3 are associated with active or poised-to-transcribe genes. In mammalian cells, H3 methylated at K9 recruits binding of the HP1 proteins and functions in the assembly of constitutive heterochromatin. In addition, H3 methylated at K27 corresponds to facultative heterochromatin formation and functions to recruit polycomb repressor complexes to mediate transcriptional silencing of many developmentally regulated genes. As mentioned already, H2A.Z is required for the expression of genes at the euchromatin/heterochromatin boundaries in yeast, and it has been suggested to have a role in maintaining the integrity of euchromatin and preventing spreading of heterochromatin into the actively transcribing regions (Meneghini, Wu, and Madhani, 2003). To mediate this function, there is evidence that H2A.Z preferentially associates with specific H3 methylation states. Genome-wide localization studies of H2A.Z and various histone PTMs in human T cells showed that H2A.Z-containing promoters are also enriched with mono-, di-, and tri-methylated H3K4 (Barski et al., 2007). Furthermore, high-resolution analyses of positioned nucleosomes show that the distribution of H2A.Z nucleosomes around transcription start sites overlaps the footprint of tri-methylated H3 nucleosomes (Schones et al., 2008). Consistent with these studies, our lab has found that H2A.Z-containing mononucleosomes purified from human cells are enriched for di- or tri-methylated H3K4, as compared to nucleosomes containing H2A (Sarcinella et al., 2007). This preferential association of H2A.Z with K4-methylated H3 raises two interesting possibilities: Either H2A.Z-containing nucleosomes are preferred substrates for H3K4 methyltransferases, or H2A.Z is
preferentially deposited into chromatin that has K4-methylated H3. Giving support to the latter possibility, it is known that in yeast, acetylation of H4 and/or H2A stimulates the activity of the SWR1 deposition complex (Altaf et al, 2010). However, it is not known if these nucleosomes are also enriched with H3K4 methylation. If either of these testable scenarios were true, it would suggest that the deposition of H2A.Z could influence the levels of post-translational modifications on other histone components within the nucleosome, and establish an H2A.Z-specific histone code that mediates downstream functions.

In addition to its association with open-euchromatin histone modifications, H2A.Z can also alter local chromatin structure by affecting the positioning of nucleosomes. In yeast, the GAL1 promoter is characterized by positioned nucleosomes and the one that specifically covers the transcription start site (TSS) has been shown to be instrumental in regulating transcription of this gene (Camilloni et al., 1986; Topalidou et al., 2004). Indeed, deletion of the H2A.Z gene in yeast resulted in attenuated GAL1 activation as well as a concomitant shift of the nucleosome positioned over the TSS of the gene (Guillemette et al., 2005). In mammalian cells, mapping of DNase hypersensitive (HS) sites based on sensitivity to micrococcal or other nuclease digestion is used to define open chromatin regions of the genome that often correspond to enhancers and promoters (Gross and Garrard, 1988). Overlaying these HS sites with ChIP-chip data from human T cells revealed an enrichment of H2A.Z at DNase hypersensitive sites (Barski et al., 2007). Analogously, genome-wide analyses of chromatin marks at GR-binding sites showed that the GR binds to pre-existing regions of open chromatin and these binding sites are also enriched for H2A.Z (John et al., 2008). Collectively, these studies show both direct and indirect evidence that point to a strong correlation between open/poised chromatin and H2A.Z localization in both yeast and mammalian systems.
1.2.6.3.1 Post-translational modifications of H2A.Z further define its functions

While H2A.Z is associated with chromatin that has architecture open for activation or repression, commitment to one of these transcriptional states may be determined by specific post-translational modifications on this variant. For example, in general, acetylation is one of the histone modifications most commonly linked to transcriptional activation (Shahbazian and Grunstein, 2007), and, in yeast, H2A.Z can be acetylated at K3, K8, K10 and K14 at the N-terminus of the protein (Babiarz, Halley, and Rine, 2006; Keogh et al., 2006; Millar et al., 2006). Of these sites, K14 is the predominant acetylation site on yeast H2A.Z, and several groups have reported that Esa1, the histone acetyltransferase (HAT) found within the NuA4 HAT complex, as well as Gcn5, the HAT that is part of the SAGA complex, can both acetylate H2A.Z in vivo. Importantly, using an antibody specific for yeast H2A.Z acetylated at K14 in ChIP assays, Millar et al. found that this acetylated form of H2A.Z is specifically found at the promoters of actively transcribing genes whereas the non-acetylated form of H2A.Z is associated with the inducible but silenced genes (Millar et al., 2006). Similar studies in chicken erythroblast cells using a metazoan acetyl-H2A.Z-specific antibody also found that the acetylated form of this variant is concentrated at the 5′ end of active genes, but is absent at inactive genes (Bruce et al., 2005). Recent studies in prostate cancer cells have also demonstrated the presence of acetylated H2A.Z at the promoter of transcriptionally active genes regulated by the androgen receptor (AR) (Valdés-Mora et al, 2011; Dryhurst et al, 2011).

It is as yet unknown how H2A.Z acetylation is mechanistically linked to transcriptional activation; however, studies of the Drosophila H2AvD suggested that acetylation of this variant is coupled to the exchange of the modified form of H2AvD in chromatin with the non-modified form (Kusch et al., 2004). In addition, a number of studies have shown that activation of several genes in mammalian cells is accompanied by the loss of H2A.Z at the 5′ end of the genes.
These include the transcription of the c-myc gene upon activation by IL-2 (Farris et al., 2005), as well as GR-regulated genes upon hormone activation (John et al., 2008). Given that H2A.Z acetylation has been suggested to de-stabilize nucleosome integrity based on in vitro salt-dependent dissociation assays (Thambirajah et al., 2006), it is possible that this modification of H2A.Z at active genes triggers eviction of H2A.Z upon transcriptional activation. However, direct in vivo evidence for this mechanistic pathway is still lacking at present.

Apart from acetylation, our lab has shown that H2A.Z can also be monoubiquitylated (Sarcinella et al., 2007). Addition of the single ubiquitin group predominantly occurs on K120 at the C-terminus of human H2A.Z, but can also occur on redundant sites, such as K121 or K125, when K120 is mutated. The ubiquitylated form of H2A.Z is enriched at facultative heterochromatin-silenced regions such as the inactive X chromosome in human female cells, and its ubiquitylation is dependent on the RING1b E3 ligase that is part of the polycomb repressor PRC1 complex. Therefore, in contrast to H2A.Z acetylation, monoubiquitylation of H2A.Z is associated with the polycomb-mediated transcriptional silencing pathway. We note that in our studies examining the epigenetic signatures of H2A.Z- versus H2A-containing nucleosomes, we found that the former fraction is enriched for K4-methylated H3, but we also found that both H2A.Z- and H2A-containing nucleosomes have equal propensity for being associated with K27-methylated H3. Therefore, a significant fraction of H2A.Z in mammalian cells is associated with facultative heterochromatin and polycomb gene silencing. In fact, from analysis in our own laboratory of H2A.Z in mammalian cells, we estimated that up to 25% of H2A.Z is ubiquitylated, whereas by acid urea gel analyses, we found that less than 10% of H2A.Z is acetylated (unpublished observations). Taken together, we favour a model where the bulk of H2A.Z in human cells is unmodified and associated with K4-methylated H3 to maintain an open chromatin structure that is poised for transcriptional activation (Figure 1-5). On top of
Figure 1-5. **Functional states of H2A.Z are associated with distinct PTMs.** The bulk of H2A.Z nucleosomes contain the euchromatin mark H3K4me3. Acetylation of H2A.Z has been reported to be associated with transcriptional activation (see text). In contrast, nucleosomes containing the monoubiquitylated form of H2A.Z are found in facultative heterochromatin, which includes the H3K27me3 mark, and are therefore also predicted to be associated with transcriptional repression.
this ground or default state, ubiquitylation of H2A.Z leads to gene silencing through the facultative heterochromatin formation whereas acetylation of H2A.Z occurs at the promoters of genes once they are committed to activate.

### 1.3 Thesis Rationale

The proposal of the Histone Code Hypothesis suggested that the structural readout of histone PTMs is a complex combinatorial language. Studies of H2A.Z have revealed its function to be somewhat enigmatic given its diverse, and sometimes seemingly contradictory roles. However, using concepts from the Histone Code Hypothesis, our laboratory has proposed that histone PTMs participate in dictating the functional role of H2A.Z. Consequently, my research presented in this thesis tested the following hypotheses: 1) Deubiquitylation of H2A.Z would be part of the transcriptional activation process, and therefore deubiquitylating enzymes that mediate this process would act as transcriptional co-activators; 2) H2A.Z nucleosomes differ from H2A nucleosomes through a collective signature of histone PTMs and these PTMs act as unique binding platforms for the recruitment of effector proteins that participate in downstream functional outcomes. In chapter 2, I present evidence describing the ubiquitin specific protease 10 (USP10) as a deubiquitylase of H2A.Z and that deubiquitylation of H2A.Z by USP10 is part of the transcriptional activation program of androgen receptor (AR)-regulated genes. In chapter 3, I describe a proteomics approach used to characterize the proteins that associate with H2A.Z nucleosomes within the chromatin fibre. I characterized one such protein, Brd2, as a this ground or default state, ubiquitylation of H2A.Z leads to gene silencing through the facultative heterochromatin formation whereas acetylation of H2A.Z occurs at the promoters of genes once they are committed to activate.
Chapter 2

Identification and Characterization of USP10 as an H2A.Z Deubiquitylase and a Transcriptional Co-Activator

A version of this chapter has been published as:


Elizabeth Sarcinella analyzed the slides for immunofluorescence in Figure 2-4 and generated the recombinant deubiquitylases used in Figure 2-3. I performed all other experiments.
2 IDENTIFICATION AND CHARACTERIZATION OF USP10 AS AN H2A.Z DEUBIQUITYLASE AND A TRANSCRIPTIONAL CO-ACTIVATOR

2.1 INTRODUCTION

H2A.Z is a highly conserved variant of the canonical histone H2A. Various functions have been attributed to H2A.Z, including prevention of heterochromatin spreading (Meneghini, Wu, and Madhani, 2003), suppression of antisense RNAs (Zofall et al., 2009) and maintenance of chromosome stability and segregation (Ahmed et al., 2007; Hou et al., 2010; Rangasamy, Greaves, and Tremethick, 2004). By far, the strongest evidence links H2A.Z function to regulation of gene transcription (reviewed in: Draker and Cheung, 2009; Svotelis, Gevry, and Gaudreau, 2009). For example, ChIP-microarray and ChIP-sequencing (ChIP-seq) studies showed that many genes have H2A.Z deposited around their transcription start sites. Various studies have also highlighted a dual role of H2A.Z in transcriptional regulation, wherein it can act in either a negative or positive manner (reviewed in: Guillemette and Gaudreau, 2006). How a single histone variant can perform such contrasting functions is still unclear. However, its ability to either repress or promote transcription could be explained by differential post-translational modifications that occur on H2A.Z. For example, fractions of total H2A.Z are acetylated or monoubiquitylated, and we have previously hypothesized that these modifications distinguish H2A.Z’s functions in activation or repression (Draker and Cheung, 2009). In that regard, the enzymes that modulate these respective modifications represent key regulatory components that dictate H2A.Z functions.

Mammalian H2A.Z is known to be multiply acetylated at several lysine residues (including K4, K7 and K11) at the N-terminus (Beck et al., 2006; Bonenfant et al., 2006; Ishibashi et al., 2009). In contrast, monoubiquitylation predominantly occurs at K120 at the C-terminus, although K121
and K125 can serve as alternative ubiquitylation sites (Sarcinella et al., 2007). In yeast, H2A.Z is acetylated by the NuA4 and GCN5 acetyltransferases (Babiarz, Halley, and Rine, 2006; Keogh et al., 2006; Millar et al., 2006). Deficiency in H2A.Z acetylation leads to defects in chromosome transmission and cell proliferation. Furthermore, the acetylated form of H2A.Z is found at the 5′ end of active genes, suggesting a positive function for this modified form of H2A.Z in transcriptional regulation (Bruce et al., 2005; Millar et al., 2006). Much less is known about H2A.Z ubiquitylation other than that ubiquitylated H2A.Z (H2A.Zub1) is enriched at facultative heterochromatin in mammalian cells (Sarcinella et al., 2007). Ubiquitylation of H2A.Z is mediated by Ring1b, the E3 ligase member of the polycomb repressive complex 1 (PRC1), suggesting that this modified form of H2A.Z is part of the polycomb silencing pathway. Available evidence suggests that ubiquitylation of H2A.Z is part of the polycomb silencing pathway conserved in more complex eukaryotes.

Like many post-translational modifications (PTMs), ubiquitylation is also a reversible reaction. A specialized class of enzymes called isopeptidases mediates removal of ubiquitin. These deubiquitylating enzymes (DUBs) act by cleaving the isopeptide bond that links ubiquitin to its substrate. These enzymes are generally classified into five families based on their catalytic domains: ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), ovarian tumour proteases (OTUs), Josephins and Jab1/MPN/MOV34 metalloenzymes (JAMM/MPN+) (reviewed in: Komander, Clague, and Urbe, 2009). USPs, UCHs, OTUs and Josephins are all cysteine proteases, whereas the JAMM/MPN+ enzymes are zinc metalloproteases.

To date, several mammalian DUBs have been identified to target H2Aub1, and many have been linked to transcriptional activation (reviewed in: Vissers et al., 2008). For example, USP16 was first described as a mitotic DUB targeting H2Aub1 (Cai, Babbitt, and Marchesi, 1999), and a
recent report suggested that this enzyme also regulates Hox gene expression (Joo et al., 2007). Similarly, USP21 was found to promote transcription of developmentally regulated genes in the liver presumably through its deubiquitylation of H2Aub1 (Nakagawa et al., 2008). Finally, USP22 and the 2A-DUB (KIAA1915), both target H2Aub1 (although USP22 can also deubiquitylate H2Bub1), and both were described as co-activators of androgen receptor-mediated transcription (Zhao et al., 2008; Zhu et al., 2007).

Thus far, an H2A.Z deubiquitylase has not been reported. Given that H2A.Zub1 has also been linked to repression of gene expression, our lab predicted that the deubiquitylating enzyme for H2A.Z would have a transcriptional activation function. In that regard, USP10 has been implicated as a transcription co-activator for androgen receptor (AR)-regulated genes (Faus et al., 2005) and, therefore, I tested whether it might target H2A.Zub1. Indeed, in this study I provide evidence that USP10 deubiquitylates H2A.Zub1 and H2Aub1 \textit{in vitro} and \textit{in vivo}. Importantly, knockdown of USP10 in prostate cancer cells increases the global levels of H2A.Zub1 and H2Aub1. USP10 and H2A.Z are both required for AR-regulated gene transcription, and ChIP assays showed that H2A.Z is directly associated with the promoter and enhancer of the PSA and KLK2 genes. Finally, knockdown of USP10 impairs eviction of H2A.Z at the regulatory regions of PSA and KLK2 that normally occurs in DHT-treated cells. Collectively, these data support a novel role for H2A.Z in the regulation of AR-mediated transcription that also involves the deubiquitylating activity of USP10 towards histones.

\section{2.2 Materials and Methods}

\subsection{2.2.1 Cell culture, transfection, plasmids and antibodies}

293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. LNCaP cells were obtained from ATCC and PC-3 cells stably expressing AR
[PC-3(AR)] were courtesy of Dr. Theodore J. Brown (Mount Sinai Hospital, Toronto). Both cell types were grown in RPMI 1640 media supplemented with 10% fetal bovine serum. For culturing in the absence of hormone, cells were grown in phenol-red free RPMI 1640 supplemented with 5% charcoal-stripped fetal bovine serum (Invitrogen) for 72 hrs prior to treatment with hormone. Dihydrotestosterone (DHT) was obtained from Sigma and re-suspended in absolute ethanol; DHT was added to cells at a final concentration of 10nM, or for control samples, an equivalent volume of ethanol was added. All transfections were carried out using Lipofectamine 2000 (Invitrogen). All expression constructs used were based on the pcDNA 3.1 (+) (Invitrogen) backbone with the Flag tag cloned in-frame. In co-transfection experiments, the ratio of the Flag-H2A/Flag-H2A.Z to Flag-USP10 plasmids were either 1:1 or 1:3. H2A.Z antibody directed against the L1 loop was described previously (Sarcinella et al., 2007), H2A acidic patch antibody was from Upstate, H3 (ab1791) and USP10 antibodies were from Abcam, H2Bub1 monoclonal antibody (NRO3) was from MédiMabs, Flag M2 and anti-His monoclonal antibodies were from Sigma, and AR antibody (PG-21) was from Millipore. Anti-HA Affinity Matrix (Roche), containing rat monoclonal antibody (clone 3F10) conjugated to agarose beads, was used for HA ChIP experiments.

2.2.2 Immunoprecipitation of HA-ubiquitin-conjugated proteins

293T cells were transiently transfected with an HA-ubiquitin-expressing construct or an empty vector. Cell lysates were harvested in RIPA buffer, briefly sonicated, then used for immunoprecipitation overnight at 4°C, using anti-HA monoclonal antibody conjugated to agarose beads. Following seven washes in RIPA buffer, immunoprecipitated material was eluted by boiling for 10 minutes in 2X sample buffer, then used for SDS-PAGE and Western blotting using antibodies against H2A.Z, H2A or HA.
2.2.3 **In vitro deubiquitylation assay**

Deubiquitylase cDNAs were cloned into the pVL1393 vector (BD Biosciences) and His tag sequences were added in-frame to the DUB cDNA. His-tagged recombinant protein was generated using BaculoGold (BD) packaging vector in the baculovirus expression system using standard techniques, and purified via Ni-NTA agarose beads. Equal amounts of purified DUBs (1.5µg each) were mixed with 2 µg of total acid-soluble protein harvested from cells expressing either Flag-tagged H2A.Z or Flag-tagged H2A in a buffer containing 60mM HEPES (pH 7.5), 5mM MgCl₂, 4% glycerol, 1mM EDTA, 1mM DTT plus fresh protease inhibitors. Reactions were incubated at 37°C for 30 minutes. The reactions were stopped by the addition of 2X sample buffer and the samples were boiled for 5 minutes.

2.2.4 **Immunofluorescence analysis**

LNCaP cells were seeded on glass coverslips and grown as described above. Immunofluorescence analysis was performed as described in (Sarcinella et al., 2007) using anti-USP10 and anti-AR antibodies.

2.2.5 **Small-scale biochemical fractionation**

Following treatment of cells with ethanol or 10nM DHT for 2hrs, subcellular fractions of LNCaP cells were harvested using the Subcellular Protein Fractionation Kit (Thermo Scientific) according to manufacturer’s instructions. Equal percentages (by volume) of each fraction were analyzed by SDS-PAGE and Western blotting using standard techniques.

2.2.6 **Stable shRNA and protein expression via retroviral transduction of LNCaP cells**

H2A.Z (cagctgtccagtgttggtg) and USP10 (gaatatcagagaattgagt) shRNA target sequences were cloned into the pSUPER-retro-puro vector (Oligoengine) as per manufacturer’s instructions. To
generate stable LNCaP cells, 7.0 x 10^5 cells were seeded per well in 6-well dishes in RPMI 1640, 10% fetal bovine serum. 24hrs later, culture media was replaced with 4 ml of viral supernatant (containing 4 µg/ml of polybrene) and plates were centrifuged at 1,500 x g for 4 hrs at 20°C. Following centrifugation, viral supernatant was removed and replaced with fresh culture media. 24hrs later, cells were selected in 0.9 µg/ml puromycin for a minimum of 4 days, after which cells were maintained in media containing 0.6 µg/ml puromycin.

2.2.7 Luciferase assays

Empty vector (pcDNA3.1/pLNCX2), USP10 or P/CAF expression constructs were transfected into PC-3(AR) cells along with the AR-dependent luciferase reporter, which contains 3 repeats of the androgen response element in its promoter. Cells were lysed 48 hrs after transfection in cell culture lysis reagent (Promega). Luciferase activities were measured using the Luciferase assay system (Promega) according to the manufacturer’s instructions. Co-transfection of a β-gal-expressing plasmid, with subsequent measurement of β-gal activity, was used to normalize luciferase data.

2.2.8 RT-qPCR analysis

LNCaP cells were grown in the absence of hormone and then treated with DHT or ethanol as described above. Twenty-four hours following treatment, RNA was harvested in TRIzol reagent (Invitrogen) according to manufacturer’s instructions. RNA was re-suspended in molecular biology grade ddH2O and 500ng was used in a reverse transcription reaction to synthesize cDNA using Oligo (dT)12-18 and SuperScript II (Invitrogen) . Quantitative polymerase chain reactions (qPCR) were assembled in triplicate using PerfeCta SYBR Green SuperMix (Quanta Biosciences) and transcript-specific primers. Reactions were run on an Applied Biosystems SDS7900HT thermal cycler in a 384-well format. Gene expression was normalized to
expression of the housekeeping gene RPL27 as described in (de Jonge et al., 2007). Data are presented as means ± standard deviations and are representative of at least three independent experiments using independently generated batches of stable cells. Primers for the PSA gene were described previously (Zhu et al., 2007). KLK2 primers were as follows: forward 5’ gctggagagtgaagattc 3’, reverse 5’ gttcaggctcaacaggttg 3’. Primers for RPL27 were described in (de Jonge et al., 2007).

2.2.9 Chromatin immunoprecipitation (ChIP) and sequential ChIP assays

LNCaP cells were grown in 15cm plates, in the absence of hormone, and then treated with DHT or ethanol as described above. Cells were fixed by adding formaldehyde directly to the culture medium to a final concentration of 1% and incubated at room temperature for 10 min. Formaldehyde was quenched by adding glycine to a final concentration of 125mM with incubation at room temperature for an additional 5 min. Cells were washed 3 times in cold 1X PBS plus protease inhibitors. Cells were then resuspended in a nuclei isolation buffer comprising 50mM HEPES (pH 8.0), 1mM EDTA, 0.5mM EGTA, 140mM NaCl, 10% glycerol, 0.5% Igepal CA-630, 0.25% Triton X-100, plus protease inhibitors. Following a 10 min incubation on ice, nuclei were pelleted via centrifugation and resuspended in a sonication buffer composed of 10mM HEPES (pH 8.0), 1mM EDTA, 0.5mM EGTA, 200mM NaCl and incubated on ice for 10 min. Nuclei were pelleted via centrifugation and resuspended in 50mM HEPES (pH 8.0), 140mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholic acid. Chromatin was sonicated using a Branson Sonifier 450 to produce chromatin fragments with an average size of 600bp. Samples were then centrifuged at max speed for 10min, 4°C. Supernatant was used for immunoprecipitations (IP) after pre-clearing with Protein G Dynabeads (Invitrogen). 2 x 10^6 cells were used per IP in a total volume of 200 µl of
sonication buffer at 4°C overnight, with rotation. The following amounts of antibodies were used per IP: AR (PG-21), 1.2 μg; H3 1.5 μg; H2A.Z L1 loop, 2 μl serum. Chromatin-antibody complexes were captured using Protein G Dynabeads for 1hr at 4°C with rotation. Captured chromatin was washed sequentially, for 10 min each, in the following buffers containing fresh protease inhibitors: RIPA [150mM NaCl, 50mM Tris-HCl (pH 8.0), 0.1% SDS, 0.5% Na-deoxycholic acid, 1% Igepal CA-630], High Salt [500mM NaCl, 50mM Tris-HCl (pH 8.0), 0.1% SDS, 1% Igepal CA-630], LiCl buffer [250 mM LiCl, Tris-HCl (pH 8.0), 0.5% Na-deoxycholic acid, 1% Igepal CA-630], followed by 2 washes in TE buffer [10mM Tris-HCl (pH 8.0), 1mM EDTA]. For Sequential ChIP assays, following the final TE wash, chromatin complexes were eluted in 1% SDS, 50mM Tris-HCl pH 7.5, 10mM EDTA for 30min at room temperature, then diluted ten-fold in sonication buffer (minus SDS), and subjected to a second round of ChIP using anti-HA affinity matrix. Following overnight incubation, chromatin complexes were subjected to four 10-minute washes in sonication buffer, followed by two washes in TE buffer. For both ChIP and re-ChIP, chromatin was eluted in 2 x 200 μl of Elution Buffer (2% SDS, 10mM DTT, 0.1M sodium bicarbonate). 5M NaCl (16μl ) was added to each sample followed by reverse cross-linking at 65°C for 4-6hrs. 95% ethanol (1ml) was added to each sample and placed at –20°C overnight. Chromatin was pelleted via centrifugation, washed once in 70% ethanol and re-suspended in TE buffer. Following treatment of samples with RNAse A and Proteinase K, DNA was extracted using standard phenol-chloroform procedures and re-dissolved in 100 μl of molecular biology-grade water. An aliquot (5 μl) of each sample was used for qPCR analysis, in triplicate, as described above. The following primers were used for ChIP analyses: PSA Enhancer, forward 5' agatccaggcttgcttactgtcct 3', reverse 5' acctgetcagcetttgtetgtgat 3'; PSA (-)2Kb forward 5' caaccaaaacctgacccaac 3', reverse 5' tttgcctggcccgtagt 3'; PSA Promoter, forward 5' tgggtcttggagtgaacagactct 3', reverse 5' tgggtcttggagtgiacagactct 3'.
agacagccccagatgaaacagaa 3’; KLK2 Enhancer forward 5’ gttgaagcagactcgtaa 3’, reverse 5’ ctgaccacatctctcaagct 3’; KLK2 Promoter, forward 5’ gggaatgcctccagatg 3’, reverse 5’ ctgaccctgtgccacct 3’. Data are presented as means ± standard deviation and are representative of at least three independent experiments. H2A.Z ChIP data are presented as “H2A.Z enrichment”, to account for nucleosome density, which was calculated by dividing the % INPUT of the H2A.Z IPs by the % INPUT of the total H3 IPs.

2.3 RESULTS

2.3.1 Detection of monoubiquitylated histones

Our lab has previously determined that the monoubiquitylated form of H2A.Z can be detected as a slower migrating protein band using our H2A.Z-L1 antibody in Western blots (Sarcinella et al., 2007). We have also observed the slower migrating form of H2A using antibodies against both the endogenous and tagged versions of H2A. For both H2A.Z and H2A Western blots, I confirmed that the slower migrating species are the monoubiquitylated forms of the respective histones by conducting anti-HA immunoprecipitations using lysates from cells transfected with HA-ubiquitin alone, or in combination with either Flag-H2A or Flag-H2A.Z (Figure 2-1). Anti-HA Western blotting (bottom panels) shows a characteristic smear of HA-ubiquitin-conjugated proteins, demonstrating that the tagged form of ubiquitin can be added to target proteins. Compared to untransfected cells, cells expressing the HA-ubiquitin construct revealed an additional slower migrating protein band that was detected using both anit-H2A.Z and anti-H2A antibodies (see INPUT samples in 2-1A). Given its size of approximately 26 kDa, this protein band likely represents histones monoubiquitylated by HA-ubiquitin. Probing the HA-immunoprecipitated samples with anti-H2A.Z or anti-H2A antibody revealed only a single protein band that corresponded in size to the upper most protein band in HA-ubiquitin-
expressing cells. This suggested that monoubiquitylated histones, modified with HA-ubiquitin were efficiently immunoprecipitated. The identity of the second upper protein band as being HA-tagged monoubiquitylated H2A/H2A.Z was confirmed by performing immunoprecipitations with either wildtype or non-ubiquitylatable mutants of the two histones. The non-ubiquitylatable mutants contain lysine to arginine substitutions in their C-termini rendering them non-modifiable by ubiquitylation; we have previously described the use of these constructs (Sarcinella et al., 2007). In cells co-transfected with HA-ubiquitin and the wildtype H2A/H2A.Z, two upper protein bands were observed, as was seen when probing for the endogenous histones in Figure 2-1A (compare INPUT fractions). Anti-Flag Western blots from IP’ed material from these samples show a single band that also corresponds, in size, to the second shifted band. In contrast, no shifted protein bands were detectable in samples containing the non-ubiquitylatable mutants, and no protein bands were detected in the immunoprecipitated samples (Figure 2-1B & C). From these data, we can be confident that our Western blotting methods reliably detect the monoubiquitylated forms of H2A and H2A.Z.

2.3.2 USP10 specifically deubiquitylates H2A.Zub1 and H2Aub1

To test whether USP10 targets histones as substrates, I co-expressed Flag-tagged USP10 (Flag-USP10) with tagged H2A.Z or H2A in 293T cells and measured the levels of ubiquitylated histones by Western blot. Increasing expression of Flag-USP10 resulted in a corresponding reduction of the ubiquitylated histone levels in a dose-dependent manner (Figure 2-2A). Moreover, expression of USP10 alone effectively reduced the endogenous H2A.Zub1 and H2Aub1 levels (Figure 2-2B). Importantly, increased USP10 expression did not affect the levels of endogenous H2Bub1 (Figure 2-2B), indicating that USP10 specifically targets the H2A family of histones. Finally, this effect was dependent on the enzymatic activity of USP10 since expression of the catalytically inactive mutant containing a cysteine 424-to-alanine substitution
Figure 2-1. Detection of mono-ubiquitylated H2A.Z and H2A by Western blot. (A) Lysate from 293T cells transfected with HA-tagged ubiquitin (HA-Ub) was used to immunoprecipitate ubiquitylated proteins. Eluted material was assessed by Western blot using anti-H2A.Z, anti-H2A, and anti-HA antibodies. (B & C) Lysates from cells transfected with HA-ubiquitin alone, or co-transfected with Flag-tagged versions of either wild type (WT), or non-ubiquitylatable mutant versions (MUT) of H2A/H2A.Z where the C-terminal lysines have been mutated to arginines, were used for immunoprecipitating ubiquitylated histones. Eluted material was assessed by Western blot using anti-Flag, and anti-HA antibodies. The asterisk, “*”, marks a non-specific band detected by the Flag antibody. Molecular weight (in kDa) ranges are indicated.
Figure 2-2. Expression of USP10 causes a reduction in H2A.Zub1 and H2Aub1 levels. (A) A Flag-tagged version of USP10, or empty vector, was transiently co-transfected with either Flag-H2A.Z or Flag-H2A, in 293T cells. Total protein lysate was harvested and used for Western blotting. (B) Flag-USP10, or empty vector, was transiently transfected into 293T cells and levels of endogenous mono-ubiquitylated H2A.Z and H2A were assessed by Western blotting using antibodies recognizing the endogenous proteins. The level of monoubiquitylated H2B was assessed using an antibody specifically recognizing the monoubiquitylated form of H2B—the membrane used for H2A.Z Western blotting was stripped and re-probed with anti-H2Bub1 antibody. (C) Levels of Flag-H2A.Zub1 and Flag-H2Aub1 were assessed using lysate from cells co-transfected with either wild-type USP10 or a catalytically inactive mutant containing a cysteine to alanine mutation at residue 424 (C424A). Short exposure of the Flag blot shows the non-ubiquitylated forms of Flag-H2A.Z/Flag-H2A for loading purposes.
(C434A) mutation (Soncini, Berdo, and Draetta, 2001) did not alter the levels of ubiquitylated histones (Figure 2-2C). Together, these findings show that expression of USP10 leads to a specific reduction of H2A.Zub1 and H2Aub1 levels in vivo.

I next tested whether USP10 directly targets H2A.Zub1 and H2Aub1 by using an in vitro deubiquitylation assay. Acid-extracted histones from 293T cells expressing either Flag-H2A or Flag-H2A.Z were incubated with a panel of recombinant deubiquitylases generated using the baculovirus expression system. Using an anti-Flag antibody in Western blots, I then measured the changes in the levels of Flag-H2A.Zub1 and Flag-H2Aub1 brought about by the activities of the recombinant enzymes. As shown in Figure 2-3, His-tagged USP10 directly deubiquitylated H2A.Z in this assay (left panel). Consistent with the in vivo results (Figure 2-2C), the enzymatically inactive C424A mutant was impaired in its ability to deubiquitylate the incubated histones. In addition to USP10, I also tested the in vitro activities of two other DUBs, USP16 and 2A-DUB, previously identified to target H2Aub1 (Joo et al., 2007; Zhu et al., 2007). Of all the enzymes tested, USP16 consistently displayed the highest efficiency in deubiquitylating both H2Aub1 and H2A.Zub1. USP10 and 2A-DUB have similar activities towards H2A.Zub1, but both are less efficient than USP16. Interestingly, while USP16 has similar activity towards both H2A.Zub1 and H2Aub1, neither USP10, nor 2A-DUB, showed significant activities towards H2Aub1 in this assay. The previous report on 2A-DUB suggested that, in vitro, H2A deubiquitylation was enhanced by hyper-acetylation of the histone substrates via TSA treatment (Zhu et al., 2007). I therefore examined the deubiquitylation activities of the DUBs on histone substrates harvested from TSA-treated cells. Interestingly, I found that TSA-treatment not only increased acetylation levels of H4, but it also led to a small but consistent increase in the overall H2A.Zub1 and H2Aub1 levels (Figure 2-3). However, in contrast to the previous report, hyper-
**Figure 2-3.** USP10 deubiquitylates H2A.Zub1 and H2Aub1 *in vitro*. *In vitro* deubiquitylation assays were assembled using a panel of His-tagged deubiquitylases that were generated using the baculovirus protein expression system. Purified deubiquitylases were incubated with acid-soluble protein extracts from nuclei of 293T cells expressing either Flag-tagged H2A.Z or H2A. Levels of the various deubiquitylases were compared by Western blotting using an anti-His antibody (top panel) and levels of the monoubiquitylated tagged histones were assessed using the Flag M2 antibody—short exposures of the Flag blots show the unmodified band of the tagged histones for loading purposes. In order to determine if hyper-acetylated histones are better substrates for in vitro deubiquitylation, acid-soluble protein was harvested from cells treated with trichostatin A (TSA) and hyper-acetylation of histones was confirmed using an anti-acetyl H4 antibody (bottom panel).
acetylation of H2A or H2A.Z did not increase the deubiquitylation of these substrates under these assay conditions.

In summary, the in vitro assay showed that H2A.Zub1 is a direct substrate for three histone deubiquitylases: USP16, USP10 and 2A-DUB. While USP16 deubiquitylates both H2A.Zub1 and H2Aub1, both USP10 and 2A-DUB prefer H2A.Zub1 in vitro. It is possible that additional co-factors, not present in the assay system, are needed for these enzymes to target H2A. Moreover, given that both USP10 and 2A-DUB were reported to function in activating AR-regulated genes, whereas USP16 was found to be important for deubiquitylating H2Aub1 during mitosis, their differences in activities and substrate preferences may reflect their different functions in vivo.

2.3.3 USP10 is localized to both the cytoplasm and nucleus

Insofar as USP10 was previously identified as a transcriptional co-activator for AR-regulated genes, I wanted to examine the subcellular distribution of this enzyme, particularly in the androgen-responsive LNCaP cell line. By immunofluorescence (IF) examination, USP10 was observed in both the cytoplasm and the nuclei of LNCaP cells (Figure 2-4). USP10 was also previously suggested to physically interact with AR and therefore, the localization of this hormone receptor was examined in the IF studies as well. While the majority of AR is in the cytoplasm of the hormone-deprived cells, treatment with 10nM dihydrotestosterone (DHT) dramatically re-localized the majority of AR to the nucleus. In contrast, DHT did not significantly alter USP10’s distribution in the cytoplasm and nuclei of the hormone-treated cells. These data suggest that, if USP10 and AR do physically interact, the interaction only occurs within a small nuclear fraction of USP10.
**Figure 2-4. USP10 localizes to the cytoplasm and nucleus.** LNCaP cells were grown on glass coverslips in charcoal-stripped, phenol red-free RPMI 1640 media for 72 hrs. Cells were then treated for 2hrs with either DHT at a final concentration of 10nM, or an equivalent volume of absolute ethanol (EtOH). Cells were fixed and co-stained with DAPI and antibodies recognizing either androgen receptor (AR) (A) or USP10 (B). (C) Total protein levels of AR and USP10 were compared, via Western blot, between LNCaP cells treated either with DHT, or EtOH, for 2hrs (H3 used as a loading control). (D) Sub-cellular localization of USP10 and AR was determined by Western blot using cytoplasmic and nuclear fractions (S1 – membrane-associated fraction; S2 – soluble cytoplasmic fraction; S3 – soluble nuclear fraction; S4 – chromatin-associated fraction) of LNCaP cells treated with EtOH or DHT for 2hrs. Tubulin, PARP and H3 were used as loading controls for the cytoplasmic, soluble nuclear, and chromatin fractions, respectively. Sub-cellular fractions were harvested as described in Materials and Methods. The asterisk, “*”, marks a band in the ladder that cross-reacted with the AR antibody.
To examine the sub-cellular localization of USP10 with a more quantitative method, I used a biochemical fractionation assay to separate the cytoplasmic and nuclear protein fractions from LNCaP cells. I first compared total protein levels of AR and USP10 in EtOH- or DHT-treated LNCaP cells (Figure 2-4C). Additionally, total levels of H2A and H2A.Z, both substrates of USP10, were also assessed. Hormone treatment induced a slight but consistent increase in the total levels of AR, whereas it did not have any effect on the levels of USP10, H2A or H2A.Z in these cells. Consistent with the IF results, USP10 remained mostly cytoplasmic under both conditions, whereas AR was re-localized to the nucleus upon DHT-treatment. However, a small but distinct fraction of USP10 does reside in the nucleus and its abundance is not affected by hormone treatment (Figure 2-4D). Insofar as significant amounts of USP10 are in the cytoplasm, it likely has cytoplasmic substrates and functions. Indeed, USP10 regulates the stability of transmembrane ion channels in epithelial cells (Bomberger, Barnaby, and Stanton, 2009; Boulkroun et al., 2008), and it has recently been reported to target cytoplasmic p53 and regulate its protein stability (Yuan et al., 2010). In contrast, the nuclear fraction of USP10 likely has a different function, such as transcription regulation, and since very little USP10 fractionates with chromatin, it suggests that the interaction between USP10 and chromatin is highly dynamic and transient.

### 2.3.4 USP10 and H2A.Z regulate AR-mediated transcription

Since a change in the distribution of USP10 following stimulation with DHT was not observed, I wanted to confirm that USP10 does affect AR-regulated gene expression as reported. To this end, I first used a luciferase-based reporter assay in which the reporter gene is under the control of three androgen response elements (AREs). This is a conserved DNA sequence to which AR binds, and is found in the regulatory regions of androgen-responsive genes (Heery et al., 1997).
In prostate cancer PC3 cells that stably express the androgen receptor [PC-3(AR)], expression of the AR-dependent luciferase reporter gene was indeed induced following treatment with DHT (Figure 2-5A). Co-transfection of USP10 along with the reporter construct showed that USP10 enhances the DHT-induced luciferase expression in a dose-dependent manner and its effect is comparable to that of another AR co-activator, the histone acetyltransferase P/CAF. More importantly, when the PC3-AR cells were pre-treated with USP10 shRNA to knockdown expression of endogenous USP10, a clear reduction in the DHT-induced luciferase level was observed, supporting the conclusion that USP10 is a bone fide co-activator of AR-regulated transcription.

To further test the dependency of endogenous AR-regulated gene expression on USP10, I examined the expression of the well-characterized AR-activated PSA (KLK3) gene, as well as the KLK2 gene, in LNCaP cells. Additionally, since USP10 targets H2A.Z for deubiquitylation, I asked if H2A.Z is involved in regulating hormone-induced gene expression mediated through AR. To do so, I generated stable LNCaP cell lines expressing shRNAs that target USP10 or H2A.Z, and confirmed that USP10 and H2A.Z expression were knocked down in these cells by Western blot (Figure 2-6A). Endogenous USP10 expression levels in the USP10 knockdown cells were less than 20% of the wild type control cells, whereas the H2A.Z expression was approximately 50% in the H2A.Z knockdown cells (semi-quantitative analysis by Western blot in Figure 2-6B). Since the H2A.Z shRNA is highly specific, as it targets the 3’ UTR of the transcript, the residual amount of H2A.Z could be related to the recently discovered additional isoform of H2A.Z (Dryhurst et al., 2009; Eirin-Lopez et al., 2009), the transcript of which is not predicted to be targeted by the shRNA construct. In support of the finding that USP10 targets histones, cells with stable knockdown of USP10 consistently had higher levels of H2A.Zub1 and H2Aub1, whereas the levels of H2Bub1 remain unchanged (Figure 2-6C). Characterization
Figure 2-5. USP10 acts as a co-activator of AR in luciferase-based assays. (A) PC-3 cells, stably expressing AR, were grown in the absence of hormone, then transiently transfected with a firefly luciferase-expressing plasmid containing 3X androgen response elements in the promoter, along with a plasmid expressing USP10, the histone acetyltransferase P/CAF, or empty vector. In all cases, an additional plasmid expressing beta galactosidase (β-gal) was co-transfected for normalization. Cells were then stimulated with DHT (10nM) or EtOH for 24hrs, after which lysates were collected for measuring luciferase activity. Since USP10 and P/CAF are in different vector backbones, their respective empty vectors were used as negative controls. The relative amounts of each plasmid are 1X and 3X. (B) Cells were grown as in (A) then co-transfected with the luciferase reporter plasmid, β-gal-expressing plasmid, and either a plasmid expressing an shRNA targeting USP10 mRNA, or a scrambled shRNA sequence control, followed by treatment with hormone or EtOH. Luciferase activity was measured 24hrs after treatment.
Figure 2-6. **USP10 and H2A.Z are required for full expression of androgen-responsive genes.** LNCaP cells were transduced with retrovirus expressing shRNAs targeting USP10 or H2A.Z mRNA, or a non-targeting scrambled control sequence. (A) Whole-cell lysates were used to assess USP10 and H2A.Z knockdown efficiencies by Western blot. Additionally, AR and H2A protein levels were examined in the knockdown cells. Tubulin and H3 were used as loading controls. (B) Whole cell lysates from LNCaP cells with stable knockdown of USP10, H2A.Z, or expressing a scrambled control shRNA were used for Western blot analysis of US10 and H2A.Z protein levels. Decreasing amounts of the Scramble control sample were loaded in order to estimate the decrease in USP10 and H2A.Z protein levels. The loading of the Scramble sample, from left to right, was as follows, in lanes 1-5: 100%, 80%, 60%, 40%, 20%. Tubulin and H3 Westerns are shown as loading controls. Note that equal loading is demonstrated in lanes 1 & 6 for both panels. (C) Total protein lysates from LNCaP cells with stable knockdown of USP10 were used to examine the levels of H2A.Zub1, H2Aub1 (upper bands in the respective blots) and H2Bub1 by Western blot. Shorter exposures of the H2A.Z and H2A blots, showing the non-ubiquitylated band only (lower band), as well as H3, are used to demonstrate equal loading. (D) Transduced cells were grown in the absence of hormone for 72 hrs then treated with 10nM DHT or an equivalent volume of EtOH for 24hrs. RNA was harvested using TRIzol, and cDNA was synthesized and used as template for quantitative PCR (qPCR) using primers specific for PSA or KLK2 mRNA. Each PCR reaction was performed in triplicate with each experiment repeated at least three times independently, using different batches of stable cells, also generated independently. Values are presented as means ± standard deviations.
of the H2A.Z and USP10 knockdown cells by reverse transcription-quantitative PCR (RT-qPCR, Figure 2-6D) revealed a reduction of the induced levels of PSA and KLK2 mRNA relative to the scrambled control in both cells lines. This effect was not due to a reduction in AR expression (Figure 2-6C), but rather suggests that H2A.Z and USP10 are each required in the transcriptional activation of these genes.

To test whether H2A.Z and USP10 directly regulate expression of the AR-responsive genes, I used chromatin immunoprecipitation assays (ChIP) to examine the localization of these proteins at the PSA and KLK2 genes. PCR primers targeting the PSA gene are shown schematically in Figure 2-7A. Primers targeted the promoter, enhancer, or an additional region in between the two regulatory elements that was used as a control region, which is approximately 2,000 bp upstream of the transcriptional start site (labeled as “(-)2kb” in graphs). Consistent with current literature, I found that, upon DHT treatment, AR is recruited to the AREs present at the enhancers and promoters of the PSA and KLK2 genes (Figure 2-7B). Also, AR enrichment is much higher at the upstream enhancer than at the promoter of the PSA gene (note that the scale of the percentage input for the enhancer is much higher than that for the promoter), whereas AR levels are low and did not increase upon DHT treatment at a control region located between these AREs. H2A.Z has been implicated in the regulation of other hormone-induced genes, such as glucocorticoid receptor (GR), and the estrogen receptor (ER), and is thought to localize at hormone receptor binding sites (Gevry et al., 2009; John et al., 2008). Therefore, I examined the enrichment of H2A.Z at the AREs before and after DHT treatment. Since changes in H2A.Z levels may simply reflect changes in total nucleosome levels, the H2A.Z ChIP signal was normalized to H3 ChIP signal to account for this possibility. As a result, the data are presented as “H2A.Z enrichment” and represent net changes in H2A.Z levels. My analysis showed that a
Figure 2-7. H2A.Z localizes to androgen response elements at AR-regulated genes.

LNCaP cells were grown in the absence of hormone for 72 hrs then treated with DHT (10nM final concentration) or an equivalent volume of EtOH for 2hrs. Cells were then fixed in 1% formaldehyde and chromatin was harvested for chromatin immunoprecipitation analysis. (A) Schematic of the PSA gene. Arrows represent approximate locations of primers used in qPCR. (B) ChIP analysis of AR recruitment to the PSA enhancer and promoter following stimulation of cells with DHT. The “(-) 2kb” label on the graphs represent the region in between the promoter and enhancer of the PSA gene and is approximately 2kb from the transcriptional start site. (C) & (D) ChIP analysis of H2A.Z at the PSA and KLK2 genes. H2A.Z ChIP data are presented as “H2A.Z enrichment”, to account for nucleosome density, which was calculated by dividing the % INPUT of the H2A.Z IPs by the % INPUT of the total H3 IPs (data not shown). Each PCR reaction was performed in triplicate with each experiment repeated at least three times independently. Values are presented as means ± standard deviations.
significant amount of H2A.Z was present at the regulatory elements compared to the control region (Figure 2-7C). Interestingly, although the net loss of H2A.Z at the enhancer regions is minimal, a more dramatic change was observed at the promoter regions following DHT treatment. The H3 ChIP signals, used to normalize the H2A.Z ChIP, decreased at both regions (not shown). The H2A.Z signal decreased by a comparable amount at the enhancer, resulting in a small net change in H2A.Z, which is interpreted as total nucleosome loss at this region. However, at the promoters, the decrease in H2A.Z is greater than the decrease observed for H3, resulting in a net loss of H2A.Z, which is interpreted as a loss of H2A.Z-H2B dimers (see discussion). A similar observation was made at the KLK2 gene (Figure 2-7D). The transcription activation-induced loss of H2A.Z was confirmed using two additional commercially available antibodies against H2A.Z (data not shown), and is not due to a change in the total protein level in response to hormone (see Figure 2-4C). Recently, another group has independently confirmed this observation. A study by Dryhurst et al. (Dryhurst et al., 2012) reported the transcription induced loss of H2A.Z at the PSA gene in both LNCaP and C4-2 cells. Such transcription-induced loss of H2A.Z was previously reported for a number of other genes in mammalian cells (Farris et al., 2005; John et al., 2008), as well as in yeast cells (Adam et al., 2001; Larochelle and Gaudreau, 2003; Santisteban, Kalashnikova, and Smith, 2000; Zhang, Roberts, and Cairns, 2005), suggesting that this is a common feature of H2A.Z following gene activation. However, this observation is in contrast to the study by Gèvry and colleagues (Gevry et al., 2009) where hormone activation induced deposition of H2A.Z at the ER binding sites. At present, the exact role of H2A.Z in regulation of AR-regulated genes is not clear; however, the expression studies showed that H2A.Z is required for maximal induction of PSA and KLK2 expression upon DHT-treatment.
In addition to AR and H2A.Z, I made multiple attempts to ChIP USP10 to the PSA or KLK2 genes using two different commercially available antibodies. However, despite my efforts, I was unable to consistently ChIP USP10 at the AREs of PSA and KLK2. One possibility is that the antibodies are not suitable for chromatin immunoprecipitation. On the other hand, given that the biochemical fractionation assay (Figure 2-4D) showed that only a small amount of USP10 resides in the nucleus, it is likely that the limited amount, as well as the transient nature of USP10 binding to chromatin, precluded robust immunoprecipitation of this enzyme at the AR-regulated genes. In support of this interpretation, a study of USP3, characterizing the enzyme as an H2Aub1 and H2Bub1 DUB, reached similar conclusions (Nicassio et al., 2007). Despite its prominent nuclear localization, wild type USP3 could not immunoprecipitate its histone substrates, and by FRAP (fluorescence recovery after photobleaching) USP3 was shown to have a very transient and dynamic interaction with chromatin. Therefore, it is likely that the engagement of USP10 with chromatin is also transient.

Since there is no antibody that specifically detects H2A.Zub1, I generated LNCaP cells stably expressing HA-tagged ubiquitin to assess changes in the levels of ubiquitylated proteins, including H2A.Zub1, at the PSA and KLK2 genes before and after hormone induction. ChIP analyses using anti-HA antibody showed that ubiquitylated proteins are present at all of the upstream regions of both PSA and KLK2 (Figure 2-8A). Interestingly, total levels of ubiquitylated proteins decreased at the PSA enhancer and both the promoter and enhancer regions of KLK2 following hormone stimulation. This suggests that hormone induction resulted in deubiquitylation of various chromatin-bound proteins, including H2Aub1 and H2A.Zub1, at these regulatory regions of the AR-regulated genes. Since H2A.Zub1 was readily immunoprecipitated using HA-tagged ubiquitin (Figure 2-1), I took advantage of this strategy to test whether H2A.Zub1 is present at the PSA and KLK2 genes by first generating LNCaP cells...
Figure 2-8. USP10 regulates levels of ubiquitylated proteins, including ubiquitylated H2A.Z, at AR-regulated genes. (A) LNCaP cells stably expressing HA-tagged ubiquitin were used for ChIP analysis following treatment of cells with ethanol or 10nM DHT for 2hrs, as described in Materials and Methods. (B) Sequential ChIP was performed as described in Materials and Methods using LNCaP cells stably expressing HA-tagged ubiquitin with an anti-H2A.Z antibody followed by anti-HA antibody. Fold enrichment represents the % INPUT expressed relative to the control region, (-)2Kb. (C) ChIP was performed as in (A) using cells stably expressing both USP10 shRNA and HA-ubiquitin. “(-)” represents the no antibody, negative control samples. (D) H2A.Z ChIP was performed using Scramble- or USP10 shRNA-expressing cells. Data were first calculated as % INPUT then expressed as a percentage relative to the ethanol-treated samples.
that stably expressed HA-ubiquitin, then used lysate from these cells in a sequential ChIP assay by first immunoprecipitating chromatin using an H2A.Z antibody, followed by a second ChIP using the HA antibody to immunoprecipitate ubiquitin-conjugated H2A.Z (Figure 2-8B). An enrichment of the HA-ubiquitin signal from H2A.Z-containing chromatin at the regulatory regions of the PSA and KLK2 genes was observed, suggesting that H2A.Zub1 is present at these regions in the absence of hormone. More importantly, the H2A.Zub1 levels are reduced following hormone induction, suggesting that deubiquitylation of H2A.Z is associated with transcriptional activation of these genes.

In order to test whether USP10 affects the levels of ubiquitylated proteins at the PSA and KLK2 genes, we generated LNCaP cells stably expressing both HA-tagged ubiquitin and USP10 shRNA for use in ChIP assays (Figures 2-8C & 2-8D). In cells with USP10 knockdown (Figure 2-8C), the relative enrichment of HA-ubiquitin at the various regions in the absence of hormone (white bars) was comparable to the trend observed in cells expressing HA-ubiquitin alone (Figure 2-8A). In contrast, knockdown of USP10 expression increased the retention of ubiquitylated proteins at both the PSA and KLK2 genes following stimulation with hormone (black bars), suggesting that USP10 affects the levels of ubiquitylated proteins at these genes in response to hormone stimulation. Furthermore, compared to cells expressing a scrambled control shRNA, we found a consistent defect in the eviction of H2A.Z (normally seen following hormone stimulation) from both the PSA and KLK2 regulatory regions in the USP10 knockdown cells (Figure 2-8D). Since expression of H2A.Z is unaffected by knockdown of USP10 (see Figure 2-6A) this finding strongly suggests that USP10 is required for the transcription-induced loss of H2A.Z at AR-regulated genes. Collectively, these data suggest that USP10 regulates the levels of ubiquitylated proteins at AR-regulated genes, such as
H2A.Zub1 and H2Aub1, and that eviction of H2A.Z upon transcriptional activation is
dependent on this deubiquitylase.

2.4 DISCUSSION

This study identified USP10 as a DUB that targets both H2A.Zub1 and H2Aub1. Co-
transfection studies showed that USP10 specifically deubiquitylates H2A.Zub1 and H2Aub1,
but not H2Bub1, in a dose-dependent manner. More significantly, stable knockdown of USP10
expression in LNCaP cells led to a global increase in the total H2A.Zub1 and H2Aub1 levels in
these cells, as well as an increase in the levels of ubiquitylated proteins at AR-regulated genes.
In addition, knockdown of USP10 prevented full eviction of H2A.Z at the regulatory elements
of AR-regulated genes. Finally, in vitro assays confirmed that H2A.Z is a direct target of
USP10, as well as other DUBs such as USP16 and 2A-DUB. Interestingly, while USP16 has
robust activity towards both H2A.Zub1 and H2Aub1 in this assay, neither USP10, nor 2A-DUB
debiquitylated H2Aub1 in vitro. USP16, also known as Ubp-M, was first described as the
enzyme responsible for deubiquitylating H2A during mitosis (Cai, Babbitt, and Marchesi,
1999). Early studies showed that the bulk of H2A is deubiquitylated during mitosis and it was
hypothesized that removal of the ubiquitin group is required to allow maximal condensation of
chromatin into chromosomes during this stage of the cell cycle. In contrast, USP10 and 2A-
DUB have been functionally linked to transcriptional regulation, a process that requires a large
number of activators and co-activators. Since USP10 and 2A-DUB affect H2A ubiquitylation
levels in vivo, but not significantly in our in vitro assay, it is possible that additional cofactors
are required to increase the efficiencies or expand the substrate specificities of these enzymes in
the cellular context.
H2A.Z is a highly conserved variant of H2A specifically deposited at the promoters of a large proportion of genes in the human and yeast genomes. This variant has been directly linked to both transcriptional activation and repression; however, its exact functions in these processes have not yet been clearly elucidated. Our lab has previously found that a fraction of H2A.Z in mammalian cells is monoubiquitylated and this form of the variant is associated with facultative heterochromatin (Sarcinella et al., 2007). We also found that the transcription-repressing PRC1 component Ring1b mediates monoubiquitylation of H2A.Z and hypothesized that the enzyme that removes this modification likely has an opposite function, such as that of a transcriptional co-activator (Draker and Cheung, 2009). In that regard, USP10 fulfills this prediction since I showed that USP10 is needed for maximal induction of AR-regulated genes. For example, knockdown of USP10 not only reduced induction of the AR-dependent luciferase reporter gene, but it also reduced expression of the endogenous AR-regulated PSA and KLK2 genes in DHT-treated LNCaP cells. Recently, Zhu and colleagues (Zhu et al., 2007) showed that the 2A-DUB enzyme also functions in the activation of AR-regulated genes by deubiquitylating H2Aub1. The findings presented here support this model and further suggest that H2A.Z deubiquitylation is also part of this process. Moreover, three separate DUBs, USP10, USP22 and 2A-DUB are involved in this pathway. At present, it is not known whether they have redundant or separate functions in AR-regulated gene expression. In addition, it is possible that their specific functions may differ temporally or in the recruitment of unique binding partners or co-activators. This concept of histone modifying enzymes with overlapping functions mediating AR-induced transcription is not without precedence. The histone demethylases LSD1 and JHDM2A (JMJD1A) can both catalyze the removal of mono- and di-methyl groups from H3 lysine 9, yet both function prominently in the transcriptional activation of AR-regulated genes (Metzger et al., 2005; Yamane et al., 2006). The large number of co-activators identified in this
pathway attests to the fact that AR-mediated transcriptional regulation is highly complex and that multiple histone-modifying activities are functionally required for this process (Heemers and Tindall, 2007).

By ChIP analysis, I observed that H2A.Z is enriched at the enhancers and promoters of the PSA and KLK2 genes under non-inducing conditions, an observation that has recently been confirmed by another laboratory (Dryhurst et al., 2012). Furthermore, results from the sequential ChIP analysis of HA-tagged ubiquitin from H2A.Z-enriched chromatin indicate that the monoubiquitylated form of H2A.Z is present at these regions in the absence of hormone, as is the case with H2Aub1. Induction of these genes upon DHT treatment led to a significant loss of H2A.Z at these regulatory regions, a process that is at least partially dependent on USP10. Consistent with the conclusion that H2A.Z is directly involved in AR-regulated gene expression, its requirement has also been reported for other hormone-regulation pathways such as GR- and ER-induced genes (Gevry et al., 2009; John et al., 2008). In addition, the chromatin remodeling complex, SRCAP, which catalyzes incorporation of H2A.Z into chromatin (Ruhl et al., 2006), has also been described as a co-activator of AR-mediated transcription (Monroy et al., 2003; Slupianek et al., 2010). Interestingly, while findings presented here are consistent with the GR-regulated genes in that a substantial amount of H2A.Z is enriched at the regulatory regions of these genes prior to hormone induction, they are opposite to the observations of Gévry and colleagues (Gevry et al., 2009) that showed H2A.Z is deposited at the ER-binding sites only after hormone induction. In that case, they found that p400, another H2A.Z deposition complex, is required for the hormone-induced recruitment of H2A.Z to the ER-binding sites. This differential usage of the chromatin remodeling complexes (SRCAP vs. p400) in the AR- vs. ER-regulated gene activation may explain the disparate timing of H2A.Z deposition in these hormone receptor-signaling pathways. However, the functional roles of H2A.Z in these two
different hormone regulation pathways are currently unclear. Furthermore, given that there are two isoforms of H2A.Z that differ by only three amino acids (Dryhurst et al., 2009; Eirin-Lopez et al., 2009), it would be interesting to determine the contribution of each isoform in the regulation of these pathways. Given that expression of the two isoforms are differentially regulated in response to hormone, in both LNCaP and C4-2 cells, it would appear that H2A.Z-1 and H2A.Z-2 may have unique roles in androgen-responsive cells (Dryhurst et al., 2012).

The observed loss of H2A.Z upon DHT treatment and AR recruitment is consistent with multiple studies that showed a loss of H2A.Z upon gene activation (Adam et al., 2001; Farris et al., 2005; John et al., 2008; Larochelle and Gaudreau, 2003; Santisteban, Kalashnikova, and Smith, 2000; Zhang, Roberts, and Cairns, 2005). Genome-wide studies showed that, in yeast, H2A.Z preferentially regulates inducible genes and this variant is often deposited at sites that flank nucleosome-free regions in promoters (Guillemette et al., 2005; Li et al., 2005; Millar et al., 2006; Raisner et al., 2005; Zhang, Roberts, and Cairns, 2005). In mammalian cells, H2A.Z is also frequently found at regulatory elements such as promoters, enhancers, and insulators, which often display DNase I hypersensitivity (Barski et al., 2007; Gross and Garrard, 1988; John et al., 2008). Moreover, for GR-regulated genes, enrichment of H2A.Z at the DNase I hypersensitive sites decreases upon GR recruitment. This frequent loss of H2A.Z upon transcriptional activation may be related to biochemical studies showing that H2A.Z-containing nucleosomes are less stable (Abbott et al., 2001; Henikoff, 2009; Jin and Felsenfeld, 2007; Placek et al., 2005). Collectively, these and other studies suggest that H2A.Z functions to maintain a chromatin structure that is amenable to rapid remodeling and poised for transcriptional activation (Bruce et al., 2005; Millar et al., 2006). It is of further interest to note that acetylation of H2A.Z has been suggested to correlate with transcriptional activation and eviction of H2A.Z from promoter elements. Thus far, it is not known how H2A.Z
ubiquitylation might affect this process. One hypothesis is that ubiquitylation of H2A.Z may alter nucleosome stability and impede H2A.Z eviction and thus deubiquitylation of H2A.Zub1 is a pre-requisite step for transcription activation. Further studies testing this hypothesis will be important for determining how H2A.Z deubiquitylation might function in transcriptional regulation.
Characterization of Brd2 as a Transcriptional Co-Activator that Engages H2A.Z Nucleosomes

A version of this chapter is under revision in PLoS Genetics as:


The samples used for mass spectrometry analysis in Figure 1 were prepared by Elizabeth Sarcinella; mass spectrometry and data analysis were performed by Dr. Thomas Kislinger and Vladimir Ignatchenko. Elizabeth Sarcinella also performed experiments shown in figures 3-2B, 3-3A, 3-3C, and 3-4. I performed all other experiments.
3 CHARACTERIZATION OF Brd2 AS A TRANSCRIPTIONAL CO-ACTIVATOR THAT ENGAGES H2A.Z NUCLEOSOMES

3.1 INTRODUCTION

H2A.Z is a variant of the canonical histone H2A. Amongst the different variants of core histones that have been identified to date, H2A.Z is unique in that it is the only variant that is essential for viability and development in a number of organisms (Clarkson et al., 1999; Faast et al., 2001; Liu, Li, and Gorovskyma, 1996; Ridgway et al., 2004). H2A.Z has been implicated to function in multiple cellular pathways, including maintenance of chromosome stability and segregation (Ahmed et al., 2007; Hou et al., 2010; Rangasamy, Greaves, and Tremethick, 2004), prevention of the spread of heterochromatin (Meneghini, Wu, and Madhani, 2003), as well as regulation of transcription (for review see: (Draker and Cheung, 2009; Guillemette and Gaudreau, 2006)). Currently, the essential function of H2A.Z is unknown. Although H2A.Z participates in diverse cellular pathways, its mechanisms are yet to be fully elucidated. Like all other histones, H2A.Z is subjected to post-translational modifications (PTMs), which may further modulate its function in these different pathways. For example, acetylation of residues in the N-terminus has been linked to transcriptional activation (Bruce et al., 2005; Halley et al., 2010; Millar et al., 2006; Valdes-Mora et al., 2011) whereas ubiquitylation of the C-terminus is associated with transcriptionally inactive chromatin (Draker, Sarcinella, and Cheung, 2011; Sarcinella et al., 2007). These studies have led us, and others, to propose that H2A.Z physically poises chromatin at transcription start sites and that PTMs on H2A.Z further specify its function in transcriptional activation or repression (Draker and Cheung, 2009; Svotelis, Gevry, and Gaudreau, 2009).

At the amino acid level, mammalian H2A and H2A.Z share about 60% identity. Knockout of the H2A.Z gene is lethal in mice, which suggests that the unique regions of H2A.Z are required
for its essential function in complex eukaryotes. Moreover, these unique regions likely engage effector proteins that mediate H2A.Z-specific functions. A number of studies have examined and identified H2A.Z-interacting proteins; however, these studies have focused on purifying soluble, tagged H2A.Z, often from whole-cell extracts, to identify proteins interacting directly with this variant (Kobor et al., 2004; Luk et al., 2007; Mizuguchi et al., 2004; Straube, Blackwell, and Pemberton, 2010). Not surprisingly, these studies mostly identified H2A.Z chaperone proteins and chromatin remodeling complexes that deposit H2A.Z into the chromatin fibre. While these studies have yielded invaluable information regarding the biology and regulation of the targeting and incorporation of H2A.Z into chromatin, they were not as informative in elucidating the functions of H2A.Z downstream of its deposition. Our lab was interested in identifying effector proteins that engage H2A.Z on chromatin since these proteins likely contribute to the physiological functions associated with H2A.Z. We therefore used a mass spectrometry-based approach to identify proteins that preferentially associate with H2A.Z nucleosomes for this purpose. We specifically chose mononucleosomes, instead of soluble H2A.Z, as the bait for two reasons: First, histones exist in the context of nucleosomes in vivo and there are now many examples of proteins that contact multiple sites on nucleosomes to stabilize their interactions with chromatin (for recent examples see: Agricola et al., 2011; Eustermann et al., 2011; Nady et al., 2011; Ruthenburg et al., 2011). Second, we have previously found that a distinct pattern of H3 methylation status on H2A.Z nucleosomes, compared to H2A nucleosomes (Sarcinella et al., 2007). This suggested that H2A.Z nucleosomes have unique histone PTM signatures that could collectively influence the engagement of downstream effector proteins. This idea is consistent with the concept of multivalency whereby multiple histone PTMs contribute to the overall binding and stability of
chromatin-binding proteins, or complexes that contain multiple histone PTM-binding motifs (Ruthenburg et al., 2007).

Using a nucleosome purification-mass spectrometry analyses, we identified a large number of proteins that preferentially associated with H2A.Z nucleosomes over H2A nucleosomes. Gene ontology (GO) analyses showed that the majority of the interacting proteins are chromosome- or chromatin-associated proteins. Consistent with the transcription-related functions of H2A.Z, many of the identified proteins have putative transcription-associated functions. Of the top 21 identified proteins, we focused our follow-up studies on Brd2 because of its transcriptional co-activator function, and because it contains a double bromodomain which binds to acetyl-lysines.

Brd2 belongs to the BET family of proteins, all of which contain tandem bromodomains in their N-termini and an extraterminal domain of unknown function in their C-termini (Florence and Faller, 2001). Brd2 has essential cellular functions as evidenced by the early embryonic lethality of homozygous null mice (Gyuris et al., 2009; Shang et al., 2009). Moreover, Brd2 hypomorphic mice become extremely obese when placed on a regular diet, and yet they avoid the development of insulin resistance and diabetic disease (Wang et al., 2010). In contrast, over-expression of Brd2, specifically in the B-cell compartment of mice, leads to development of leukemia (Greenwald et al., 2004). Although the exact role of Brd2 in these cellular processes is not clear, the importance of its function may have to do with its ability to act as a transcriptional co-activator. It has been reported that, in combination with Ras or MEKK, Brd2 participates in the transcriptional activation of cell cycle regulatory genes cyclin A, D1 and E (Denis et al., 2000). Furthermore, Brd2’s role in transcription is also evidenced by its reported associations with components of transcriptional machinery such as E2F, TBP and the largest
subunit of Pol II (Crowley et al., 2002; Denis et al., 2006; Peng et al., 2007; Sinha, Faller, and Denis, 2005).

In this study, we found that Brd2 preferentially interacts with H2A.Z nucleosomes through the recognition of acetylated lysines by the bromodomains of Brd2. Peptide competition assays suggest that acetylated H4 is the primary site of interaction between Brd2 and H2A.Z nucleosomes, but additional points of contact likely exist to stabilize this interaction. *In vivo* experiments showed that Brd2 is recruited to androgen receptor (AR)-regulated genes following hormone stimulation, in a manner that is dependent on H2A.Z. Finally, chemical inhibition of Brd2 binding to chromatin through the use of the small molecule inhibitor JQ1 blocked recruitment of Brd2 to AR-regulated genes and prevented transcriptional activation of these genes. Our analysis of proteins that specifically interact with H2A.Z nucleosomes has yielded novel insights into how H2A.Z containing-chromatin engages and recruits effector proteins to mediate downstream functions in transcriptional regulation.

3.2 MATERIALS AND METHODS

3.2.1 Cell culture, reagents, plasmids, and antibodies

293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. LNCaP cells were obtained from ATCC and were grown in RPMI 1640 media supplemented with 10% fetal bovine serum. For culturing in the absence of hormone, cells were grown in phenol-red free RPMI 1640 supplemented with 5% charcoal-stripped fetal bovine serum (Invitrogen) for 72 hrs prior to treatment with hormone. Dihydrotestosterone (DHT) was obtained from Sigma and resuspended in absolute ethanol; DHT was added to cells at a final concentration of 10nM, or for control samples, an equivalent volume of ethanol was added. For treatment of cells with trichostatin A (TSA), cells were treated with TSA (200nM) or an
equivalent volume of DMSO for 2 hours prior to harvest. The JQ1 reagent was kindly provided by Dr. Jay Bradner, Dana-Farber Cancer Institute. All transfections were carried out using Lipofectamine 2000 (Invitrogen). All expression constructs used were based on the pcDNA 3.1 (+) (Invitrogen) backbone with the Flag tag cloned in-frame. H2A.Z antibody directed against the L1 loop was described previously (Sarcinella et al., 2007), H3 (ab1791) and Brd2 (ab3718) from Abcam, GFP from Santa Cruz (sc-8334), H4 tetra acetyl was a kind gift from the lab of C. David Allis, H4K12Ac from Upstate, H3K9Ac from Upstate, H3K14Ac from Upstate, pan-acetyl Ab from Cell Signaling, AR antibody (PG-21) was from Millipore and Flag M2 monoclonal antibody from Sigma.

3.2.2 Mononucleosome immunoprecipitation

Generation of mononucleosomes was performed as described previously (Sarcinella et al., 2007) with slight modifications. 293T cells were grown in 15 cm-diameter plates and were transfected with a construct that expresses either Flag-H2A or Flag-H2A.Z. Cells were trypsinized, counted, and washed in 1X PBS, 48hrs following transfection. Cellular pellets were resuspended in Buffer A (20mM HEPES, pH 7.5, 10mM KCl, 1.5mM MgCl2, 0.34M sucrose, 10% glycerol, 1mM dithiothreitol, 5mM sodium butyrate, 10mM NEM, and protease inhibitors), pelleted and then resuspended in buffer A containing 0.2% Triton X-100 and incubated on ice for 5 min. The nuclear suspension was centrifuged at 1300 x g; nuclei were then washed once in Cutting Buffer (10mM Tris-HCl, pH 7.5, 15mM NaCl) then resuspended in Cutting Buffer with 2mM CaCl2. Microccocal nuclease (MNase; Worthington) was added at a concentration of 10 units/1.0 x 10^7 cells the incubated at 37°C for exactly 30 min. The reaction was stopped by the addition of 1mM EGTA. The MNase-digested nuclei were centrifuged at 1300 x g and then subjected to hypotonic lysis by resuspension in TE buffer (10mM Tris-HCL, pH 8.0, 1mM EDTA). Samples were incubated on ice for 30 min, with
occasional mixing by pipette. The suspension was then centrifuged at 16 000 \times g and the supernatant was transferred to a new tube. Salt was adjusted to 150mM NaCl by adding a 3X Buffer D (60mM HEPES, pH 7.5, 450mM NaCl, 4.5mM MgCl2, 0.6mM EGTA, 0.6% Triton X-100, 30% glycerol) drop-wise, with constant mixing on a vortex set to low speed. Insoluble material was pelleted via centrifugation. The clarified supernatant was then used for immunoprecipitation by adding M2-agarose beads and incubated overnight at 4°C on an end-over-end rotator. Beads were washed 4 times in 1X Buffer D, followed by 3 washes in 1X Buffer D containing 0.5% Triton X-100. Proteins were eluted from the beads by resuspension in 2X SDS sample buffer and boiled for 10min. For Western blot analysis, samples were run on SDS-polyacrylamide electrophoresis gels according to standard practices. Due to a difference in expression between Flag-H2A and Flag-H2A.Z, the overall nucleosome levels were normalized between immunoprecipitated samples by H3 Western blotting. For mass spectrometry analysis, on average, an equivalent of approximately $3.0 \times 10^6$ cells were loaded on NuPAGE® Novex® 4–12% Bis-Tris (1.5-mm thick, 10-well) pre-cast polyacrylamide gels, and separated by molecular mass. Gel lanes were cut into 10 gel blocks of equal size and in-gel digested as previously described (Shevchenko et al., 2006). Extracted peptides were C-18 purified using Varian OMIX cartridges (Mississauga, ON, Canada) and analyzed by 1D-LC-MS/MS on a LTQ-Orbitrap XL as previously described (Taylor et al., 2009).

### 3.2.3 Mass spectrometry analysis and proteins identification

Raw data was converted to m/z XML using ReAdW and searched by X!Tandem against a locally installed version of the human UniProt complete human proteome protein sequence database (release date 2009, 20,323 sequences). The search was performed with a fragment ion mass tolerance of 0.4 Da and a parent ion mass tolerance of ±10 ppm. Complete tryptic digest was assumed. Carbamidomethylation of cysteine was specified as fixed and oxidation of
methionine as a variable modification. A target/decoy search was performed to experimentally estimate the false positive rate and only proteins identified with two unique high quality peptide identifications were considered as previously reported (Taylor et al., 2009) (FDR ~0.5%). An in-house protein grouping algorithm was applied to satisfy the principles of parsimony (Drake et al., 2010; Elschenbroich et al., 2011).

3.2.4 Peptide competition assays

Immunoprecipitations were performed essentially as described above, with the following modification: Peptides were added to the input material giving a final peptide concentration of 30 µg/ml, then incubated at 4°C with rotation for 30min. M2-agarose beads were then added as described in 3.2.2.

3.2.5 Stable knockdown of H2A.Z in LNCaP cells

Cells stably expressing the H2A.Z (cagctgtccagtgttggtg) shRNA target sequence were generated as described in Chapter 2.2.6 and in Draker et al. (2011). Cells were maintained in media as described in 3.2.1, with the addition of puromycin (0.6µg/ml).

3.2.6 RT-qPCR analysis and Chromatin immunoprecipitation (ChIP) assays

RT-qPCR or ChIP analysis of LNCaP cells was performed as previously described in Chapter 2.2.8, and in Draker et al. (2011). All experiments included a ‘no antibody’ negative control sample, which was processed identical to, and parallel with, samples containing antibody. All samples containing antibody displayed a signal at least 10-fold higher than the no antibody control.
3.3 RESULTS

3.3.1 Identifying proteins that interact with H2A.Z nucleosomes

To understand the physiological functions of chromosomal H2A.Z, we took an unbiased proteomics approach to identify proteins preferentially interacting with H2A.Z-containing nucleosomes. We first transfected 293T cells with either Flag-tagged H2A.Z (or Flag-tagged H2A as a control), digested the chromatin down to mononucleosomes with micrococcal nuclease (MNase) and then immunoprecipitated intact nucleosomes with anti-Flag antibody. By this method, we isolated and analyzed the co-purifying proteins by LC-MS/MS (Figure 3-1A; see Materials and Methods for details). Following three independent purifications and MS analyses, the cumulative data were filtered using stringent cutoffs to generate a list of proteins that preferentially interact with H2A.Z nucleosomes (Figure 3-1A). Gene ontology analysis of the top 21 identified proteins (Figure 3-1D) revealed that most of the proteins are chromatin-associated proteins involved in chromatin organization and transcription. For example, our screen identified components of the H2A.Z remodeling complex SRCAP, including DMAP1, RUVB1, RUVB2 and VPS72. In addition, the transcriptional regulator WDR5 was also one of our top hits. WDR5 is involved in transcription via its association with mammalian H3K4 methyltransferase complexes (Hughes et al., 2004; Nakamura et al., 2002; Wysocka et al., 2003; Yokoyama et al., 2004). Since WDR5 preferentially binds di- and tri-methylated H3K4 (Wysocka et al., 2005), this is consistent with our previous finding that H2A.Z nucleosomes are enriched with di- and tri-methylated H3K4 (Sarcinella et al., 2007).

Interestingly, many of the other identified proteins have conserved domains that can recognize various histone PTMs. For example, PHF6 and PHF14 have PHD fingers, which are motifs that can recognize methylated lysine residues (Sanchez and Zhou, 2011). Similarly, PWWP2A
Figure 3-1. Nucleosome IP/Mass spectrometry approach and analysis of H2A.Z-nucleosome interacting proteins. (A) Schematic of the nucleosome IP & mass spectrometry protocol used. (B) Heat map of spectral counts from amalgamated data showing the top 21 protein hits. (C) Venn diagram summarizing the number of unique and overlapping hits between H2A.Z nucleosomes, H2A nucleosomes, and the Flag-tagged GFP control protein. (D) Gene ontology analysis of the proteins identified in our MS analysis.
contains a PWWP domain, which is part of the Royal Family of domains that also recognize methylated lysine residues (Maurer-Stroh et al., 2003). Finally, PHIP and Brd2 both have bromodomains, which are well-characterized acetyl-lysine binding motifs (Mujtaba, Zeng, and Zhou, 2007; Zeng and Zhou, 2002). Given that many of the interacting proteins contain motifs known to bind post-translationally modified histones, the overall PTM signature of H2A.Z nucleosomes likely mediates the interactions. In order to validate interactions identified through mass spec analysis of H2A.Z- and H2A nucleosomes, I generated HA-tagged versions of two of the hits from our list, USP39 (SNUT2) and PWWP2A. These constructs were then co-transfected in 293T cells with Flag-H2A, Flag-H2A.Z, or Flag-NLS-GFP control protein. Mononucleosomes were immunoprecipitated from these cells and interactions between the HA-tagged proteins and the nucleosomes were assessed via HA Western blots. As shown in Figure 3-2A, both USP39 and PWWP2A prefentially interact with H2A.Z nucleosomes compared to H2A nucleosomes, validating our mass spec analysis.

### 3.3.2 Brd2 engages H2A.Z nucleosomes through the recognition of acetylated H4 residues by its bromodomains

One of the proteins identified in our mass spec analysis was Brd2, which is a double-bromodomain protein known to be involved in transcriptional activation. Given that both H2A.Z and Brd2 are known to have transcription-related functions, we chose to perform follow-up experiments to further validate and characterize the interaction between Brd2 and H2A.Z nucleosomes. First, we confirmed this interaction by repeating our mononucleosome IP experiments using cells expressing Flag-tagged H2A.Z or Flag-tagged H2A, and then compared the levels of endogenous Brd2 immunoprecipitated by Western blot, using anti-Brd2 antibody (Figure 3-2B). In addition, since Brd2 contains 2 bromodomains, which have been shown to
Figure 3-2. Validation of H2A.Z-nucleosome-interacting proteins. (A) N-terminal HA-tagged versions of USP39 (SNUT2) and PWWP2A were generated and co-transfected with either Flag-H2A, Flag-H2A.Z, or Flag-NLS-GFP as a control. Mononucleosomes were immunoprecipitated as described in Materials and Methods with anti-Flag antibody and the eluted proteins were used for Western blot analysis with anti-Flag and anti-HA antibodies, as indicated. (B) Mononucleosomes were prepared from cells treated with TSA (200nM for 2 hours) or DMSO and eluted material was used for Western blot analysis with the indicated antibodies. (C) Mononucleosomes were immunoprecipitated from cells co-transfected with Flag-H2A.Z and either HA-tagged VPS72 or YFP-tagged Brd2, and treated with TSA (200nM for 2 hrs) or DMSO. INPUT fractions represent 1% of immunoprecipitated material. The IP fractions represent 30%, for Brd2 blots, and 5%, for histone blots, of the total eluted volume.
bind acetylated H4 tails (Huang et al., 2007; Kanno et al., 2004; Nakamura et al., 2007; Umehara et al., 2010a; Umehara et al., 2010b), we tested whether hyperacetylating the histones, prior to harvest and immunoprecipitation, would enhance the interaction between Brd2 and chromatin. In control cells (treated with DMSO), Brd2 was immunoprecipitated with H2A.Z nucleosomes, whereas no detectable levels of Brd2 were observed on H2A nucleosomes (Figure 3-2B—compare lanes 1 & 3 from IP fraction). Treatment of cells with the histone deacetylase inhibitor trichostatin A (TSA) resulted in an overall increase in histone acetylation as confirmed by acetyl-H4 Western blots (lanes 2 & 4 in both the INPUT and IP fractions). More importantly, hyperacetylation greatly enhanced the interaction of Brd2 with H2A.Z nucleosomes. The hyperacetylated H2A nucleosomes also pulled down some Brd2 but the amount is minor compared to that found on H2A.Z nucleosomes (compare lanes 2 & 4 in the IP fraction). All together, these results confirmed our MS data and showed that Brd2 is a novel H2A.Z-nucleosome-interacting protein. Moreover, this interaction is enhanced when the histone acetylation levels are increased. To determine if the enhancement of Brd2 binding to H2A.Z nucleosomes following hyperacetylation is specific to Brd2, I compared the binding properties of Brd2 to that of a known H2A.Z-binding protein, VPS72. VPS72 is the human homologue of Swc2, which is a component of the H2A.Z deposition complex in yeast, Swr1, and directly interacts with H2A.Z within the Swr1 complex (Wu et al., 2005). I first co-transfected Flag-H2A.Z together with YFP-Brd2 or HA-VPS72, and then performed mononucleosome immunoprecipitations. Like the endogenous protein, YFP-Brd2 was immunoprecipitated with H2A.Z nucleosomes, and the amount of protein immunoprecipitated was greatly increased in cells treated with TSA (Figure 3-2C). In contrast, the same amount of HA-VPS72 was immunoprecipitated with H2A.Z nucleosomes regardless of the acetylation status of the chromatin. Therefore, increased binding under hyperacetylated conditions is not a
general property of H2A.Z nucleosome-interacting proteins. Interestingly, over-expression of the YFP-Brd2 construct increases levels of H4ac, which has been reported previously (Rafalska-Metcalf et al., 2010).

Since the interaction between Brd2 and H2A.Z nucleosomes greatly increased when the chromatin is hyperacetylated, this suggests that the interaction is mediated through the bromodomains of Brd2 and the acetylated lysines on histones. To test this, we compared the ability of H2A.Z nucleosomes to immunoprecipitate wild type (WT) Brd2, to a mutant version (BD) of Brd2 that contains point mutations in each of its bromodomains, rendering the domains incapable of binding acetylated lysine residues (Rafalska-Metcalf et al., 2010). Consistent with our prediction, the BD mutant was not immunoprecipitated with H2A.Z nucleosomes, under basal or hyperacetylated conditions (compare IP fractions in lanes 3 & 4 with lanes 5 & 6 in Figure 3-3A). It is noteworthy that endogenous Brd2 was also immunoprecipitated in both sample sets at comparable levels, as detected by probing the membrane with anti-Brd2 antibody. Therefore, the interaction of Brd2 with H2A.Z nucleosomes is completely dependent on the bromodomains of Brd2.

Previously, it has been reported that the bromodomains of Brd2 bind acetylated lysine residues on H4 (Huang et al., 2007; Kanno et al., 2004; Nakamura et al., 2007; Umehara et al., 2010a; Umehara et al., 2010b). Interestingly, the H2A.Z N-terminal tail also harbours multiple acetylated lysines at similar intervals as those found on the H4 tail (Bonenfant et al., 2006; Bruce et al., 2005; Ishibashi et al., 2009; Millar et al., 2006). Therefore, it is possible that the bromodomains of Brd2 may also bind acetylated H2A.Z. To test this, we initially performed peptide pull-down assays using recombinant BD2 of Brd2 and acetylated peptides of H4, H2A.Z and H2A. However, under our assay conditions, we could not detect binding of BD2 to any of
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293T nuclei digestion → MNase digestion → mononucleosomes → lyse nuclei → collect soluble mononucleosomes → IP INPUT → add Flag M2 beads → Flag IP

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Figure 3-3. Brd2 binds to H2A.Z nucleosomes through the recognition of acetylated H4.

(A) Mononucleosomes IPs were prepared from cells expressing Flag-H2A.Z alone (UT) or either wild type (WT) YFP-Brd2 or a mutant version of YFP-Brd2, which contains a single point mutation in each of the bromodomains (BD). Levels of the tagged proteins were detected using both anti-GFP antibody as well as anti-Brd2 antibody. INPUT fraction represent 1% of immunoprecipitated material. The IP fraction represent 30%, for Brd2 blots, and 5%, for histone blots, of the total eluted volume. (B) A schematic of the workflow for mononucleosome immunoprecipitations performed in the presence of competing peptide. (C) Peptide competition assay: Western blots of eluted material from H2A.Z mononucleosome immunoprecipitations performed in the presence of various competing peptides. Sites of acetylation of the various peptides are indicated (UN, unacetylated peptide). “Tetra” represents H4 peptide acetylated at lysines residues 5, 8, 12, and 16.
the tested peptides (data not shown). It is possible that our recombinant BD2 did not include enough flanking sequences for proper folding. Alternatively, as the reported binding co-efficient for Brd2 BD2 binding to H4ac peptides is in the mM range (Huang et al., 2007; Umehara et al., 2010b), this suggests that this interaction is very weak, and, therefore, our peptide pull-down conditions may not have been optimized for efficient detection. As an alternative approach, we used the same series of peptides in peptide competition assays to ask whether any of them can compete Brd2 binding from the purified nucleosomes (see competition scheme depicted in Fig 3-3B). Consistent with the reported binding of Brd2 to acetylated K12 on H4 (Huang et al., 2007; Kanno et al., 2004; Nakamura et al., 2007; Umehara et al., 2010a; Umehara et al., 2010b), and the reported preference of Brd2 for tri- or tetra-acylated H4 peptides (Bock et al., 2011), the addition of acetylated H4 peptides (K12, or tetra acetylated at K5, K8, K12 & K16) efficiently competed away binding of Brd2 to H2A.Z nucleosomes (Figure 3-3C). However, neither H2A.Zac nor H2Aac peptides were able to perturb the interaction between Brd2 H2A.Z nucleosomes. Therefore, these data suggest that the acetylated H4 on H2A.Z nucleosomes is a critical contact site mediating the interaction between Brd2 and H2A.Z nucleosomes.

In light of this finding, we directly examined the acetylation levels on H2A.Z and H2A nucleosomes. We immunoprecipitated either H2A.Z, or H2A nucleosomes, and we compared the levels of acetylated H4 and H3 residues by Western blots. As shown in Figure 3-4, the acetylation status of all residues tested on H4 and H3 was indeed higher on H2A.Z nucleosomes compared to H2A nucleosomes. This difference was most apparent under basal (DMSO) conditions for most of the acetylation marks examined, suggesting that H2A.Z nucleosomes have inherently higher levels of acetylation. As histone acetylation is generally associated with
Figure 3-4. **H2A.Z nucleosomes are enriched with H3 and H4 acetylation, compared to H2A nucleosomes.** 293T cells were transfected with either Flag-H2A.Z or Flag-H2A. H2A.Z and H2A nucleosomes were immunoprecipitated following treatment with DMSO or TSA (200nM for 2 hrs)—eluted material was analyzed by Western blot for various acetylation marks on H4 (A) and H3 (B) using the indicated antibodies. “AcLys”, an anti-pan-acetyl lysine antibody, which detects both acetyl H4 and H3 bands in the Western blot, as indicated.
“open”, or euchromatin, and transcription, this observation is consistent with our previous work that showed that the H3 associated with H2A.Z nucleosomes have distinct methylation profiles compared to those associated with H2A nucleosomes. For example, we found an enrichment of euchromatic marks (H3K4me3) and a depletion of heterochromatin marks (H3K9me3) on H2A.Z nucleosomes, compared to H2A nucleosomes (Sarcinella et al., 2007). Acetylated lysine 12 on H4 has been reported to be a primary residue for recognition by the bromodomains of Brd2 (Huang et al., 2007; Kanno et al., 2004; Nakamura et al., 2007; Umehara et al., 2010a; Umehara et al., 2010b). Although we observed slightly higher levels of H4K12ac on H2A.Z nucleosomes, compared to H2A nucleosomes, the biggest difference appeared to be in the tetra-acetylated H4 modification, particularly in control-treated cells (compare lanes 1 & 3 in Figure 3-4A). This observation is consistent with the recent finding that Brd2 has a higher affinity for tri- or tetra-acetylated H4 peptides, compared to singly modified peptides (Bock et al., 2011).

In summary, our data suggest that Brd2 preferentially binds to H2A.Z nucleosomes, and that this interaction is primarily mediated through the recognition of acetylated H4 residues in the H2A.Z nucleosome. Furthermore, the preference of Brd2 for H2A.Z nucleosomes, over H2A nucleosomes, is likely due, in part, to the higher levels of acetylated H4 in the H2A.Z nucleosomes, providing increased binding sites for Brd2. On the other hand, since H2A nucleosomes also contain significant levels of H4 acetylation, the much higher preference of Brd2 for H2A.Z nucleosomes over H2A nucleosomes suggest that additional regions of contact or stabilization may be present in H2A.Z nucleosomes.
3.3.3  **Brd2 is recruited to AR-regulated genes in a manner dependent on H2A.Z and the bromodomains of Brd2**

To test the biological significance of the interaction between H2A.Z nucleosomes and Brd2, I examined their relationship in the context of androgen receptor (AR)-regulated genes. Work presented in Chapter 2 demonstrated that H2A.Z is required for the full activation of AR-regulated genes in the prostate cancer cell line LNCaP (Draker, Sarcinella, and Cheung, 2011). A similar study has also described a role for both isoforms of H2A.Z in AR-regulated gene expression (Dryhurst et al., 2012). Therefore, I sought to test whether Brd2 is also recruited to AR-regulated genes in a hormone-dependent manner. In particular, I focused on the enhancer and promoter regions of the PSA gene since H2A.Z is enriched at these elements. By ChIP analyses, the recruitment/enrichment of AR, H2A.Z, H4ac and Brd2 to the regulatory regions of the PSA gene was examined over a time period of 120 minutes following hormone treatment (Figure 3-5B). In agreement with our previous study, a net loss of H2A.Z occurs at the promoter, but not at the enhancer, following stimulation of the cells with hormone. Analysis of AR recruitment following stimulation of cells with hormone reveals a well-characterized pattern: Namely, AR is rapidly recruited to the enhancer and promoter, initially peaking at 60 minutes, followed by a second peak at 120 minutes. Analysis of Brd2 revealed that recruitment follows a similar pattern as AR, with two peaks of enrichment at 60 minutes and 120 minutes post-stimulation. Since the preferred binding site for Brd2 is the tetra-acetylated form of H4 (see Figure 3-3C and (Bock et al., 2011)), which we also demonstrated is enriched on H2A.Z nucleosomes (see Figure 3-4A), I examined the dynamics of this histone modification throughout the ChIP time-course experiment. Although H4ac levels increase over the time course, an initial lag is observed before the 30-minute time point at the enhancer and up to the 60 minute time point at the promoter, suggesting that increases in H4 acetylation occur
Figure 3-5. Brd2 is recruited to the PSA gene in androgen-treated cells. (A) A schematic of the prostate specific antigen (PSA) gene. Arrows indicate approximate positions of primers used for qPCR analysis. (B) ChIP analysis of the PSA gene following a time-course of androgen stimulation—antibodies used for immunoprecipitation are shown on the left. The data represent the fold-enrichment in hormone-stimulated cells relative to respective ethanol-treated controls (vehicle control). H2A.Z and H4ac (tetra-acetylated H4) ChIP data were also normalized to total H3 signal (data not shown) to account for changes in nucleosome density. All ChIP antibodies showed at least 10-fold enrichment over ‘no antibody’ control samples. Each qPCR reaction was performed in triplicate with each experiment repeated at least three times independently. Values are presented as means, ± standard deviation.
following the initial events of transcriptional activation. Interestingly, the recruitment of Brd2 showed a similar lag at both regions. This suggests that the recruitment of Brd2 to PSA is dependent on H4 acetylation levels.

Given that Brd2 is recruited to AR-regulated genes upon hormone activation, I next asked whether this recruitment is dependent on H2A.Z. To this end, Brd2 recruitment was examined by ChIP analysis in the H2A.Z knockdown cells that I previously generated and described in Chapter 2 (Draker, Sarcinella, and Cheung, 2011). Since H4 acetylation is important for the binding of Brd2 to H2A.Z nucleosomes, and H2A.Z nucleosomes have high levels of H4 acetylation, I also tested if H4 acetylation levels were affected in the H2A.Z knockdown cells. I specifically focused on the time point 60 minutes post-treatment since this corresponds to the first peak of Brd2 recruitment and H4 acetylation. Finally, two AR-regulated genes, PSA and KLK2, were examined to test for consistency. As shown in Figure 3-6B, knockdown of H2A.Z reduced recruitment of Brd2 to both the promoter and enhancer regions. Similarly, enrichment of the H4ac mark is reduced at both regions in the H2A.Z knockdown cells. The reduction of Brd2 recruitment and levels of H4ac we observe by ChIP analysis is not due to reduced overall levels in H2A.Z knockdown cells, since total levels of Brd2 and H4ac, as measured by Western blot, were comparable in scramble control cells and H2A.Z KD cells (see Figure 3-6A).

The recruitment of Brd2 to AR-regulated genes has not been reported before. Moreover, I discovered that the recruitment is dependent on the presence of H2A.Z. To test the importance of Brd2 in the transcriptional activation of AR-regulated genes, I initially attempted to generate stable Brd2 knockdown LNCaP cells using Brd2-targeting shRNA constructs. However, cells
Figure 3-6. H2A.Z influences H4 acetylation and Brd2 recruitment at AR-regulated genes.  
(A) Western blot analyses of whole-cell lysates from LNCaP cells stably expressing either a 
scrambled control shRNA or an shRNA targeting H2A.Z mRNA. Tubulin and H3 were used as 
of loading controls.  (B) ChIP analysis of Brd2 and acetylated H4 at the PSA and KLK2 genes. 
Knockdown of H2A.Z reduces the recruitment of Brd2 as well as the acetylation of H4 at AR-
regulated genes following hormone stimulation. The data represent the fold-enrichment in 
hormone-stimulated cells relative to respective ethanol-treated controls (vehicle control). H4ac 
ChIP was also normalized to H3. All ChIP antibodies showed at least 10-fold enrichment over 
‘no antibody’ control samples. Each qPCR reaction was performed in triplicate with each 
experiment repeated at least three times independently. Values are presented as means, ± 
standard deviation.
that showed good knockdown of Brd2 also displayed growth defects, which precluded AR-activation studies. Brd2 is an essential gene and published literature has alluded to the fact that studying Brd2 function by shRNA knockdown is problematic (Denis, 2010). To address this question using a different approach, I took advantage of the recently developed small molecule inhibitor, JQ1, which binds the bromodomains of Brd2 (and other BET family members) and interferes with their binding to acetylated lysines (Filippakopoulos et al., 2010). By pre-treating the hormone-stimulated cells with JQ1, the binding of Brd2 to chromatin could be disrupted, allowing me to test whether this would affect AR-regulated gene expression. To first confirm that JQ1 could perturb interaction between Brd2 and H2A.Z nucleosomes, I performed H2A.Z mononucleosome IPs using lysate from 293T cells treated with vehicle control (DMSO) or JQ1 and compared the levels of Brd2 immunoprecipitated between the two samples sets. As seen in Figure 3-7A, pre-treatment of cells with JQ1 prior to immunoprecipitation reduces the amount of Brd2 associated with H2A.Z nucleosomes. JQ1 treatment does not significantly affect total levels of Brd2, although a slight decrease in total levels of H4ac was observed (3-7B). These observations validate the importance of Brd2’s bromodomains in binding H2A.Z nucleosomes in vivo. Given that JQ1 can disrupt the interaction between Brd2 and H2A.Z nucleosomes, and that both are involved in the transcription of androgen-responsive genes, I next tested the effects of JQ1 treatment on androgen-stimulated gene expression in LNCaP cells. When LNCaP cells were pre-treated with JQ1 prior to stimulation with hormone, expression of both PSA and KLK2 were reduced, in a dose-dependent manner (Figure 3-7C). This suggests that BET family members, such as Brd2, make a significant contribution to the expression of AR-regulated genes. This conclusion is in agreement with the ChIP data in Figure 3-5B, showing recruitment
Figure 3-7. JQ1 interferes with the interaction between Brd2 and H2A.Z nucleosomes, and inhibits expression of AR-regulated genes. (A) H2A.Z mononucleosome immunoprecipitations were performed using cells transfected with Flag-H2A.Z, and pre-treated with DMSO or the small molecule inhibitor JQ1 prior to harvest—mononucleosomes were immunoprecipitated with anti-Flag antibody. Input (1%) and IP (5%, for histones, or 30%, for Brd2, of eluted volume) material was used for Western blotting. H3 and Flag blots are shown for loading purposes. (B) Whole-cell lysates of LNCaP cells treated with JQ1 (125nM or 250nM) or DMSO for 24 hrs were analyzed by Western blot. Alpha tubulin and H3 are shown as loading controls. (C) RT-qPCR analysis of LNCaP cells pre-treated with JQ1 or DMSO for 24 hrs, and then stimulated with 10nM of DHT for 24hrs. Analysis of both the PSA and KLK2 genes are shown.
of Brd2 to the PSA gene during transcriptional activation. Given the effect of JQ1 treatment on gene expression, I predicted that JQ1 treatment would inhibit the recruitment of Brd2 to AR-regulated genes. To test this, Brd2 recruitment in JQ1-treated cells was examined by ChIP analysis. In addition, I also included analysis of H4ac. As shown in Figure 3-8, Brd2 recruitment is reduced at the enhancer and promoter regions of the PSA gene in cells treated with JQ1, compared to mock-treated cells. This observation was also confirmed at the KLK2 gene. Furthermore, levels of H4ac show a slight reduction in JQ1-treated cells, which is also seen at a global level in LNCaP cells treated with JQ1 (Figure 3-7B).

Since JQ1 treatment has such a dramatic effect on expression of AR-regulated genes, and is not specific to Brd2 but targets all members of the BET family, it is possible that other BET proteins are involved in AR-regulated gene expression. Brd4 is a well-characterized transcriptional co-activator that binds acetylated histones at gene promoters to recruit the transcription elongation factor p-TEFb as well as other transcription co-activators (Rahman et al., 2011; Wu and Chiang, 2007). Therefore, by ChIP, I tested whether Brd4 is also recruited to the regulatory elements of AR-regulated genes upon stimulation with hormone. This analysis was done in both DMSO-treated and JQ1-treated cells. As shown in Figure 3-9A, Brd4 was recruited to both PSA and KLK2 genes, and its recruitment was impaired by JQ1 treatment. Since Brd4 is also recruited to these genes, and binds acetylated histones, which are enriched in H2A.Z nucleosomes, the possibility exists that Brd4 also interacts with H2A.Z nucleosomes. Although Brd4 was not identified in our mass spec analysis, I performed mononucleosome immunoprecipitations using cells expressing Flag-H2A.Z or Flag-H2A. Basal and hyperacetylated conditions were also compared by DMSO and TSA treatment, respectively. As shown in Figure 3-9B, Brd4 shows a similar enrichment on H2A.Z nucleosomes, compared to H2A nucleosomes, under both DMSO-
Figure 3-8. JQ1 treatment of LNCaP cells reduces androgen-stimulated recruitment of Brd2 and H4 acetylation at AR-regulated genes. PSA and KLK2 regulatory regions were analyzed by ChIP for Brd2 recruitment and H4 acetylation (tetra-acetylated). Cells were pre-treated with JQ1 (250nM) or DMSO for 24 hrs prior to stimulation with DHT (10nM) or ethanol for 60 min. Data represent fold enrichment of DHT-treated cells relative to the respective ethanol-treated controls. H4 ChIP data was also normalized to H3 ChIP to control for nucleosome density. All ChIP antibodies showed at least 10-fold enrichment over ‘no antibody’ control samples. Each qPCR reaction was performed in triplicate and the experiment repeated two times independently. Values are presented as means, ± standard deviation.
Figure 3-9. Brd4 is recruited to AR-regulated genes in an H2A.Z-dependent manner. (A) ChIP analysis of Brd4 at PSA and KLK2 genes in LNCaP cells treated with JQ1 (250nM) or DMSO 24 hrs prior to stimulation with 10nM DHT or ethanol for 60 min. (B) Mononucleosomes were immunoprecipitated from 293T cells transfected with Flag-H2A or Flag-H2A.Z. Input (1%) and IP (5%, for histones, or 30% for Brd2 & Brd4, of eluted volume) material was used for Western blotting. H3 and Flag blots are shown for loading purposes. (C) ChIP analysis of Brd4 in LNCaP cells treated with ethanol or DHT for 60 minutes. ChIP data represent fold enrichment of DHT-treated cells relative to the respective ethanol-treated controls. Each qPCR reaction was performed in triplicate and the experiment repeated two times independently. Values are presented as means, ± standard deviation.
and TSA-treated conditions. Given these data, it is possible that Brd4 recruitment to AR-regulated genes also depends on H2A.Z. Therefore, I also performed ChIP analysis of Brd4 in H2A.Z knockdown cells, as described for Brd2 and H4ac in Figure 3-7. Analysis of Brd4 recruitment to the PSA and KLK2 genes in H2A.Z knockdown cells suggests that the recruitment is also dependent on H2A.Z. Collectively, these experiments argue that H2A.Z nucleosomes may influence the recruitment of several BET family members to AR-regulated genes upon transcriptional activation.

3.4 DISCUSSION

In order to gain a deeper understanding of the physiological functions of H2A.Z, we reasoned that proteins acting downstream of H2A.Z must first engage this histone variant in the context of chromatin. Therefore, we specifically chose to perform a proteomics screen to identify proteins that preferentially associate with H2A.Z nucleosomes, as compared to nucleosomes containing the core histone H2A. This is a novel departure from the previously published approaches that focused on identifying and characterizing soluble H2A.Z-interacting proteins, which often led to the identification of histone chaperones and remodeling complexes, such as the Swr1 complex, which deposits H2A.Z into chromatin (Kobor et al., 2004; Luk et al., 2007; Mizuguchi et al., 2004; Straube, Blackwell, and Pemberton, 2010). Indeed, the top proteins identified by our screen represent mostly chromosomal proteins and a number of them are either known regulators of transcription, or are predicted to be involved in the regulation of gene expression. Moreover, many of the proteins contain conserved structural motifs that are predicted to recognize and bind histone PTMs, which is consistent with the idea that these proteins are recruited to H2A.Z-containing chromatin to mediate downstream functions. The idea of histone modifications acting to recruit downstream effector proteins/complexes to mediate specific biological outcomes was the basis for the proposed Histone Code Hypothesis (Strahl and Allis,
While the use of the word “code” in this context has been a matter of debate, it is nevertheless clear that specific combinations of PTMs do cluster and function together. Moreover, many examples have shown that effector proteins can engage chromatin in a multivalent manner, recognizing multiple PTMs and multiple histone proteins at once (Agricola et al., 2011; Eustermann et al., 2011; Nady et al., 2011; Ruthenburg et al., 2011). Previous work from our lab has shown that H2A.Z nucleosomes contain a unique set of methylation marks on H3, compared to H2A nucleosomes (Sarcinella et al., 2007). The work presented in this chapter has extended this observation and showed that H2A.Z nucleosomes are also enriched for various acetylation marks on H3 and H4. Furthermore, initial structural studies of the H2A.Z nucleosome revealed that the presence of the H2A.Z-H2B dimer altered the docking domain with the H3-H4 tetramer, and the H2A.Z-H2B dimer contains an extended acidic patch, which is displayed on the surface of the octamer and could serve as a unique site of interaction with other proteins (Suto et al., 2000). From these data it can be concluded that H2A.Z nucleosomes display a combination of unique surfaces, sequences, and PTMs, which increases the complexity of potential binding platforms for recruiting downstream effector proteins.

Since H2A.Z incorporation into chromatin creates unique surfaces within the context of the entire nucleosome, understanding the physiological function of H2A.Z must take this into consideration. Effector proteins engaging H2A.Z nucleosomes could use a multitude of surfaces on the nucleosome, either alone or in combination, potentially without making direct contact with H2A.Z itself. In support of this view, several of the proteins identified in our screen correlated with an enrichment of specific PTMs on the H2A.Z nucleosomes. For example, WDR5 has been shown to recognize H3K4me3 (Wysocka et al., 2005), and our lab has previously showed that this mark is enriched on H2A.Z nucleosomes (Sarcinella et al., 2007). This concept is further supported by a very recently published study examining binding partners
of H2A-, H2A.X- and H2A.Z-nucleosome-interacting proteins through mass spectrometry analysis, whereby the authors used recombinant nucleosomes, lacking any PTMs, to identify nucleosome-interacting proteins from HeLa cell nuclear lysate (Fujimoto et al., 2011). Of the proteins identified by Fujimoto et al. that interacted with H2A.Z nucleosomes, only one appears in our list of top interacting proteins, SF3B1, which is a component of the SF3B spliceosome complex. This interaction is likely to depend mainly on recognition of nucleic acids and therefore may not be heavily influenced by histone PTMs. The remaining proteins in our list of top interactors, many of which contain motifs that recognize histone PTMs, were not identified by Fujimoto et al. This suggests that histone PTMs significantly influence the engagement of nucleosome-interacting proteins in vivo. Since H2A.Z can uniquely affect PTMs on the nucleosome, preservation of these marks may be critical in characterizing effector proteins that bind H2A.Z nucleosomes. Therefore, our protocol using endogenous nucleosomes provides a significant advantage. Indeed, in characterizing the interaction between Brd2 and H2A.Z nucleosomes we showed that recognition of H4 acetylation by the bromodomains of Brd2 is critical for the interaction.

Many of the hits identified in our screen contain domains that recognize histone PTMs. However, with the exception of WDR5 and Brd2, which have been more extensively characterized, many of the proteins, such as PWWP2A and PHF6, currently have no known functions. Given the potential for these proteins to interact with H2A.Z nucleosomes through their respective domains, characterization of the PTMs that these domains recognize on H2A.Z nucleosomes may provide important clues regarding their biological functions.

In this study, we have identified Brd2 as a novel interactor with H2A.Z nucleosomes. The data suggest that Brd2 binds to H2A.Z nucleosomes mainly through acetylated H4. Indeed, we
found that H2A.Z nucleosomes have increased levels of H4 acetylation, but the difference in the levels of H4 acetylation between H2A.Z and H2A nucleosomes does not seem to account for the large difference in Brd2 binding between the two types of nucleosomes. This suggests that additional contact points may exist on the H2A.Z nucleosome, or additional factors help to stabilize Brd2 once bound via acetylated H4 contacts. This hypothesis is in agreement with previous studies characterizing the bromodomains of Brd2, BD1 and BD2. First, Brd2 appears to form a homodimer through BD1-BD1 interactions, resulting in an additional negatively charged binding pocket at the dimer interface, which may serve as an additional surface for interaction with chromatin (Nakamura et al., 2007). This observation also implies that the physiological interaction of Brd2 with chromatin may involve the engagement of up to four bromodomains simultaneously. Second, although both BD1 and BD2 preferentially bind acetylated H4 tails, recognition of acetylated H3 and H2B has also been reported (Kanno et al., 2004; Umehara et al., 2010b). Furthermore, although H2A.Z peptides were not able to compete away Brd2 binding in the peptide competition assay, we cannot rule out that Brd2 also makes contact directly with other regions of H2A.Z. Taken together, these data suggest that Brd2 engages H2A.Z nucleosomes in a complex, multivalent manner. The complexity of this interaction also highlights the importance of our approach in characterizing the ability of H2A.Z to influence the recruitment of proteins to chromatin, i.e. the use of nucleosomes as opposed to peptides. A recent study (Nikolov et al., 2011) also supports this view; the authors compared the recruitment of H3 methyl-binding proteins using either peptides or recombinant nucleosomes. Only a small number of the H3 methyl-binding proteins were recruited by both peptides and nucleosomes. Importantly, this suggests that many effector proteins recognize histone PTMs specifically in a nucleosome or chromatin context.
To complement the characterization of Brd2 as an H2A.Z nucleosome-binding protein, I was able to demonstrate that Brd2 is a novel regulator of androgen-responsive genes in LNCaP cells. My ChIP analysis of the PSA gene showed that Brd2 is recruited to the promoter and enhancer regions following stimulation of cells with hormone. Accordingly, increases in the acetylation of H4 were also observed. Since Brd2 preferentially binds H2A.Z nucleosomes, and H2A.Z is deposited at promoters and enhancers of AR-regulated genes, I tested the dependence of Brd2 recruitment to the PSA and KLK2 genes using H2A.Z knockdown cells that I described in Chapter 2 (Draker, Sarcinella, and Cheung, 2011). In H2A.Z knockdown cells, both Brd2 recruitment and H4ac levels are reduced at both genes. Furthermore, chemical inhibition of the bromodomains of Brd2 using the small molecule inhibitor JQ1 reduced expression of AR-regulated genes, and also inhibited recruitment of Brd2. Taken together, these data support an in vivo model whereby, at AR-regulated genes, H2A.Z establishes a unique platform for the recruitment of transcriptional co-activators, such as Brd2. A similar analysis of Brd4 further suggests that H2A.Z nucleosomes also facilitate the recruitment of additional BET family members.

The recruitment of Brd2 to H2A.Z nucleosomes depends on the recognition of acetylated lysines; however, a complex multivalent interaction is likely to occur in vivo. Indeed, recent studies have reported that bromodomains often exhibit low affinities for their acetylated targets (VanDemark et al., 2007; Zeng et al., 2008). However, as suggested by Voigt and Reinberg (2011), a chromatin template within the cell nucleus could provide local high concentrations of PTMs. In turn, this could enhance the binding of low affinity interactions, yet would maintain their dynamic nature, allowing for rapid responses to cellular signals. Therefore, we hypothesize that the unique binding surfaces of H2A.Z nucleosomes act as integral platforms for early critical nucleation events, such as the recruitment of transcriptional co-activators. Based
on our data, H2A.Z nucleosomes may provide localized sites of H4ac, to promote the early stages of recruitment of factors such as Brd2 and Brd4. Recruitment of histone acetyltransferases (HATs) would then allow for subsequent increases and spreading of histone acetylation, which would promote further recruitment of Brd2, and other factors dependent on histone acetylation. It has previously been reported that Brd2 is associated with acetyltransferase activity towards H4 and H2A (Sinha, Faller, and Denis, 2005). If initial engagement of Brd2 also recruits HAT activity, this would provide a positive feedback loop for Brd2 recruitment to AR-regulated genes. This model is therefore also compatible with the observation that there is a loss of H2A.Z at the promoters of AR-regulated genes, following hormone stimulation. In this model (Figure 3-10) H2A.Z nucleosomes participate in early recruitment events, but not the subsequent retention of the factors involved, such as Brd2. Following initial activation, nucleosome loss or H2A.Z-H2B dimer ejection would then also provide access to underlying DNA sequences that are necessary for transcription factor binding, such as the AR. Alternatively, residual levels of H2A.Z nucleosomes may be sufficient to maintain the gene in a transcriptionally active state, following the initial recruitment of critical transcription factors.

The importance of Brd2 recruitment in the transcriptional activation of AR-regulated genes was demonstrated in the experiments using the inhibitor JQ1. Treatment of LNCaP cells with JQ1 had a dramatic effect on the expression of AR-regulated genes, and also significantly inhibited the recruitment of Brd2 to those genes. This suggests that Brd2 recruitment is a critical early event in the activation of these genes. Although the experiments with JQ1 were focused on its effect on Brd2, JQ1 inhibits other BET family members as well (Filippakopoulos et al., 2010), but the role of other BET proteins in AR-regulated transcription has not been reported. However, I did test for recruitment of Brd4 to PSA and KLK2, and it showed a similar trend
Figure 3-10. A model of Brd2 recruitment to AR-regulated genes. Upon hormone stimulation, Brd2 is recruited to H2A.Z nucleosomes containing acetylated H4 lysines. Association of Brd2 with histone acetyltransferase (HAT) activity promotes acetylation of neighbouring nucleosomes. Recruitment of chromatin remodeling activity causes eviction of H2A.Z nucleosomes, which promotes the recruitment of DNA-binding transcription factors, such as AR, and spreading of the acetylated H4 mark promotes a subsequent spreading of Brd2. A similar mechanism for Brd4 recruitment may also exist.
observed for Brd2, which was also blocked by JQ1 treatment (Figure 3-9A). Since both Brd2 and Brd4 participate in expression of AR-regulated genes, this may explain the dramatic effect JQ1 has on the expression of these genes. Furthermore, I also found that Brd4 is enriched on H2A.Z nucleosomes compared to H2A nucleosomes. Accordingly, Brd4 recruitment to AR-regulated genes was abrogated in H2A.Z knockdown cells. Together, these data suggest that H2A.Z influences the recruitment of multiple BET family members to AR-regulated genes. Given that JQ1 has such a dramatic effect on AR-regulated transcription, it may have potential therapeutic uses in the treatment of prostate cancer. The use of JQ1 has already shown promise in the treatment of leukemia, based on several recent studies, which reported dramatic effects on the growth and differentiation of leukemia cells (Delmore et al., 2011; Zuber et al., 2011). In light of these results, the dramatic effects of JQ1 on cancer cells underscores a critical role for the docking domains of effector proteins, and their engagement with chromatin, in the translation of cellular signaling pathways.

In conclusion, this study has provided novel insights into the physiological functions of H2A.Z, in its ability to engage chromatin-binding proteins through its influence on nucleosome PTMs. Characterization of the interaction between Brd2 and H2A.Z nucleosomes also furthered our understanding of the role H2A.Z plays in promoting AR-regulated transcription in prostate cancer cells, yielding potential new targets for therapy. Our data generated from MS analysis of proteins binding to H2A.Z nucleosomes will serve as a useful tool in future studies of H2A.Z’s role in various chromatin-templated processes.
Chapter 4

Thesis summary, General Conclusions and Future Directions
4 CONCLUSIONS AND FUTURE DIRECTIONS

4.1 THESIS SUMMARY

The central aim of this thesis was to characterize the role of H2A.Z in the regulation of transcription. In particular, I was interested in studying how histone PTMs contributed to this process. This approach led to the identification of a ‘writer’ (Ring1b–see Appendix I), ‘eraser’ (USP10), and ‘reader’ (Brd2), of histone PTMs that participate in transcriptional regulation by H2A.Z.

We had previously hypothesized that PTMs on H2A.Z are critical in committing the variant to distinct functional roles within chromatin (Draker and Cheung, 2009). Specifically, in a previous study from our lab (Sarcinella et al., 2007), we noted that H2A.Zub1 was present in heterochromatin, and RNAi studies revealed that the ubiquitin E3 ligase Ring1b targets H2A.Z for ubiquitylation (see Appendix I). This finding was significant in that Ring1b is part of a transcriptional repression complex, PRC1, providing further evidence that H2A.Zub1 is directly involved in transcriptional repression. The work presented in Chapter 2 was based on our hypothesis that the removal of ubiquitin from H2A.Zub1 would be involved in transcriptional activation. To test the hypothesis, I used a candidate approach to identify the deubiquitylase USP10 as an enzyme that regulates levels of H2A.Zub1, as well as H2Aub1. Two other DUBs that had previously been reported to have activity towards H2Aub1, USP16 and 2A-DUB, also showed activity towards H2A.Zub1. Furthermore, I demonstrated that H2A.Z is present at the enhancer and promoter regions of AR-regulated genes and that deubiquitylation of H2A.Zub1 by USP10 is required for the full activation of AR-regulated genes. Collectively, these data support our hypothesis that ubiquitylation of H2A.Z occurs during transcriptional repression, and
that removal of this mark is an important step of transcriptional activation at genes regulated by H2A.Z.

In Chapter 3, we moved away from a focus on the PTMs of H2A.Z, and the enzymes that regulate those marks. Instead, we expanded our view of how H2A.Z regulates chromatin by considering the entire H2A.Z nucleosome as a cohesive surface for recruiting proteins to chromatin. In particular, we identified proteins that preferentially associate with H2A.Z nucleosomes, compared to H2A nucleosomes, using a proteomics approach. We noted that many of the interacting proteins contain domains that potentially bind specific histone PTMs. Indeed, the characterization of Brd2 demonstrated that its interaction with H2A.Z nucleosomes is critically dependent on the recognition of H4 acetylation in H2A.Z nucleosomes, and that H4 and H3 acetylation are enriched on H2A.Z nucleosomes. The interaction between Brd2 and H2A.Z nucleosomes was validated \textit{in vivo} by demonstrating that Brd2 is recruited to AR-regulated genes upon transcriptional activation in a manner dependent on H2A.Z and the bromodomains of Brd2.

These studies suggest the existence of a unique H2A.Z ‘code’. Distinct collections of PTMs on both H2A.Z, and the H2A.Z nucleosome, define this code and specify distinct functional roles of H2A.Z in chromatin. The specific writers, erasers, and readers, associated with a particular functional role, are crucial in mediating downstream functional outcomes, as demonstrated by the characterization of USP10 and Brd2. Therefore, future studies characterizing the spectrum of PTMs, and their corresponding effector proteins, will be central to defining specific functions of H2A.Z.
4.2 GENERAL DISCUSSION AND FUTURE DIRECTIONS

4.2.1 Characterizing a role of H2A.Zub1 in transcriptional regulation

At the inception of this thesis, very little was known about H2A.Zub1, including the identity of the enzymes that regulate this modification. Similarly, very little was also known about H2Aub1. Ring1b had been identified as an E3 ligase, linking H2A.Zub1 to polycomb-mediated transcriptional silencing (de Napoles et al., 2004; Fang et al., 2004). However, at the time, the only DUB described for H2A was Ubp-M (USP16), which was characterized as a mitotic DUB of H2Aub1 (Cai, Babbitt, and Marchesi, 1999). No evidence was yet available linking H2Aub1 deubiquitylation to transcriptional activation.

We had previously observed that H2A.Zub1 was found in heterochromatin, suggesting that H2A.Zub1 might participate in transcriptional repression. Therefore, we predicted that deubiquitylation of H2A.Zub1 would function in transcriptional activation. Testing this prediction, I used a candidate approach to identify USP10 as the first described DUB of H2A.Zub1. More importantly, deubiquitylation of H2A.Zub1 by USP10 was demonstrated at AR-regulated genes during transcriptional activation, providing in vivo evidence for our prediction that deubiquitylation of H2A.Zub1 is part of transcriptional activation. These studies yielded novel insight into transcriptional regulation by H2A.Z, particularly since much of the field was, and still is, focused on studying H2A ubiquitylation and deubiquitylation.

During the course of this thesis work, while characterizing the role of USP10 as a deubiquitylase of H2A.Zub1, several DUBs were reported for H2Aub1, many of which were also linked to transcriptional activation. Deubiquitylation of H2Aub1 by USP21, for example, was shown to be important for liver regeneration in the mouse (Nakagawa et al., 2008), and USP16, was also described to have a transcriptional activation role by deubiquitylating H2Aub1 during expression
of Hoxd10 in HeLa cells (Joo et al., 2007). USP22 was identified as a component of the human SAGA transcriptional coactivator complex, with activity against H2Aub1 and H2Bub1 (Zhao et al., 2008). Interestingly, USP22 was also reported to participate in the transcriptional activation of AR-regulated genes. Another group has also reported a role for H2Aub1 in the regulation of androgen-responsive genes. Zhu et al. (2007) found that H2Aub1 was present at the promoter of the PSA gene in LNCaP cells, the levels of which decreased upon gene activation in a manner dependent on the H2Aub1 DUB, 2A-DUB.

A curious observation arising from my work is that H2A and H2A.Z share an E3 ligase in Ring1b, and, at least several DUBs (USP10, USP16, and 2A-DUB). Two DUBs of H2A have also been described in the transcriptional activation of AR-regulated genes: USP22 (Zhao et al., 2008) and 2A-DUB (Zhu et al., 2007). Although USP10 was previously described as a transcriptional co-activator of AR-regulated genes (Faus et al., 2005), by identifying H2A.Zub1 and H2Aub1 as substrates of USP10, we were able to define a specific role for USP10 in the transcriptional activation of AR-regulated genes. More importantly, although many groups have focused on studying H2Aub1 by identifying novel enzymes that regulate H2A ubiquitylation, our studies have provided a novel perspective by focusing on ubiquitylation and deubiquitylation of the variant H2A.Z. Through the studies presented here, it is now clear that, in addition to H2Aub1, H2A.Zub1 functions in transcriptional regulation. Since there is overlap in the enzymes that regulate H2A.Zub1 and H2Aub1, the mechanistic role of each may also have some overlap.

Since current evidence suggest that H2Aub1 and H2A.Zub1 may have overlapping functions in transcriptional regulation, testing this prediction and teasing apart potential differences between H2Aub1 and H2A.Zub1 will require the development of new reagents. Studies characterizing
H2Aub1 in transcriptional regulation have been greatly facilitated by the availability of a commercial antibody that specifically recognizes H2Aub1 (Vassilev et al., 1995). In contrast, no such reagent is currently available for H2A.Zub1.

4.2.2 Utility of an H2A.Zub1-specific antibody

A major limitation in studying H2A.Zub1 has been the lack of an antibody that specifically recognizes H2A.Zub1. Development of this reagent would provide a significant technical advancement, greatly facilitating the study of H2A.Zub1. In the absence of such a reagent, we have depended on more indirect methods of analysis. For example, our initial characterization of H2A.Zub1 in vivo relied on immunofluorescence-based assays, comparing the differential staining patterns of our two H2A.Z antibodies—one that recognizes both the ub1-modified and non-ubiquitylated forms (L1 antibody), and another that only recognizes the non-ubiquitylated form (see Appendix I for a more detailed summary). Analysis of H2A.Zub1 at the PSA and KLK2 genes presented in Chapter 2 was done using an indirect approach by using ChIP re-ChIP analysis in cells that were stably expressing HA-tagged ubiquitin. Several studies have also applied this approach to study H2Aub1 (Joo et al., 2007; Wei et al., 2006; Zhao et al., 2008), although a monoclonal IgM antibody does exist that recognizes H2Aub1 (Vassilev et al., 1995). Perhaps the most viable approach to generating a highly specific antibody would be through the use of branched peptide based on the C-terminus of H2A.Z containing the isopeptide bond of the ubiquitin moiety at lysine 120 (Sarcinella et al., 2007) as an immunogen. This approach was used to generate a highly specific monoclonal antibody against the monoubiquitylated form of H2B (Minsky et al., 2008). In contrast, the H2Aub1 antibody was generated using “residual nuclear pellet proteins”. Consequently, the specificity of this antibody is questionable, particularly against whole-cell lysate; indeed, my own experience with this antibody in Western blotting applications has revealed that it can recognize all of the core histones.
Beyond its utility in reliably assessing the levels of H2A.Zub1 at individual genes using standard ChIP assays, an antibody specific for H2A.Zub1 would facilitate genome-wide studies of H2A.Zub1’s distribution. ChIP-seq based approaches would greatly expand our current knowledge of genes that are regulated by H2A.Zub1. Studies in ES cells have shown that H2A.Z is present at polycomb-repressed genes, and co-localizes with polycomb group proteins such as Ring1b and Suz12 (Creyghton et al., 2008; Illingworth et al., 2012). Since we have identified Ring1b as an H2A.Z E3 ligase, we would predict that polycomb-repressed genes in ES cells are enriched with H2A.Zub1. Therefore, an H2A.Zub1 antibody could aid in testing this prediction. Similar types of studies would ideally be conducted in a specific cell-type or system chosen for in-depth follow-up. For instance, probing androgen-responsive cells for genome-wide distribution of H2A.Zub1 could be combined with AR ChIP-seq data; overlapping genes could potentially identify a unique subset of genes that are regulated by both H2A.Zub1 and AR.

A link between H2A.Z and cancer progression has previously been reported. Overexpression of H2A.Z has been observed in breast cancer cells (Hua et al., 2008; Svolcis et al., 2010) and the acetylated form of H2A.Z is associated with gene deregulation in prostate cancer cells (Valdes-Mora et al., 2012). Moreover, overexpression of several polycomb group proteins has been reported in a variety of tumors (Richly, Aloia, and Di Croce, 2011). Hence, changes in H2A.Zub1 levels may also occur in the diseased state, and would be predicted to associate with aberrant gene silencing. Since there is also accumulating evidence that global levels of certain histone modifications correlate with specific stages of disease, such as cancer (see Chapter 1.2.1.5), analysis of the global levels of H2A.Zub1 in disease versus normal tissues could be of significant prognostic value.
4.2.3 Identifying novel E3 ligases and DUBs

Identifying the collection of E3 ligases and DUBs that regulate H2A.Zub1 levels would also deepen our understanding of how this PTM is regulated, and would provide insight into its function within chromatin. Although a candidate approach was used in this thesis to identify Ring1b and USP10, a genome-wide approach would offer the advantage of a large-scale assessment in an unbiased manner. Additionally, a specific cell-type and system could prove to be most informative since many of these enzymes may have cell-specific functions.

Commercially available siRNA libraries targeting the E3 ligase family or the DUB family of enzymes provide a method for large-scale analysis. Used in combination with Western blotting to measure the levels of H2A.Zub1 would provide an unbiased method for identification of specific ‘readers’ and ‘writers’ that contribute to regulating H2A.Zub1 levels. Parallel assessment of H2Aub1 and H2Bub1 would also provide insight regarding enzyme specificity.

Following the preliminary identification of enzymes, follow-up studies would be required to validate the results, including the use of in vitro assays. Additional genome-wide approaches could also prove useful in follow-up studies of individual enzymes. For example, if a suitable antibody were available, ChIP-seq studies of the specific enzyme, combined with ChIP-seq analysis of H2A.Zub1 would provide a candidate list of genes that are regulated through both the enzyme and H2A.Zub1. Alternatively, RNA-seq analysis in control and knockdown cells (using a specific knockdown of the enzyme of interest) would serve a similar purpose when combined with the with H2A.Zub1 ChIP-seq data. If knockdown studies were used, combining the data from genome-wide analysis of H2A.Zub1 would be more critical. This is because there are now many examples of well-characterized histone modifying enzymes having non-histone substrates (Chuikov et al., 2004; Ea and Baltimore, 2009; Huang et al., 2006a; Rathert et al.,...
Therefore, genes affected by the knockdown of a particular enzyme would also need to be validated for the presence of H2A.Zub1 at the gene.

Following the identification of individual enzymes, a mass spec approach to identifying interacting proteins may yield information regarding the specific complexes with which the enzyme associates. This would be useful in identifying factors necessary for regulating activity and also targeting the enzyme. For example, it was found that both USP12 and USP46 interact with the protein WDR48, which is required for the enzymatic activity of the two DUBs towards H2Aub1 (Joo et al., 2011). A similar explanation may exist regarding the lack of activity of USP10 and 2A-DUB towards H2Aub1 in the in vitro deubiquitylation assay (Figure 2-3). Therefore, it is possible that the DUBs involved in AR-regulated gene expression may exist in distinct complexes to provide unique substrate specificity and temporal regulation. Indeed, a 2009 study employing a global proteomic analysis of the interacting proteins of 75 DUBs from the human genome revealed that 36% of these enzymes were associated with WD proteins (Sowa et al., 2009). The authors found that DUBs are frequently found in multi-protein complexes, the components of which were predicted to contribute to the regulation of DUB activity and substrate specificity.

### 4.2.4 Defining an H2A.Z ‘code’

It is well established that H2A.Z regulates various chromatin-based processes, and as such has distinct localization throughout the chromatin fibre (see Chapter 1.2.3.1). Accordingly, H2A.Z nucleosomes are unique in their signature of PTMs. Bulk H2A.Z nucleosomes from 293T cells contain a relative enrichment of euchromatic marks such as H3K4me2/3 and depletion of heterochromatic marks such as H3K9me2/3 compared to H2A nucleosomes (Sarcinella et al., 2007). Subsequent analysis described in Chapter 3 showed that H2A.Z nucleosomes also
contain higher levels of H4 and H3 acetylation. Our mass spec analysis revealed that H2A.Z nucleosomes preferentially or specifically interact with a unique set of proteins compared to H2A nucleosomes. Many of the interacting proteins also have conserved domains that can recognize histone PTMs. Indeed, analysis of the interaction between Brd2 and H2A.Z nucleosomes revealed a dependence on H4 acetylation, a PTM enriched on H2A.Z nucleosomes. Collectively, these data suggest that H2A.Z influences PTMs of the histone octamer, which then influences the specific recruitment of downstream effector proteins. Since PTMs of H2A.Z can also modify its function, the collective signature of H2A.Z-nucleosome PTMs may specify an H2A.Z-based ‘code’. How important is this ‘code’ in specifying H2A.Z’s function? To answer this question, future studies that fully characterize this ‘code’ will significantly extend our current understanding of the unique functions of H2A.Z.

Although we have characterized a subset of PTMs enriched on H2A.Z nucleosomes, our approach relied on Western blotting to evaluate individual modification on eluted nucleosomes by SDS-PAGE. Reliance on antibody-based approaches to fully assess PTM signatures of H2A.Z nucleosomes has several disadvantages. Firstly, the use of antibodies for PTM detection requires an a priori knowledge of the histone PTM. Second, inherent technical difficulties associated with antibody-epitope interaction, such as cross-reactivity and epitope occlusion can interfere with data interpretation. Specificity of antibodies generated against histone PTMs have recently come into question. A study of over 200 different antibodies directed against 57 different histone PTMs revealed that more than 25% of the antibodies failed specificity tests (Egelhofer et al., 2011). Epitope occlusion is also of particular concern since the co-existence of nearby PTMs can occlude recognition of individual modifications by antibodies. For example, antibody recognition of phosphorylated H3S10 is influenced by acetylation of the nearby K9 and K14 residues (Cheung, 2004). As a result, an alternative, unbiased approach to fully
characterizing the histone PTMs on H2A.Z nucleosomes would be preferable. In recent years, various quantitative mass spectrometry approaches have been employed to characterize histone proteins and their associated PTMs (Zee, Young, and Garcia, 2011). For example, a top-down approach analyzes full-length proteins, whereas the traditional bottom-up approach analyzes samples that have been enzymatically digested. Analysis of full-length protein offers the advantage of preserving combinations of PTMs present on the same histone protein, although this approach can suffer from poorer resolution. In contrast, SILAC (stable isotope labeling by amino acids in cell culture) uses a bottom-up approach that compares samples grown in the presence of heavy isotopes to samples grown under standard conditions. The spectral shift of labeled proteins facilitates a high-resolution quantification with low background. Regardless of the specific technique used, a mass spec-based approach would provide an unbiased analysis of all PTMs associated with H2A.Z nucleosomes.

Analysis of H2A.Z-nucleosome PTMs using a quantitative mass spec approach would be amendable to extensive inquiry into how specific modifications influence the nucleosome PTM signature. The use of epitope-tagged histones to purify mononucleosomes enables the use of mutant forms of H2A.Z. In particular, point mutations in key residues would allow for the generation of non-modifiable mutants. For example, mutation of the lysine residues 120, 121, and 125 to arginine in the C-terminus of H2A.Z prevents monoubiquitylation, as detected by Western blotting (Sarcinella et al., 2007). These types of mutants could be used in mass spec analysis of H2A.Z nucleosomes to assess their effect on the level of other histone PTMs, potentially identifying novel cis- or trans-histone PTM crosstalk. Moreover, they could also be used to test binding specificity of interacting proteins identified by mass spec analysis.

Comparison of proteins that interact with wild-type H2A.Z vs. a non-ubiquitylatable mutant, for example, could aid in identifying proteins that preferentially bind in the presence, or absence of
the monoubiquitin modification. This could be of particular interest with the ubiquitin PTM since little is known of proteins that bind ubiquitylated histones, whether directly or indirectly.

4.2.5 Mechanisms of transcriptional regulation by H2A.Z at androgen-responsive genes

My analysis of Brd2 recruitment to the PSA and KLK2 genes has yielded some insight into the existence of an H2A.Z code at AR-regulated genes. The ChIP data in Chapter 3 suggest that the recruitment of Brd2 depends on H2A.Z, and Brd2’s recognition of H4 acetylation. Extrapolating on our in vitro evidence of the interaction between Brd2 and H2A.Z nucleosomes, we interpret the in vivo data to suggest that H2A.Z nucleosomes at AR-regulated genes are localized sites of H4 acetylation. Considering that evidence presented in Chapter 2 argued for the presence of H2A.Zub1 at AR-regulated genes, an interesting possibility exists: At AR-regulated genes, under repressed conditions, H2A.Z nucleosomes may be modified with both monoubiquitin on H2A.Z and acetylation on H4. Ubiquitylation of H2A.Z may therefore function to prevent binding of co-activators such as Brd2 in the absence of activating signal. This hypothesis is analogous to the role described for H2Aub1 in transcriptional repression by antagonizing the binding of FACT (Zhou et al., 2008). Once transcription is initiated, H2A.Z is deubiquitylated allowing for localized recruitment of effector proteins, such as Brd2 through the recognition of pre-acetylated H4. Later events would result in amplification of the signal through recruitment of HATs, spreading of acetylation, which would lead to additional Brd2 recruitment, as depicted in Figure 3-10. The co-existence of both H2A.Zub1 and H4 acetylation on the same nucleosome agrees with the observation that, in yeast, incorporation of H2A.Z is stimulated by pre-acetylation of H2A and H4, i.e., H2A.Z is preferentially deposited into nucleosomes containing H2A and H4 acetylation (Altaf et al., 2010). Following deposition, H2A.Z would then need to be targeted for ubiquitylation at repressed genes. Alternatively, early
events post activation lead to preferential acetylation of H2A.Z nucleosomes, providing an initial binding platform for the early recruitment of Brd2, and other factors. These two proposed models of H2A.Z’s role in regulating gene expression at hormone-responsive genes is summarized in Figure 4-1. Both of these possibilities are readily testable, although testing the first possibility would be greatly facilitated by the availability of an H2A.Zub1-specific antibody.

In addition to USP10 and Brd2, it is likely that H2A.Z nucleosomes engage additional effector proteins during the regulation of androgen-responsive genes. Although an unbiased approach to identifying additional proteins would certainly be useful (as described in 4.2.4), known H2A.Z-nucleosome-interacting proteins could readily be tested in a manner similar to Brd2 and USP10. I have already validated preferential interaction between H2A.Z nucleosomes and both USP39, and PWWP2A (Figure 3-2A). Very little is known about the cellular functions of these two proteins. Since we have now characterized them as potential H2A.Z effector proteins, a simple first step in studying their functions could be to test the possibility for a potential role in AR-regulated gene expression.

4.3 CLOSING REMARKS

H2A.Z has a variety of functions within chromatin, a characteristic that perhaps contributes to it being essential for viability in more complex organisms. However, since it is not clear which function of H2A.Z is essential for viability, understanding the intricacies of each of its roles will help define its biological significance. Furthermore, although H2A.Z has distinct roles within chromatin, common mechanisms of how H2A.Z regulates these processes might exist. Therefore, uncovering the details of one particular role may provide novel insight into how H2A.Z regulates chromatin in general. In this vein, the research presented in this thesis on
Figure 4-1. Model of H2A.Z’s regulation of androgen-responsive genes. Upper pathway: H2A.Zub1 participates in transcriptional repression by blocking recruitment of HATs to the promoters and enhancers of AR-regulated genes. Activation signals lead to recruitment of USP10, causing the deubiquitylation of H2A.Zub1, which allows for recruitment of HATs to H2A.Z nucleosomes. Acetylation of H2A.Z nucleosomes provides docking sites for co-activators such as Brd2, which then promotes additional recruitment of HATs and amplification of histone acetylation in surrounding nucleosomes. Bottom pathway: H2A.Z nucleosomes are both ubiquitylated on H2A.Z and acetylated on H4 (and perhaps other residues). Recruitment of factors such as Brd2 is blocked by the ubiquitin moiety, maintaining the genes in a transcriptionally silent state in the absence of hormone. Activation signals recruit USP10 to the regulator elements, leading to deubiquitylation of H2A.Zub1 and exposure of the docking sites for Brd2 recruitment.
H2A.Z’s role in transcriptional regulation will also hopefully provide insight into H2A.Z’s other roles within chromatin. Put in context with the rest of the field, this thesis emphasizes the importance of PTMs on H2A.Z nucleosomes in dictating specific functional roles of H2A.Z. Therefore, testing the possibility of an H2A.Z code could help define distinct sets of H2A.Z nucleosome PTMs, and their cognate effector proteins, that are associated with specific functional roles. With new types of post-translational modifications still being discovered, in addition to the many that already exist, the possibility of an H2A.Z-based code is daunting in its potential complexity. However, in the current era of omics-based research, many more powerful tools and techniques are at our disposal to delve into the complexity H2A.Z codes, and I am proud to have contributed, in some way, to its humble beginnings.
Identification of Ring1b as an E3 Ubiquitin Ligase of H2A.Z

The data from this Appendix has been published as:


I performed all experiments.
Summary of the Study: “Monoubiquitylation of H2A.Z Distinguishes its Association with Euchromatin or Facultative Chromatin”

In this study, we found that a fraction of H2A.Z is monoubiquitylated. Expression of tagged forms of H2A.Z revealed a slower migrating band in SDS-PAGE and Western blotting analyses, which corresponded to a molecular weight consistent with monoubiquitylation. The identity of the shifted band as monoubiquitylated H2A.Z was confirmed by co-transfection of a GFP-tagged version of H2A.Z with HA-ubiquitin. Anti-GFP Western blot analysis of lysates revealed a second shifted band, corresponding to HA-ubiquitin-GFP-H2A.Z, and anti-HA Western blotting confirmed this interpretation. Moreover, mutation of lysine residues to arginine in the C-terminus of GFP-H2A.Z eliminates the presence of the shifted band, further suggesting that H2A.Z, like H2A, is monoubiquitylated at its C-terminus. By mutating the individual lysines in the C-terminus of H2A.Z, we also showed that all three lysines, 120, 121, and 125, could be targeted for ubiquitylation since only when all three residues were mutated to arginine was the shifted band eliminated in Western blotting. However, mutation of lysine 120 alone caused the biggest decrease in the level of the shifted band, suggesting that this residue is the preferred site in vivo.

In parallel with the above studies, we also generated two different antibodies directed against H2A.Z. Peptides corresponding to two regions where H2A.Z differs from H2A the most—the L1-loop (refer to Figure 1-3) and the C-terminus—were used as immunogens. In agreement with our conclusion that the C-terminus is monoubiquitylated, our antibody directed against this region does not detect the shifted band in Western blots, which we interpreted as epitope exclusion caused by the ubiquitin moiety. A similar observation was made using a commercially available antibody that is directed against the C-terminus. In contrast, our
antibody directed against the L1-loop region was able to detect both forms of H2A.Z. By comparing the staining patterns of the two antibodies in immunofluorescence studies of metaphase chromosomes from 293T cells, we showed that the monoubiquitylated form of H2A.Z is enriched on the inactive X chromosome. Silencing of the inactive X chromosome in female cells is known to occur through the polycomb silencing pathway, during which monoubiquitylation of H2A occurs by the activity of the polycomb protein, and E3 ligase, Ring1b (de Napoles et al., 2004; Fang et al., 2004). Since we detected H2A.Zub1 on the inactive X chromosome, we asked if Ring1b also mediates the monoubiquitylation of H2A.Z. To address this question, I co-transfected Flag-tagged versions of H2A or H2A.Z with a Ring1b shRNA construct, or a control shRNA (Scramble), then assessed changes in the levels of Flag-H2Aub1 and Flag-H2A.Zub1 by Western blot. As shown in Figure A-1A, knockdown of Ring1b protein levels caused a specific loss of Flag-H2A/H2A.Zub1. Moreover, overexpression of Ring1b, together with Bmi1 (a cofactor belonging to the PCR1 complex, of which both Ring1b and Bmi1 belong), increases the level of endogenous H2A.Zub1 (compare lanes 1 & 3 in Figure A-1B). Knockdown of Ring1b also specifically reduced levels of endogenous H2A.Zub1, as measured by Western blot using our L1 H2A.Z antibody.

In conclusion, this study identified and characterized the monoubiquitylated form of H2A.Z. The presence of H2A.Zub1 on the inactive X chromosome, as demonstrated by immunofluorescence analysis, and identification of Ring1b as an E3 ligase of H2.Z, suggest that ubiquitylation of H2A.Z is part of the transcriptional silencing pathway.
Figure A- 1. **Ring1b regulates levels of ubiquitylated H2A.Z.** (A) 293T cells were co-transfected with Flag-H2A/H2A.Z and either a control shRNA (Scramble) or a Ring1b-targeting shRNA construct. Both shRNA constructs were based on the pSUPER.retro.neo vector. 72 hours after transfection, whole-cell lysates were harvested and used for SDS-PAGE and Western blotting. H3 is shown for loading purposes. (B) Analysis of endogenous H2A.Z levels from cells transfected with shRNA constructs alone, or cotransfected with Bmi1 and Flag-Ring1b constructs. Lysates were harvested 72 hours after transfection and levels of H2A.Zub1 were assessed using our L1 H2A.Z antibody. Commassie-stained gel, showing the histones, was used for loading purposes.
References


Eirin-Lopez, J. M., Gonzalez-Romero, R., Dryhurst, D., Ishibashi, T., and Ausio, J. (2009). The evolutionary differentiation of two histone H2A.Z variants in chordates (H2A.Z-1 and
H2A.Z-2) is mediated by a stepwise mutation process that affects three amino acid residues. *BMC Evol Biol* 9, 31.


at K4 and is essential for H3 K4 methylation and vertebrate development. *Cell* 121(6), 859-72.


