H2A.Z: a molecular rheostat for gene regulation in embryonic stem cells

by

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Abstract
Chromatin regulation is a key mechanism for controlling gene expression patterns during development and differentiation. The histone H2A variant H2A.Z is highly conserved among eukaryotes and is of particular interest because it has an essential, yet unknown role in early development. H2A.Z is enriched at the promoter regions of most genes that harbor H3K4me3 in mouse embryonic stem cells (mESCs) including both active and silent, poised genes, marked additionally by polycomb-mediated H3K27me3 and compromising a large cohort of developmental regulators. How H2A.Z mediates these contrasting gene expression states is not known.

H2A.Z displays homology to canonical H2A throughout the histone fold domain, however considerable divergence exists outside of this domain, suggesting specialized functions. Here we developed a quantitative chromatin immunoprecipitation followed by mass spectrometry approach to identify downstream effectors of H2A.Z. We identified BET (bromodomain and extraterminal) transcriptional regulator proteins including Brd2 as highly enriched in H2A.Z chromatin. We demonstrate by ChIP-seq that Brd2 significantly overlap H2A.Z at the promoter region of active genes. Conversely, PRC1-dependent H2A.Z ubiquitination prevents Brd2 occupancy at poised, bivalent genes. Loss of H2A.Z ubiquitination of by mutation of C-terminal lysines results in a Brd2 recruitment and de-repression of bivalent genes. Moreover, inhibition of Brd2 by small molecule inhibition or siRNA-mediated depletion restores repression and leads to a recruitment of PRC2. In contrast, siRNA inhibition of another BET family member Brd4, does not restore repression suggesting that Brd2 and Brd4 play distinct roles in ESCs. This thesis provides novel insights into how H2A.Z acts as a molecular rheostat to regulate the balance between active and silent genes in ESCs, and more broadly a model for its role in responsive systems including development and cancer.

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Chapter 1

Introduction

H2A.Z: a molecular rheostat for transcriptional control

Author Contributions for Chapter 1:

Adapted by Paul Fields from a review written by Paul Fields, Vidya Subramanian, and Laurie Boyer.

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Introduction

A fundamental question of biology is how a single fertilized zygote can differentiate into all of the trillions of cells that make up a fully mature adult. In spite of almost every cell having the same copy of the genome, a large number of diverse functionally distinct cell types exist. Cellular diversity arises based on the regulated expression of specific genes and other transcripts, including miRNAs and lncRNAs, within the genome. Regulation of this process must be tightly coordinated during development and homeostasis as faulty regulation is the basis of many diseases including congenital disorders and cancer.

Regulation of chromatin is critical for proper control of all DNA-mediated processes including transcriptional responses to environmental and developmental cues. Chromatin is packaged in the nucleus into repeating units of nucleosomes made up of 147bp of DNA wrapped around an octamer of the core histones, two each of H2A, H2B, H3 and H4, as well as linker DNA and other DNA associated proteins (Figure 1). Chromatin can be regulated through an extensive range of modifications, from single covalent histone post-translational modifications to chromosomal domain localization within the nucleus (Figure 1) (Jenuwein and Allis, 2001; Tee and Reinberg, 2014). Each of these modifications can have significant impact on the current and potential transcriptional output of nearby genes. Furthermore, crosstalk among the many different types of chromatin modifications creates a massive combinatorial network of gene regulation. However, we currently lack an understanding of how most of these networks influence transcriptional output. Dissecting the interplay of all the levels of chromatin regulation is at the forefront of current epigenetics research.

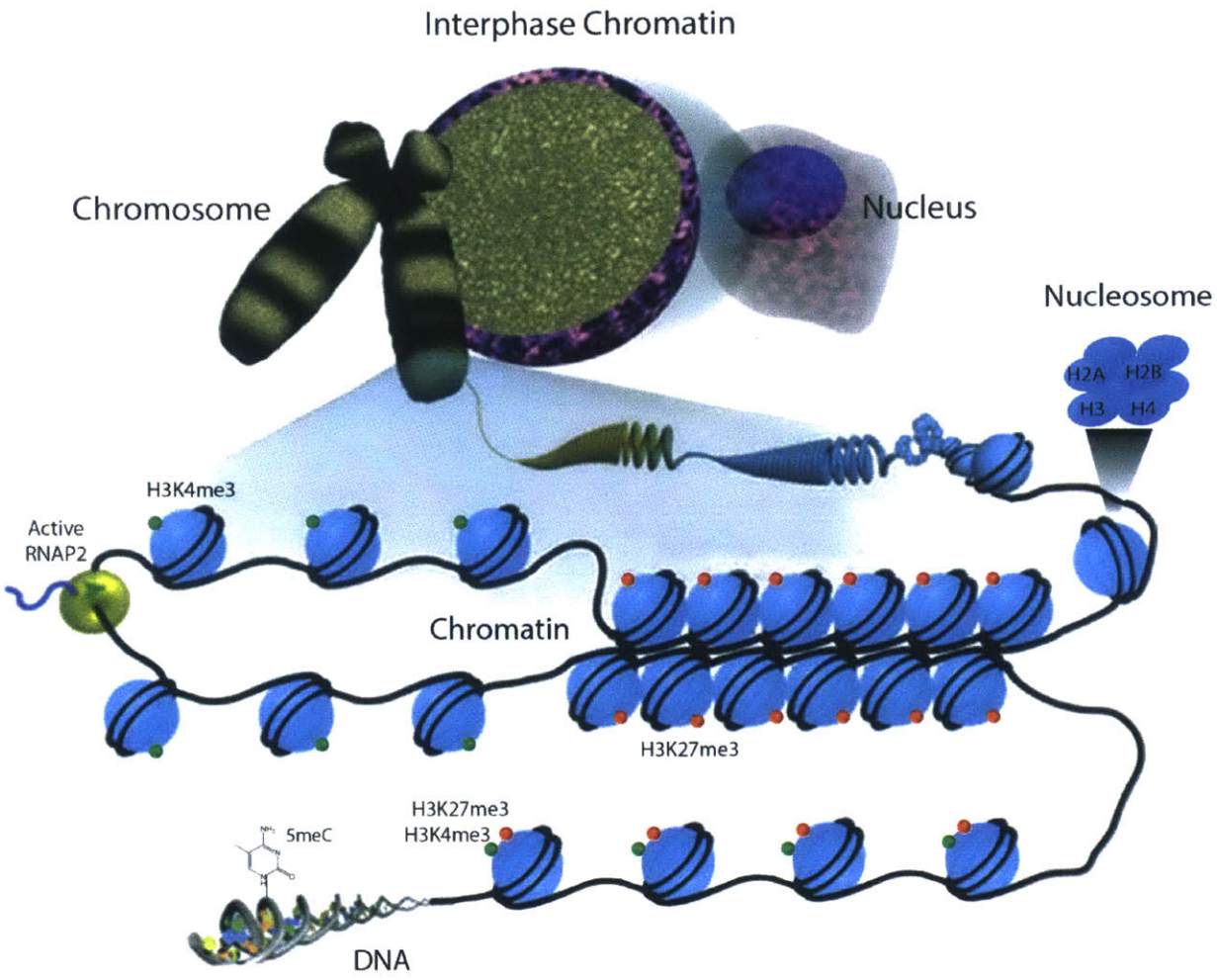

Figure 1: Chromatin is a highly regulated nuclear complex. DNA is packaged in the nucleus into repeating units of nucleosomes made up of an octamer of histones, two each H2A, H2B, H3 and H4. Nucleosomes are further assembled into higher order structures of chromatin. Active transcription is associated with regions of more open chromatin, including modifications such as H3K4me3. In contrast repressed regions of the genome are associated with H3K27me3 and DNA methylation. Moreover, bivalent regions of chromatin exist, marked by both H3K4me3 and H3K27me3 that are silent but poised to respond to cellular cues. Adapted from (Sha and Boyer, 2008).

The replication-independent incorporation of histone variants by specific ATP-dependent chromatin remodeling complexes has emerged as a critical mechanism for regulating many DNA-mediated processes including gene expression as well as DNA repair, chromosome segregation and genomic stability (Banaszynski et al., 2010; Biterge and Schneider, 2014) (Table 1). In contrast to canonical histones, which are transcribed from gene clusters and tightly coordinated with the cell cycle, histone variants are often single copy and expressed in a cell cycle independent manner. Histone variants are generally highly conserved and have evolved unique functions that cannot be accomplished by the canonical histones (Talbert and Henikoff, 2010) (Table 1). The highly conserved H2A-type variant H2A.Z has garnered

particular interest over the last several years because it has essential yet unknown roles in early metazoan development (Maze et al., 2014). H2A.Z has functions in many chromatin-regulated processes including, both gene activation and repression, DNA repair, heterochromatin formation and chromosome segregation (Altaf et al., 2009; Biterge and Schneider, 2014; Weber and Henikoff, 2014). In spite of extensive research, the mechanisms by which H2A.Z regulates these processes have remained elusive. This chapter highlights recent work that reveals mechanistic insights into how H2A.Z acts as a molecular gatekeeper for RNAPII at promoters and how H2A.Z integrates information from histone post-translational modifications (PTMs), other histone variants, and transcription factors to affect specific transcriptional outcomes during development, tissue specification, immune and hormone-mediated responses. Collectively, these data provide a framework for the work presented in this thesis, and suggest that H2A.Z acts as a molecular rheostat to integrate environmental and cellular cues to direct specific transcriptional outcomes.

Table 1: Histone Variants in Vertebrates (Adapted from (Maze et al., 2014))

Histone Variant	Genomic Location Patterns	Function	Knockout Phenotype
H2A Variants			
H2A.Z	Throughout the genome	Gene activation and silencing	Embryonic Lethal (Faast et al., 2001)
H2A.X	Throughout and Double Strand Breaks	DNA repair	Male infertility, meiosis defects (Fernandez-Capetillo et al., 2003)
MacroH2A	Inactive X Chromosome	Gene silencing	Brain malformation (Costanzi et al., 2000)
H2A.Bbd	Active X Chromosome and active chromatin	Active transcription	Unknown
H2B Variants			
TSH2B	Telomeres and Sperm chromatin	Chromatin-to-nucleoprotamine transition	Unknown
H2BFWT	Telomeres	Unknown	Unknown
H2BE	Throughout, expressed in neurons	Unknown	Olfactory receptor overexpression (Santoro et al., 2012)
H3 Variants			
H3.3	Throughout the genome	Gene activation and chromosome segregation	Infertility (Couldrey et al., 1999)

H3.4	Sperm chromatin	Unknown	Unknown
H3.X	Active chromatin	Unknown	Unknown
CENP-A	Centromeres	Chromosome segregation	Embryonic lethal (Howman et al., 2000)

Regulation of RNA Polymerase II Transcription

Epigenetic regulation, including the incorporation of histone variants such as H2A.Z, is of interest for its key roles in gene regulation and in regulating the response of RNA Polymerase II (RNAPII) to transcriptional stimuli. RNAPII transcription is highly regulated, requiring multiple temporal and spatial cues to properly activate transcription. H2A.Z and its regulation can influence many of these steps in response to developmental and cellular cues as will be discussed in depth in this chapter. Initial recruitment of RNAPII is triggered by a combination of specific transcription factors and general transcription factors including TFIIB, TFIID, TFIIE, TFIIE and TFIIH (Sainsbury et al., 2015) (Figure 2). Transcription initiation is dependent on the under-lying chromatin state and is coupled to chromatin remodeling to create a local environment permissive for activation. Prior to gene activation, in some cases, nucleosomes can act as repressive barriers at promoter regions when modified to facilitate repression (Li et al., 2007). During activation a combination of chromatin "writers" and "erasers", proteins that can change the histone modification state, in combination with ATP-dependent remodelers transition the chromatin from a repressive to an active conformation (Figure 2) (Li et al., 2007). This transition shifts promoters from being repressed by a combination of Polycomb Repressive Complexes 1 and 2, which catalyze the deposition of H2A ubiquitination (H2Aub) and H3K27me3 respectively to active chromatin marked by H3K4me3, deposited by the MLL complex, and H3K27ac, deposited by p300 (Figure 2) (Calo and Wysocka, 2013; Shilatifard, 2012; Voigt et al., 2013). Interestingly, a bivalent chromatin structure comprised of both H3K27me3, the repressive mark, together with H3K4me3, the activating mark, demarcates promoters in many mammalian cell types that are transcriptionally silent but poised to become activated upon stimuli (Voigt et al., 2013). In particular, most developmental genes harbor bivalent chromatin marks in embryonic stem cells (ESCs) (Bernstein et al., 2006). Changes in this initial chromatin environment have critical consequences on transcriptional initiation and gene regulation in response to developmental cues. Thus understanding how

chromatin-mediated signaling is translated into gene activation or repression is critical for understanding cell state regulation.

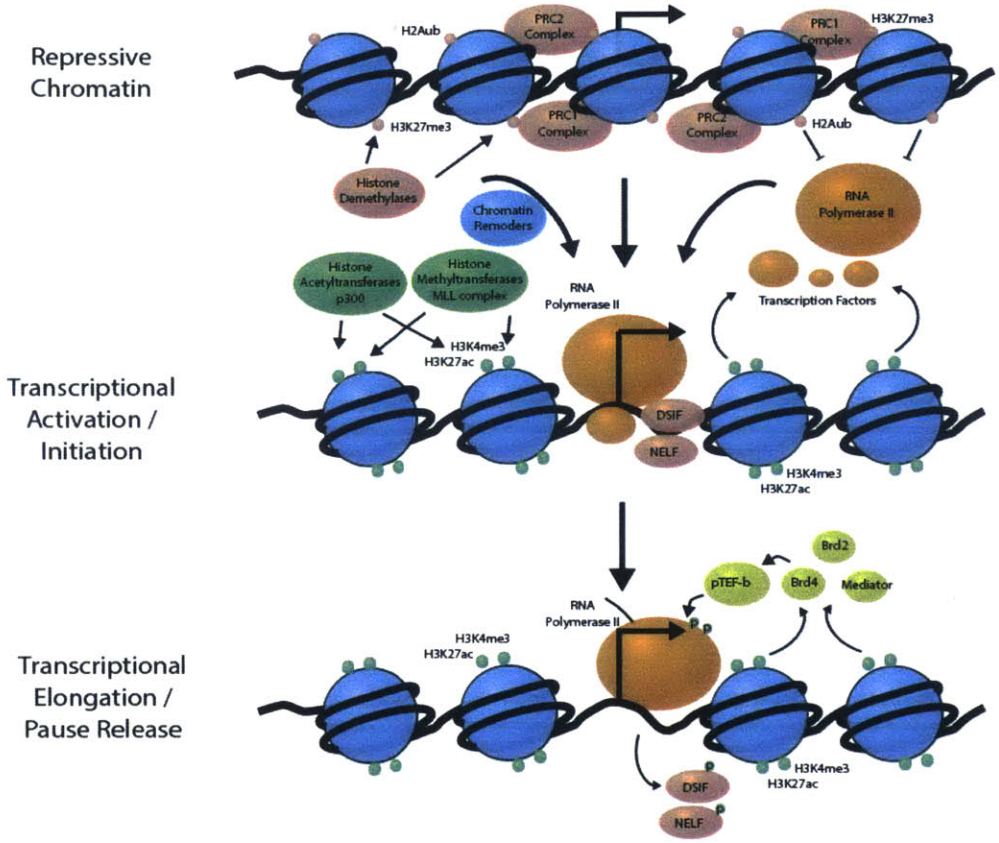

Figure 2: RNAPII Transcription is regulated by the underlying chromatin. Repressive chromatin transitions to an active state by the combination of removal of repressive marks and deposition of activating marks by chromatin "writers" and "erasers." Following RNAPII recruitment, RNAPII becomes competent for transcriptional elongation by both positive factors and removal of negative factors, which are phosphorylated by chromatin "readers."

At many promoters, RNAPII engagement is followed by an initial pausing where RNAPII is held up 30-60 nucleotides downstream of the transcriptional state site (Jonkers and Lis, 2015), and productive elongation is inhibited by a combination of NELF (negative elongation factor) and DSIF (DRB-sensitivity inducing factor) (Figure 2). In contrast, productive elongation is stimulated by positive transcription factors including p-TEFb (positive transcription elongation factor b), which phosphorylate RNAPII, NELF and DSIF. pTEF-b is activated either by directly responding to external cues, or through intermediate chromatin "reader" proteins that recognize specific epigenetic modifications, to direct transcriptional activity. A critical family of chromatin reader proteins is the BET (bromodomain and extraterminal) family of bromodomain containing proteins BRD2, BRD3 and BRD4, which possess a histone

acetylation recognition module and can both directly recruit pTEF-b (in the case of BRD4), and facilitate transcriptional elongation (Leroy et al., 2008; Patel et al., 2013). BET proteins also act to recruit other chromatin associated factors such as Mediator and cohesin to maintain transcriptional activity, potentially through promoting enhancer-promoter looping (Denis et al., 2010). Thus the chromatin environment has profound impacts on all stages of RNAPII regulation and transcriptional responsiveness to developmental and cellular cues.

H2A.Z in development

The histone H2A-type variant H2A.Z is found in all eukaryotic species from yeast to humans, and is highly conserved compared to its corresponding canonical histone H2A (~80% homology between yeast and humans) (Eirín-López et al., 2009). H2A.Z is of particular interest in gene expression due to its key roles in the regulation of RNAPII activity (Weber and Henikoff, 2014). H2A.Z was first identified in mouse cell lines and accounts for 5-10% of total H2A (West and Bonner, 1980). While structurally similar to H2A, H2A.Z shares only ~60% sequence similarity to H2A, suggesting the evolution of unique functionality (Suto et al., 2000; Thatcher and Gorovsky, 1994). Divergent regions of H2A.Z include the N- and C-terminal tails, and also the nucleosome docking domain, comprising an extended acidic patch compared to H2A (Figure 3) (Suto et al., 2000). The high degree of conservation (97% similar between mammals and *drosophila)* and broad expression pattern of H2A.Z, has led to much interest in its role in development (van Daal and Elgin, 1992), in particular through downstream effects on epigenetic regulation and gene expression patterns. In *Drosophila*, genomic deletion of the region containing H2A.Z (termed H2avD) resulted in early embryonic lethality (van Daal and Elgin, 1992). *Drosophila* H2A.Z has features of both H2A.Z and H2A.X, a histone variant which is typically associated with DNA damage, however lack of a developmental phenotype associated with mammalian H2A.X suggests key roles for H2A.Z-type features (Fernandez-Capetillo et al., 2003; Leach et al., 2000; Madigan et al., 2002). *Drosophila* lethality could be specifically rescued by the expression of a transgenic version of H2A.Z (van Daal and Elgin, 1992), demonstrating that H2A.Z is necessary for embryonic development. Subsequent studies have shown that H2A.Z is essential for the development of *tetrahymena*, mice and *C. elegans* (Faast et al., 2001; Liu et al., 1996; Whittle et al., 2008). In contrast to metazoan

development, deletion of H2A.Z in yeast is viable, but results in a slow growth phenotype and the inability to properly respond to certain external stimuli (Santisteban et al., 2000). Moreover, H2A.Z genetically interacts with over 200 genes in yeast including many chromatin regulators suggesting that it functions as a convergent point between upstream signals and downstream transcription across eukaryotes (Hang and Smith, 2011).

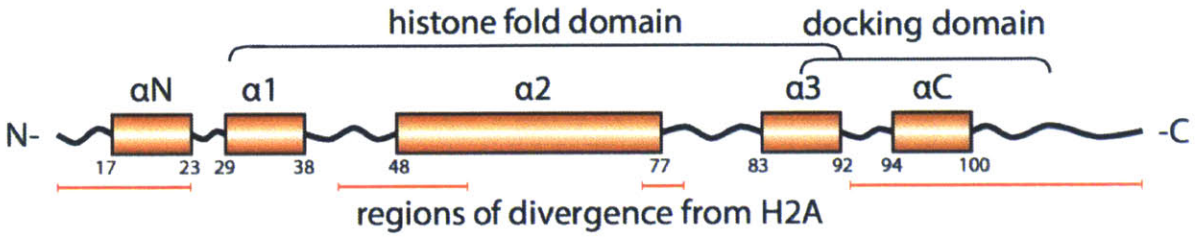

Figure 3: H2A.Z is structurally distinct from H2A. H2A.Z while structurally similar to H2A consisting of a histone fold domain of three alpha helices and a N- and C-terminal tails has regions of significant divergence through the N- and C-termini. In particular, H2A.Z diverges from H2A through the C-terminal nucleosome docking domain. (Adapted from Zlatanova 2008)

In mammals, early work demonstrated that H2A.Z knock-out mice died around the time of gastrulation, the developmental stage when the three germ layers are established (Faast et al., 2001). Explanted embryos also failed to develop *in vitro*, suggesting an essential role for H2A.Z in the maintenance of the inner cell mass and the transition from an undifferentiated blastocyst into defined germ layers. The early embryonic lethal phenotype of H2A.Z has restricted further downstream analysis in the absence of a conditional knockout. Mouse embryonic stem cells (mESCs) provide an ideal model system to investigate the mechanism of H2A.Z in early development (Creyghton et al., 2008; Hu et al., 2013; Subramanian et al., 2013). ESCs are derived from the undifferentiated inner cell mass of the blastocyst and maintain the ability to grow indefinitely in culture and differentiate into all three germ layers (Evans and Kaufman, 1981). Depletion of H2A.Z in mESCs using shRNAs results in a failure to properly differentiate including an inability to form defined germ layers and to activate key developmental genes, including many essential lineage specific transcription factors (Figure 4) (Creyghton et al., 2008). These genes in ESCs are bivalent, harboring both the active H3K4me3 and repressive H3K27me3 marks and are silent but competent to activate when

signaled to do so (Bernstein et al., 2006). Furthermore, loss of H2A.Z leads to a corresponding loss of Polycomb Repressive Complex 2 (PRC2) recruitment and to gene de-repression demonstrating a functional connection between H2A.Z and the maintenance of the bivalent, poised state (Figure 4) (Creyghton et al., 2008). More recently it was shown that in addition to PRC2, H2A.Z also facilitates the recruitment of MLL and deposition of H3K4me3 to active genes (Hu et al., 2013). Together, these data suggest that H2A.Z cooperates with other chromatin associated complexes to facilitate RNAPII-mediated transcription, as will be discussed in more depth later. Studies in ESCs have begun to elucidate how H2A.Z plays a key role in mammalian development, in particular through the regulation of the induction of key developmental genes. However a mechanistic understanding of how H2A.Z mediates these processes has only recently begun to emerge and there are still many open questions.

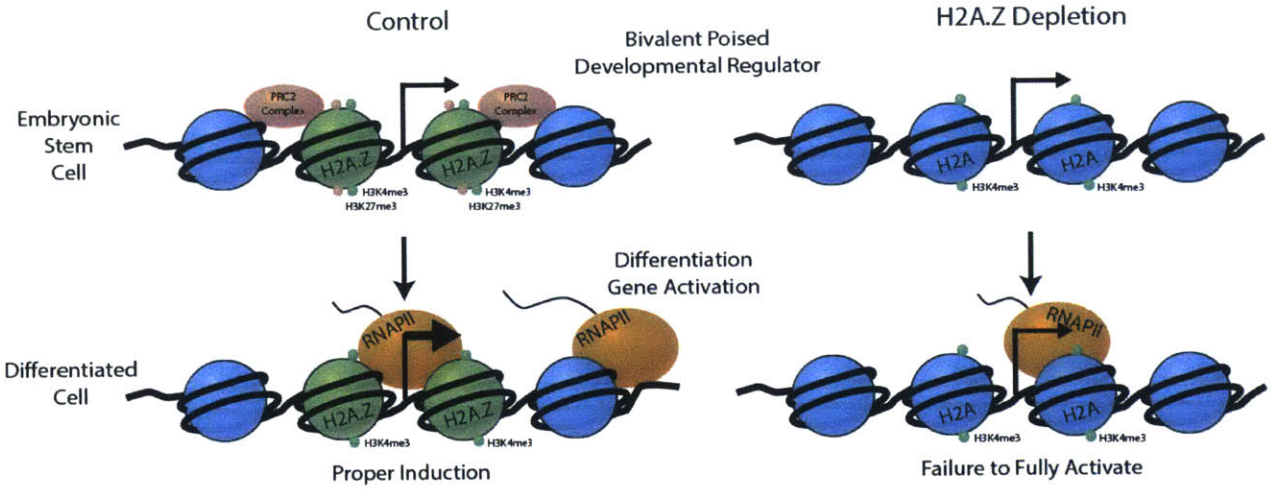

Figure 4: H2A.Z is necessary for the proper induction of developmental genes. Loss of H2A.Z in embryonic stem cells results in an inability to properly activate bivalent developmental regulators upon stimulus to differentiation. Additionally loss of H2A.Z leads to a decreased binding of PRC2 and H3K27me3 deposition in embryonic stem cells. Together these data suggest important roles for H2A.Z in the activation of developmental regulators.

Given the high degree of conservation in sequence and function of H2A.Z, studies have investigated the role of the individual divergent regions of H2A.Z, in order to better understand its mechanistic functions. The N-terminal tail of H2A.Z diverges from H2A and includes multiple lysine residues that can be acetylated, which may be recognized by specific chromatin readers. The C-terminal domain of H2A.Z includes divergent regions through the nucleosome docking domain, a key region for mediating nucleosome-protein interactions (Kato et al., 2011) (Figure 3). The H2A.Z docking domain has an extended acidic patch that

results in a more negatively charged stretch of amino acids on the surface of the nucleosome compared to H2A (Suto et al., 2000). Substitution of these residues to resemble H2A in both *Drosophila* and mESCs show that H2A.Z has specialized functions that cannot be replaced by H2A (Clarkson et al., 1999; Subramanian et al., 2013). In *Drosophila,* substitution of the extended acidic patch of H2A.Z with the equivalent H2A residues results in embryonic lethality before pupal development (Clarkson et al., 1999). Additionally, swapping the C-terminal tail, while permissive for further development, is lethal prior to adulthood (Clarkson et al., 1999). In mESCs, the extended acidic patch is also necessary for regulating proper differentiation. Using a transgenic version of H2A.Z where the three divergent residues of the acidic patch were mutated back to their corresponding H2A residues created a hyper-dynamic nucleosome that led to de-repression of bivalent developmental target genes (Subramanian et al., 2013). Together, these data suggest that the acidic patch of H2A.Z may confer stability on the nucleosome, either through intrinsic structural effects on the nucleosome, or through downstream interactions, to regulate gene expression states. However, *in vitro* modeling of the H2A.Z acidic patch mutant on the regulation of nucleosome stability remains to be tested. Residues outside the acidic patch on the C-terminal were also found to be required for proper H2A.Z function in an unbiased mutagenesis screen (Wood et al., 2013). One of these residues is conserved between H2A.Z and H2A, suggesting that there may be additional important protein dependent functions for residues that are conserved between proteins. Together, these studies demonstrate that the C-terminal region of H2A.Z is essential for its function in development, however more detailed mechanistic studies are needed to determine its precise roles.

In contrast to the C-terminal tail, the divergent residues of the N-terminal tail of H2A.Z have a less defined role in development. In *Drosophil*a, substitution with the corresponding N-terminal region of H2A, while not essential for development, resulted in decreased viability, (Clarkson et al., 1999). Moreover, evidence from *tetrahymena* demonstrates that while the N-terminal tail of H2A.Z is essential, the equivalent regions of H2A are sufficient to rescue this phenotype (Ren and Gorovsky, 2001; 2003). A direct role for the H2A.Z N-terminal tail in mammalian development remains to be elucidated. These results suggest a function for the

N-terminal tail of H2A.Z, however it may depend on conserved features between H2A.Z and H2A, potentially through post-translational modifications. While some regions of H2A can successfully compensate for the corresponding H2A.Z residues, studies clearly demonstrate that H2A.Z divergent regions play key roles in mediating developmental functions. The important functional roles of the N- and C-termini of H2A.Z are consistent with the high overall conservation of the protein. Beyond its role in early development, H2A.Z is also required for regulating gene expression in response to external cues across cell types.

H2A.Z Genome-wide Localization

Genome-wide studies have led to key insights into understanding the role of H2A.Z in regulating transcriptional response to environmental cues. Early work in yeast demonstrated that H2A.Z is localized to the promoter of most genes and is necessary for gene activation (Zhang et al., 2005). Specifically, H2A.Z showed the highest enrichment at lowly expressed genes along with a slower rate of transcriptional elongation. Upon transcriptional activation in response to either diauxic stress or heat shock, H2A.Z is lost at the promoters of responsive genes while it is gained at genes that are repressed (Zhang et al., 2005). Surprisingly, deletion of H2A.Z specifically affected the ability of genes to be activated but not repressed. These results are consistent with a key role for H2A.Z in poising of genes for activation, and that its enrichment at repressed genes could represent a ground state required for gene induction.

In mESCs, H2A.Z is enriched at the promoters of developmental regulators including many cell-type specific transcription factors (Creyghton et al., 2008). Similar to yeast, H2A.Z incorporation is essential for the proper induction of target genes. Chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-seq) show that H2A.Z localizes to the promoters of most genes in ESCs, including both active and repressed genes (Hu et al., 2013; Ku et al., 2012; Subramanian et al., 2013), largely correlating with H3K4me3 enrichment. H3K4me3 may be one determinant for the recruitment of H2A.Z, as loss of H3K4me3 by depletion of the MLL deposition complex resulted in decreased H2A.Z incorporation (Hu et al., 2013). Moreover, H2A.Z levels at gene promoters inversely correlate with expression levels (Hu et al., 2013; Ku et al., 2012), in agreement with yeast. Interestingly,

H2A.Z depletion in mESCs has little effect on the steady state levels highly expressed genes in spite of its promoter occupancy (Subramanian et al., 2013). These data suggest that redundant mechanisms may regulate active genes, or that H2A.Z is no longer necessary once genes become activated. However how H2A.Z regulates the transition between gene silencing and activation remains an open questions, as will be addressed in Chapter 2.

Beyond its role at promoters, H2A.Z is enriched at intergenic regions, including a subset of enhancers (Chen et al., 2014; 2013; Hu et al., 2013; Subramanian et al., 2013). How H2A.Z functions at enhancer regions is poorly understood. Hu et al showed that H2A.Z correlates with H3K4me3 levels at intergenic enhancers, however these regions are often excluded from enhancer annotations due to the possibly that H3K4me3 enrichment is indicative of un-annotated transcriptional start sites (Hnisz et al., 2013; Hu et al., 2013; Wamstad et al., 2012). In contrast, enhancer annotations based on H3K27ac levels and the absence of H3K4me3 show very low levels of H2A.Z enrichment (Chen et al., 2014). Further work will be necessary to clarify the functional role of H2A.Z at distal enhancers. A role for H2A.Z in the activation of enhancers or poising them for activation is an attractive hypothesis, consistent with its promoter associated role, and will likely be a direction of future study as our understanding of enhancers increases.

Genome-wide studies have now examined H2A.Z localization across a variety of cell types and organisms, and while the precise patterns of enrichment differ slightly among eukaryotes, these studies demonstrate that H2A.Z containing nucleosomes usually flank the nucleosome depleted region (NDR) at transcription start sites (TSSs). NDRs may be one factor in triggering the deposition of H2A.Z, along with H3K4me3, which together with H2A.Z reinforce a transcriptionally permissive chromatin environment (Yen et al., 2013). These results are consistent with a conserved role of H2A.Z in regulating promoter responsiveness to cellular stimuli
(Guillemette et al., 2005; Weber et al., 2010; Zilberman et al., 2008). Collectively, genome-wide studies have provided greater insights into how dynamic incorporation of H2A.Z at promoter regions regulates gene expression in response to cellular changes.

Regulation of +1 nucleosome and RNA polymerase II progression

Why is H2A.Z incorporation at the TSS important for transcriptional regulation? *In vitro* studies have demonstrated that the nucleosome immediately downstream of the TSS (i.e. denoted +1 nucleosome) poses a sizeable barrier to transcription and can direct the orientation of the pre-initiation complex (PICs) and subsequent transcriptional elongation (Nock et al., 2012; Rhee and Pugh, 2012). In *Drosophila*, the +1 nucleosome obstructs RNAPII transit, resulting in increased stalling and backtracking of the polymerase (Weber and Henikoff, 2014). Strikingly, H2A.Z enrichment at the +1 nucleosome correlates with decreased RNAPII stalling, suggesting that its incorporation reduces the high-energy barrier to RNAPII progression. Consistent with this idea, H2A.Z levels anti-correlate with nucleosome turnover, indicating that H2A.Z incorporation at the +1 nucleosome regulates productive elongation by facilitating H2A.Z/H2B dimer loss without depletion of $(H3\text{-}H4)_2$ tetramers. These findings suggest that H2A.Z incorporation at the +1 nucleosome regulates transcriptional output by modulating RNAPII kinetics and transcriptional elongation (Figure 5). *Drosophila* H2A.Z has features of both H2A.Z and H2A.X, another H2A variant with roles in DNA damage response (Leach et al., 2000; Madigan et al., 2002), thus investigations into the role of H2A.Z incorporation at the +1 nucleosome in other organisms are critical to dissecting its function. Moreover, while a large number of genes are regulated by polymerase pausing in Drosophila (Figure 2) (Min et al., 2011), not all H2A.Z target genes in other organisms are regulated in this manner. In mESCs, where H2A.Z occupies many developmental regulators (Creyghton et al., 2008; Hu et al., 2013; Ku et al., 2012; Subramanian et al., 2013), these genes harbor dramatically less paused RNAPII and are not susceptible to loss of pausing factors (Figure 2) (Hu et al., 2013; Ku et al., 2012; Subramanian et al., 2013; Williams et al., 2015). Thus, how H2A.Z functions at the +1 nucleosome to mediate contrasting transcriptional outcomes will likely vary by species, and may be influenced by other factors.

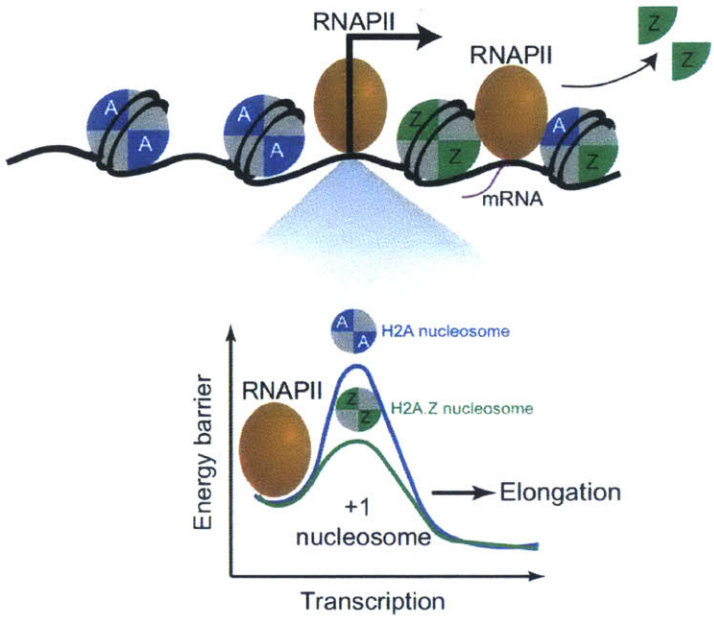

Figure 5: H2A.Z nucleosome composition at promoters influences nucleosome stability and transcriptional state. (Top) The transition from homotypic to heterotypic H2A.Z nucleosomes can regulate gene activation through regulation of transcription elongation. (Bottom) H2A.Z nucleosomes can impact the stability of the +1 nucleosome and RNA polymerase II progression. (From Subramanian et al., 2015)

H2A.Z post-translational modifications correlate with transcriptional outcome

In addition to histone variants, promoter nucleosome stability and dynamics can be regulated by histone post-translational modifications (PTMs) (Figure 6). Similar to major type histones, variants are also subject to diverse PTMs which can function to recruit downstream effectors such as histone writers, readers or erasers to target genes or influence chromatin stability and dynamics (Sevilla and Binda, 2014; Yun et al., 2011). For example, N-terminal acetylation of H2A.Z (acH2A.Z) is strongly enriched at the 5′ end of active gene promoters (Bruce et al., 2005; Hu et al., 2013; Ku et al., 2012; Valdés-Mora et al., 2012). H2A.Z acetylation is essential for gene induction in yeast (Halley et al., 2010), as well as for the activation of the estrogen receptor responsive genes (Bellucci et al., 2013). However, most studies between acH2A.Z and gene induction are largely correlative. A functional role for acH2A.Z in mammalian gene induction has recently been demonstrated in a model of myotube differentiation where exogenous expression of a non-acetylatable form of H2A.Z results in decreased induction of the master transcription factor MyoD and decreased differentiation even in the presence of endogenous

H2A.Z (Law and Cheung, 2015). A global role for acH2A.Z in regulating gene expression in response to cell stimuli remains to be elucidated. While in *S. cerevisiae* H2A.Z is acetylated by ESA1 and NuA4 (Keogh et al., 2006; Mehta et al., 2010), their mammalian counterparts have not been clearly defined. Determining the H2A.Z specific histone acetyltransferase will likely add further insight into the role of acH2A.Z is mammals.

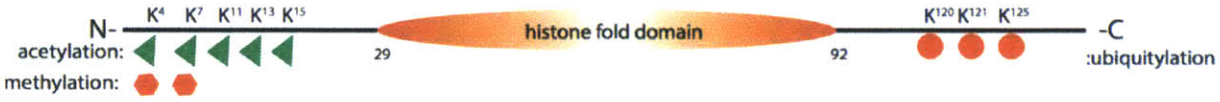

Figure 6: H2A.Z can be extensively post-translationally modified. H2A.Z can be post-translationally modified on the N- and C-terminal tails, including acetylation on the five N-terminal lysines, while methylation has only been identified on two of them. H2A.Z can also be C-terminally ubiquitinated, which can be modified on the same copy of H2A.Z as acetylation. Multiple modifications of H2A.Z with methylation has yet to be observed. (Adapted from Blinda 2013)

In addition to active genes, H2A.Z is also enriched at silent genes that are poised for activation in ESCs. In mammals, similar to H2A, H2A.Z can be ubiquitinated at K120, K121, and K125 residues by the E3 ligase activity of the Polycomb Repressive complex 1 (PRC1) component Ring1b (Figure 6) (Ku et al., 2012; Sarcinella et al., 2007). H2Aub is known to be a repressive chromatin mark and acts to both block RNAPII recruitment and activation through inhibiting chromatin remodelers, and by inhibiting H3K4 methylation (Zhou et al., 2009), suggesting a repressive role for H2A.Z monoubiquitination (H2A.Zub). Consistent with this idea, H2A.Zub appears to demarcate facultative heterochromatin (Sarcinella et al., 2007). Interestingly, loss of the H2A.Z deubiquitinating enzyme USP10 results in a failure of androgen-receptor target gene activation in LNCaP cells, suggesting that removal of this modification is necessary for gene induction (Draker et al., 2011). Recent reports showed that PRC1-mediated ubiquitination of core H2A (H2Aub) impedes RNAPII recruitment at bivalent genes (Stock et al., 2007) and may facilitate PRC2 targeting and the establishment of polycomb domain formation (Blackledge et al., 2014; Cooper et al., 2014). Moreover, H2Aub stimulates PRC2 recruitment to chromatinized templates and catalysis of H3K27me3 *in vitro* suggesting that H2Aub is a critical upstream effector of polycomb silencing (Kalb et al., 2014). Given that bivalent genes are also enriched for H2A.Z and that PRC1 catalyzes ubiquitination of both

H2A and H2A.Z (Ku et al., 2012; Stock et al., 2007; Surface, 2014), it is possible that H2A.Zub contributes to these functions.

In mouse ESCs, acH2A.Z and H2A.Zub are co-enriched with H3K27me3 nucleosomes, indicating that dually modified H2A.Z (acH2A.Zub) is present at poised developmental genes (Figure 7) (Ku et al., 2012). One attractive model is that H2A.Zub contributes to the recruitment of PRC2 and reduces nucleosome accessibility (for example, dimer loss) at the +1 nucleosome, while acH2A.Z promotes RNAPII progression perhaps through recruitment of downstream effectors. Notably, H2A.Z facilitates access to both active and repressive complexes to chromatin in ESCs to regulate the balance between self-renewal and differentiation (Hu et al., 2013). Thus, the balance of acH2A.Z and H2A.Zub at promoters may be critical for regulating the recruitment of downstream effectors and on the induction of gene expression programs in response to developmental cues, as will be examined in Chapter 2.

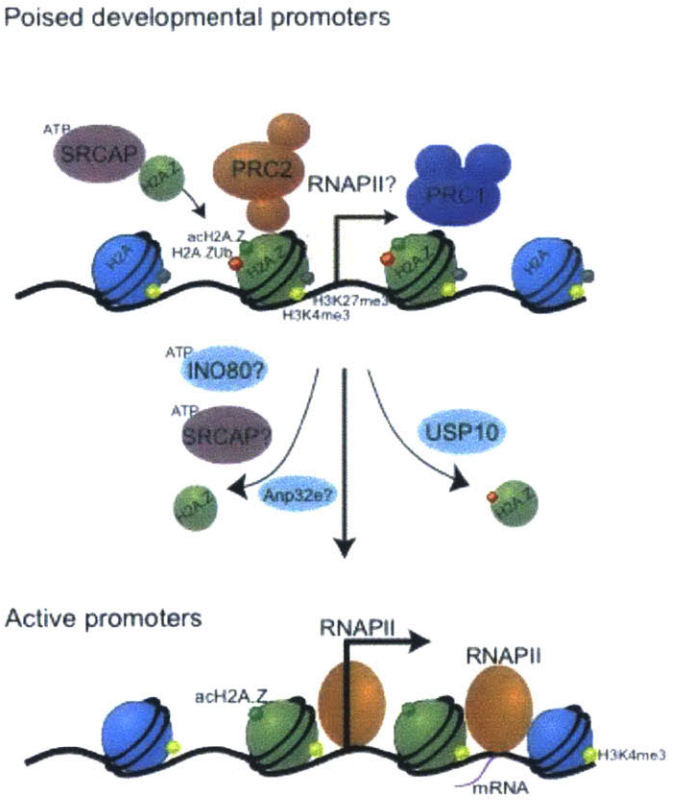

Figure 7: H2A.Z is regulated by many chromatin-associated complexes. H2A.Z is enriched at both active and silent, poised genes in embryonic stem cells. The effect of H2A.Z on transcription depends on the

balance of both activating and repressive histone post-translational modifications as well as H2A.Z-specific deposition and removal complexes in response to cellular cues. (From Subramanian 2015)

Beyond acetylation and ubiquitination, H2A.Z is also methylated on its N-terminal lysines (Binda et al., 2013). To date, this modification as yet has only been identified on H2A.Z in mESCs, but is essential for the proper maintenance of the stem cell state. Loss of the H2A.Z methyltransferase SETD6 resulted in changes in ESC morphology and up-regulation of differentiation genes, suggesting a repressive role for H2A.Zme similar to H2A.Zub. Interestingly, in contrast to ubiquitination, which decreases upon differentiation (Surface, 2014), total H2A.Zme levels increase during differentiation. While both H2A.Zme and H2A.Zub are necessary to maintain repression at target genes in ESCs, during differentiation the two marks may have distinct functions. The role of H2A.Zme must be explored more broadly and across other systems to better understand its function, however dissecting the specific functions of H2A.Z methylation will be difficult due to the overlap with acetylated residues. Together, H2A.Z PTMs play a key role in the context dependent functions of H2A.Z. Future work identifying the downstream effectors of these modifications will be essential to dissecting how H2A.Z regulates chromatin and gene expression states.

Regulation of H2A.Z incorporation is critical for gene regulation

Although H2A.Z histone PTMs provide context-dependent signals that impact functional regulation, dissecting how this variant is targeted to discrete genomic sites is also important for understanding its regulatory roles. The exchange of H2A for H2A.Z is catalyzed by ATP-dependent remodeling complexes in a replication-independent manner in all eukaryotes, namely the SWR1 complex (SRCAP in mouse and human) as well as by p400/Tip60 in higher eukaryotes (Figure 7) (Choi et al., 2009; Ruhl et al., 2006). Notably, INO80, an ATP-dependent remodeler known for its role in DNA repair, has been implicated in transcription-dependent removal of H2A.Z in yeast (Papamichos-Chronakis et al., 2011; Watanabe et al., 2013). INO80 is essential for proper regulation of mESCs, and in particular in the regulation of pluripotency gene expression and maintenance of open chromatin (Wang et al., 2014). While no direct link was shown between mammalian INO80 and H2A.Z, the authors hypothesize that INO80 could

facilitate the dynamic turnover of H2A.Z at these promoters. However a more functional connection will be necessary to dissect whether INO80 functions with H2A.Z in higher eukaryotes. Histone chaperones are additional factors that regulate histone variant incorporation (Burgess and Zhang, 2013). For example, the metazoan-specific histone chaperone, ANP32E (acidic nuclear phosphoprotein 32 kDa E), which is a member of the p400 complex, regulates variant incorporation by facilitating removal of H2A.Z dimers as evidenced by increased accumulation of H2A.Z at promoters and enhancers in ANP32E knockout cells (Figure 7) (Mao et al., 2014; Obri et al., 2014). Together, these studies suggest that the interplay between H2A.Z-specific ATP-dependent remodelers and histone chaperones is crucial for regulating H2A.Z localization.

Until recently, how H2A.Z exchange complexes are targeted to specific sites remained largely unknown. Evidence now suggests that SWR1 and INO80 complexes may recognize NDRs to remodel H2A.Z at the +1 nucleosome by selective positioning of distinct components of the remodeling complex around the NDR (Yen et al., 2013). Biochemical analyses suggest that SRCAP and ANP32E recognize specific features of H2A.Z that are divergent from core H2A such as the C terminal α-helix to facilitate the exchange reaction (Hong et al., 2014; Obri et al., 2014), providing an additional mechanism for how remodelers distinguish between the two histones. Recent work also highlights roles for core histone PTMs in proper histone exchange (Choi et al., 2009; Watanabe et al., 2013). For example, in contrast to its well-documented role in H2A.Z deposition, yeast SRCAP (SWR-C) displays altered substrate specificity in the presence of H3K56ac, a histone modification within the core domain of H3, leading to removal of H2A.Z dimers from chromatin (Watanabe et al., 2013). Interestingly, in human ESCs, H3K56ac is enriched at both active and inactive genes that largely overlap targets of the core pluripotency TFs Oct4, Sox2, and Nanog (Xie et al., 2009). Notably, H3K56ac is redistributed to developmental genes that are activated in response to retinoic acid during ESC differentiation. Thus, H3K56ac and chromatin remodelers may cross-talk to regulate H2A.Z dynamics and gene expression across eukaryotes, however a direct functional connection in higher eukaryotes remains for future investigations.

In addition to histone PTMs, H2A.Z incorporation appears to be influenced by DNA methylation levels. Studies in both plants and mammals suggest that H2A.Z and DNA methylation are mutually antagonistic at promoters and within gene bodies (Conerly et al., 2009; Zilberman et al., 2008). In mammals anti-correlation was observed at gene bodies, where H2A.Z was deposited upon gene activation concordant with de-methylation, in contrast little change in methylation was observed at gene promoters (Conerly et al., 2009). Consistent with this idea, 5-aza 2′-deoxycytidine-induced DNA demethylation stimulates SRCAP-mediated H2A.Z incorporation, facilitating nucleosome depletion and gene activation in cancer cells (Yang et al., 2012). How H2A.Z and DNA methylation cooperate in development, where permanently repressed genes must gain DNA methylation, remains to be elucidated. Although these studies point to a model whereby H2A.Z incorporation is targeted to discrete regions of the genome by a variety of mechanisms, evidence also suggests that H2A.Z can be randomly incorporated at low levels and that the removal of H2A.Z is a key event for regulating chromatin states (Hardy and Robert, 2010; Hardy et al., 2009). Thus, a balance between the targeted deposition and removal of H2A.Z is likely critical for maintaining proper chromatin states.

H2A.Z nucleosome composition affects chromatin dynamics

Modulating the number of H2A.Z copies in a nucleosome can also have consequences for nucleosome structure and function. Early structural studies suggested that, owing to steric clashes between the L1 loops of H2A.Z and H2A, formation of a heterotypic H2A.Z nucleosome is unlikely (Suto et al., 2000), but *in vitro* and *in vivo* evidence now indicates that H2A.Z can form both heterotypic and homotypic nucleosomes (Luk et al., 2010; Weber et al., 2010). *In vitro* biochemical analyses showed that two copies of H2A.Z (homotypic) result in a more stable nucleosome, which is refractory to RNAPII progression (Park et al., 2004; Thakar et al., 2010). However, homotypic H2A.Z is enriched at the +1 nucleosome in *Drosophila*, which is thought to decrease the energy barrier to RNAPII progression, and is depleted downstream of paused polymerase (Weber et al., 2010). Further biochemical analyses demonstrated that acetylation of H2A.Z additionally alters its stability. While the unacetylated form of H2A.Z showed much greater nucleosome stability, acH2A.Z induced a pronounced shift to lower

stability, greater than the shift observed for H4, a highly acetylatable histone (Thambirajah et al., 2006), suggesting a potential role for acH2A.Z in modulating how H2A.Z influences chromatin dynamics. In mouse trophoblast cells, H2A.Z appears to be redistributed to heterochromatin (for example, telomeres) during G_2/M, resulting in a shift from homotypic to heterotypic H2A.Z nucleosomes and to an expanded NDR at the TSS of active H2A.Z genes (Nekrasov et al., 2012). Surprisingly, this transition from homotypic to heterotypic H2A.Z nucleosomes during the cell cycle does not appear to correlate with cell cycle dependent transcriptional changes. Thus, the consequence of H2A.Z composition on transcription is highly complex and likely depends on its levels at the TSS relative to gene bodies as well as the presence of histone modifications or other histone variants or both.

The incorporation of other histone variants with H2A.Z can alter the functional properties of nucleosomes. H3.3 differs from major type H3 by only four or five amino acids in metazoans yet displays distinct regulation and biochemical properties. In mammalian cells, double-variant nucleosomes containing H2A.Z and H3.3 mark regions of dynamic chromatin regulation and are highly salt-labile (Jin and Felsenfeld, 2007; Jin et al., 2009; Shu et al., 2014). Moreover, in yeast, where non-centromeric H3 is most similar to vertebrate H3.3, *in vitro* reconstituted nucleosomes display release of H2A.Z dimers in low salt (Ahmad and Henikoff, 2002; Zhang et al., 2005). Together, these findings suggest a conserved mechanism in which H3.3 further regulates the stability of the H2A.Z nucleosome. Notably, whereas H2A.Z facilitates intranucleosomal folding, H3.3 counteracts the H2A.Z-mediated compaction of nucleosomal arrays *in vitro* (Chen et al., 2013; Fan et al., 2002) and can relieve the transcription repression caused by H2A.Z-containing chromatin (Chen et al., 2013). In ESCs, H3.3 depletion results in reduced nucleosome turnover and an increase in PRC2 enrichment (Banaszynski et al., 2013; Creyghton et al., 2008; Goldberg et al., 2010; Subramanian et al., 2013). Interestingly, mutation of the C-terminal H2A.Z acidic patch to resemble core H2A leads to a more dynamic nucleosome, de-repression of poised developmental genes, and increased H3.3 enrichment in ESCs (Subramanian et al., 2013). Together, these studies indicate that H3.3 cooperates with H2A.Z to regulate nucleosome stability, chromatin accessibility, and transcriptional output. Future investigations to determine how the balance between H3.3 and H2A.Z is regulated at

specific genomic locations are needed to understand how these variants regulate global gene expression programs.

H2A.Z is a critical binding platform for pioneer transcription factors

Given that transcription factor (TF) binding is influenced by nucleosome density and histone PTMs (Wan et al., 2009; Zhang et al., 2005) and that H2A.Z exhibits strong genetic interactions with TFs in yeast (Wan et al., 2009), it is likely that H2A.Z coordinates transcriptional responses in part by modulating TF accessibility. Several recent studies in mammals demonstrated that H2A.Z nucleosomes are necessary for the recruitment of specific pioneer TFs. Unlike classic TFs, pioneer TFs bind nucleosomal DNA and remain bound to their sites through mitosis, providing a level of epigenetic memory (Zaret and Carroll, 2011). FOXA2 is an example of a pioneer TF that has key roles in development and transcription activation. In ESCs, H2A.Z is required for the recruitment of FOXA2 to promoters of genes activated during early endoderm differentiation (Li et al., 2012). Moreover, binding of FOXA2 to H2A.Z nucleosomes promotes recruitment of SWI/SNF and INO80 complexes, resulting in H2A.Z removal and nucleosome depletion, establishing a platform for the binding of other TFs. Loss of either H2A.Z or FOXA2 impairs gene induction and differentiation, suggesting these factors coordinate nucleosome remodeling. Conversely, whether H2A.Z plays an active role in gene repression during differentiation or during other active processes remains to be addressed.

Recent studies showed that the bromodomain-containing protein BRD2 preferentially associates with H2A.Z-containing nucleosomes and that BRD2 recruitment is necessary for androgen receptor-regulated gene activation in LNCaP cells (Draker et al., 2012) (Figure 8). BRD2 has a higher affinity for H2A.Z nucleosomes compared to canonical H2A *in vitro*, specifically in combination with nucleosomes that also harbor H4 amino-terminal acetylation. H4 acetylation has been implicated in decondensing higher order chromatin structures to maintain an active chromatin state (Shia et al., 2006), suggesting that H4 acetylation marks the transition from a repressed to an active chromatin state. However, whether H4ac is sufficient or whether additional mechanisms are necessary to regulate recruitment of BRD2 to target promoters *in vivo* is not clear, and will be examined in depth in Chapter 2. Genome-

wide localization studies show that acH2A.Z is enriched at activated prostrate cancer genes in androgen-sensitive human prostrate cancer cells (Valdés-Mora et al., 2012). Given that bromodomains recognize acetylated lysines (Denis et al., 2010), acH2A.Z may be an additional determinant for BRD2 recruitment. More broadly, BRD2 is a member of the BET (bromodomain and extra terminal domain) family of chromatin reader proteins that also includes BRD4 (Figure 2), which has critical roles in gene activation in ESCs and in cancer (Hnisz et al., 2013; Leroy et al., 2008; Liu et al., 2014; Whyte et al., 2013). BRD4 has also been recently identified as playing roles in both androgen and estrogen receptor responsive cancers (Asangani et al., 2014; Nagarajan et al., 2014). Interestingly, both BRD4 and H4K12ac are downstream of estrogen receptor binding which is itself dependent on H2A.Z (Gévry et al., 2009; Nagarajan et al., 2014; 2015). A direct functional connection between H2A.Z and BRD4 remains unknown, however these results suggest a transitive reliance on H2A.Z for proper BRD4 recruitment in response to cellular cues. While *in vitro* acetylation of H2A.Z does not influence BRD2 nucleosome binding, whether this holds true *in vivo* will be of interest, as acH2A.Z correlates with gene activation.

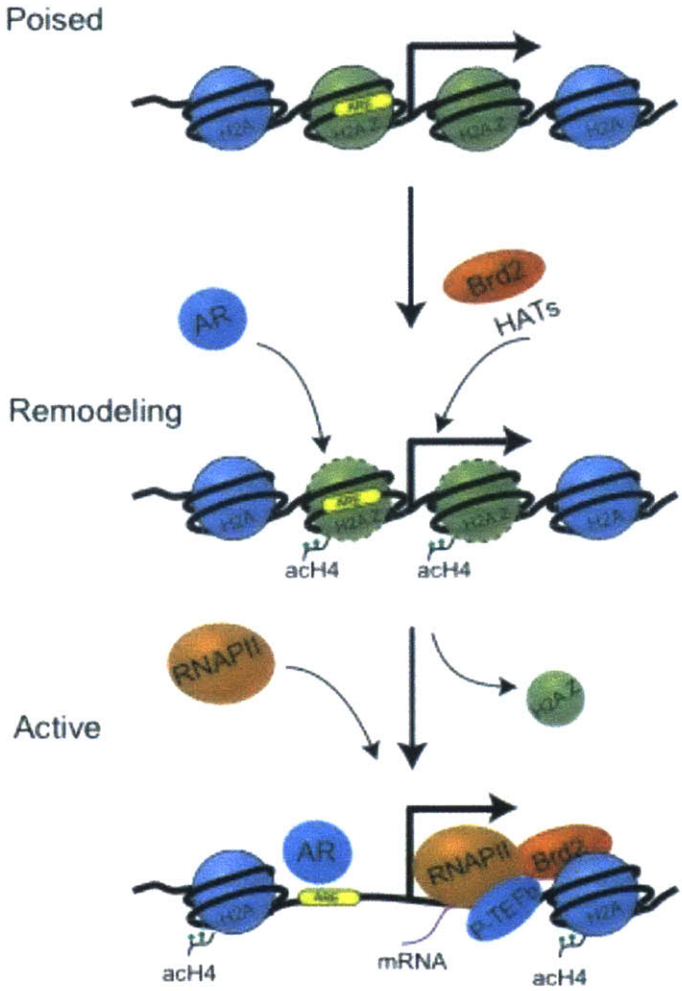

Figure 8: H2A.Z coordinates with other chromatin proteins to mediate gene activation. In response to androgen receptor (AR) signaling, H2A.Z allows binding of AR to its response elements, at both promoters and enhancers, and along with acH4 recruits Brd2 and RNA polymerase II to mediate gene activation. (From Subramanian 2015)

In addition to its requirement for regulating developmental transitions, H2A.Z is necessary for the expression of circadian-regulated genes by modulating TF binding. CLOCK and BMAL1 are pioneer TFs that bind to circadian clock-regulated genes to induce their expression in a temporally regulated manner (Mohawk et al., 2012). Like binding of Foxa2, binding of these TFs promotes gene activation and the recruitment of RNAPII. Interestingly, CLOCK:BMAL1 binding is closely tied to cyclical changes in H2A.Z occupancy and nucleosome depletion (Menet et al., 2014). Using mouse livers at different time points in the light/dark cycle, investigators demonstrated that H2A.Z-enriched nucleosomes flank CLOCK-binding sites, at both promoters and intergenic regulatory regions. Notably, H2A.Z levels oscillate on the basis

of CLOCK:BMAL1 binding. The cyclical binding of CLOCK:BMAL1 induces nucleosome depletion at promoters, allowing additional TFs to gain access to DNA. Loss of BMAL1 in mice results in decreased H2A.Z levels and higher nucleosome occupancy at CLOCK-binding sites as well as loss of circadian regulation, suggesting that H2A.Z is necessary to maintain these sites in a highly plastic state. Collectively, these studies reveal that H2A.Z nucleosomes provide a crucial binding platform for pioneer TFs and may facilitate recruitment of chromatin readers to facilitate precise activation of gene expression programs.

H2A.Z regulates higher order chromatin interactions

The function of H2A.Z at promoters has been well defined in regulating the timing and levels of transcription, however how it functions at intergenic regions is less clear (Hu et al., 2013; Jin et al., 2009; Ku et al., 2012). Many H2A.Z intergenic regions are also marked by CTCF binding sites (Fu et al., 2008; Nekrasov et al., 2012). CTCF is a zinc finger DNA binding protein that occupies thousands of sites in the genome including promoters and insulators regions, often demarcating active from silent chromatin (Herold et al., 2012). Emerging evidence suggests that CTCF is critical for the formation of DNA looping and higher order chromatin structure, in particular through connecting enhancers and promoters (Merkenschlager and Odom, 2013). Depletion of CTCF in myeloid blast cells results in a concomitant loss of H2A.Z at the promoter of regulated genes, and together this loss results in a de-repression of regulated genes and premature differentiation (Ouboussad et al., 2013). The authors propose a model where CTCF and H2A.Z cooperate to maintain the poised state by acting as an insulator at the promoter and blocking enhancer activity. Notably, recent work showed that oscillation of CTCF binding during the cell cycle correlates with changes in H2A.Z occupancy and the transition from homotypic to heterotypic H2A.Z nucleosomes in trophoblast stem cells (Nekrasov et al., 2012). These data are consistent with previous work suggesting that H2A.Z contributes to the establishment of well-positioned nucleosomes at CTCF binding sites (Fu et al., 2008). Together these results suggest H2A.Z and CTCF function together to regulate both nucleosome positioning and downstream transcriptional activity. How these proteins coordinate dynamic changes in gene expression in response to cellular signals is unknown. Interestingly, major changes in chromatin looping occur during the cell cycle as

chromosomes are condensed in M phase prior to cell division. Thus it is possible that interactions between H2A.Z and CTCF are also necessary to regulate global chromatin organization throughout the cell cycle. While the direct contribution of H2A.Z to higher order chromatin remains largely unexplored, a recent study demonstrated that H2A.Z is necessary to maintain promoter and enhancer interactions at the CyclinD1 gene and loss of H2A.Z abrogates this interaction and the ability to activate gene expression in response to estrogen receptor signaling (Dalvai et al., 2012). How H2A.Z functions at enhancers and distal regulatory regions, and whether it facilitates global chromatin structure will be an exciting area for future work. It is likely that the role of H2A.Z in regulating higher order chromatin structure depends on the integration of post-translational modifications, heterotypic vs. homotypic composition and other histone variants, thus comprehensive analysis of H2A.Z will continue to shed to insights into its functional role as a molecular rheostat for integrating diverse signals to changes in chromatin structure and ultimately transcriptional outcomes.

Conclusions

Since H2A.Z was first identified over 30 years ago, studies have begun to reveal the many layers of regulation and complex functions of H2A.Z in modulating chromatin dynamics and gene expression. Recent studies have uncovered many players involved in H2A.Z-mediated transcriptional regulation, but how H2A.Z translates upstream signals into diverse transcriptional outcomes is still largely unknown and is the focus of the work presented in this thesis. Insights into the function of H2A.Z in gene induction are critical for understanding how H2A.Z functions in other processes such as DNA repair and genomic stability. For example, p400-mediated H2A.Z incorporation during double-strand break (DSB) repair stimulates an open chromatin conformation, resulting in the formation of an efficient chromatin template for DSB repair, albeit through different effectors (Xu et al., 2012). Interestingly, H2A.Z acetylation and ubiqutination are also hallmarks of chromatin at DSBs, suggesting a general model by which H2A.Z modulates chromatin in diverse DNA-mediated processes. Given that H2A.Z has been implicated in the activation of estrogen- and androgen-responsive genes in models of breast and prostrate cancer (Dalvai et al., 2013; Draker et al., 2011; Dryhurst and Ausió, 2014; Dryhurst et al., 2012), continued mechanistic studies into H2A.Z function and in

particular how it translates upstream signaling to specific transcriptional outcomes will be critical to fully understand how H2A.Z contributes to development and disease.

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Chapter 2

H2A.Z and Brd2 coordinate the balance between active and silent genes in ESCs

Author Contributions for Chapter 2:

Experiments designed by Paul Fields and Laurie Boyer. Experiments and analysis performed by Paul Fields. Cell lines generated by Lauren Surface and Vidya Subramanian. RNA-seq performed by Lauren Surface. Mass Spectrometry performed by Namrata Udeshi and Jake Jaffe.

Summary

The histone variant H2A.Z is enriched at the promoters of active and silent genes in ESCs and is necessary for lineage commitment, yet how it contributes to these contrasting transcriptional states is poorly understood. To address this question, we developed a quantitative chromatin immunoprecipitation-mass spectrometry approach to identify specific H2A.Z chromatin-associated proteins in ESCs. We find the BET (bromodomain and extraterminal) family of dual bromodomain-containing transcriptional regulator proteins including Brd2 highly enriched in H2A.Z chromatin. We further show genome-wide that Brd2 occupies active gene promoters coincident with H2A.Z. Conversely, PRC1-dependent H2A.Z ubiquitylation prevents Brd2 occupancy at poised, bivalent genes. Loss of H2A.Zub by mutation of the three C-terminal lysines ($H2A.Z^{K3R3}$) leads to Brd2 recruitment and de-repression of bivalent genes. Moreover, inhibition of Brd2 either by siRNA or the BET inhibitor JQ1 restores gene repression in $H2A.Z^{K3R3}$ ESCs and leads to a recruitment of PRC2. In contrast, siRNA inhibition of another BET family member Brd4, does not rescue this effect suggesting that Brd2 and Brd4 play distinct roles in ESCs. Together, our study provides mechanistic insights that reveal how H2A.Z regulates the balance between active and silent genes in ESCs.

Introduction

The incorporation of histone variants is critical for regulating chromatin structure and dynamics in response to environmental and developmental cues (Biterge and Schneider, 2014; Maze et al., 2014). The histone variant H2A.Z is a highly conserved non-allelic variant of H2A that has essential but unknown roles in the development of multi-cellular organisms (Clarkson et al., 1999; Faast et al., 2001; Whittle et al., 2008). In the mouse, loss of H2A.Z is embryonic lethal around the time of gastrulation, a developmental time point when complex gene expression patterns are established (Faast et al., 2001). Consequently, pluripotent embryonic stem cells (ESCs) depleted of H2A.Z fail to activate genes during differentiation suggesting that H2A.Z is necessary for the proper induction of developmental programs. In ESCs, H2A.Z is enriched at H3K4me3-marked promoters of active genes and poised, bivalent genes that also harbor H3K27me3, and are silent yet maintain the capacity to be activated during differentiation (Creyghton et al., 2008; Hu et al., 2012; Subramanian et al., 2013). We currently lack a mechanistic understanding of how H2A.Z regulates the balance between gene activation and repression.

While H2A.Z is structurally similar to canonical H2A, divergent regions within the N- and C-terminal tails suggest that H2A.Z has evolved specialized functions and interacts with specific chromatin-associated proteins (Draker et al., 2012; Fujimoto et al., 2012; Li et al., 2012; Suto et al., 2000). Consistent with this idea, H2A.Z cooperates with steroid (e.g. glucocorticoid receptors) and nuclear hormone receptors (e.g. estrogen and androgen receptors) in responsive cell types to activate transcription (Dryhurst et al., 2012; Gévry et al., 2009; John et al., 2008). Moreover, H2A.Z interacts with Brd2, a BET (bromodomain and extraterminal domain) protein involved in transcriptional activation in 293T cells, and together they cooperate with androgen receptor to activate responsive genes in androgen-sensitive LNCaP cells (Draker et al., 2012). However, the broader consequences of the interaction between H2A.Z and Brd2 or how H2A.Z selectively recruits Brd2 to active genes is not known.

Post-translational modification of canonical histones and histone variants can affect chromatin organization and transcriptional state. For example, N-terminal H2A.Z acetylation

(acH2A.Z) strongly correlates with gene activation in a variety of systems (Bruce et al., 2005; Hu et al., 2012; Ku et al., 2012; Millar et al., 2006; Valdés-Mora et al., 2012). In ESCs, the ratio of acetylated to total H2A.Z at promoters correlates with gene activity (Hu et al., 2012; Ku et al., 2012). Conversely, H2A.Z C-terminal mono-ubiquitylation (H2A.Zub) is enriched with H3K27me3 at facultative heterochromatin on the inactive X (Ku et al., 2012; Sarcinella et al., 2007). Recent studies show that PRC1-mediated H2A.Zub is enriched at poised, bivalent genes in ESCs and is necessary to maintain PRC2 at target sites and for proper multi-lineage differentiation (Surface et al., Submitted). Bivalent genes also harbor acH2A.Z suggesting that H2A.Zub inhibits gene activation by preventing recruitment of activating factors to chromatin. However, we lack fundamental insights into how H2A.Z post-translational modifications modulate downstream effectors to regulate developmental gene expression patterns. Thus, we reasoned that determining the specific H2A.Z-interacting factors in ESCs will provide critical insights into the role of H2A.Z in regulating transcriptional output.

To determine H2A.Z-specific protein interactions in ESCs, we used SILAC (Stable Isotope Labeling of Amino acids in Cell culture) in combination with immunoprecipitation (IP) followed by quantitative mass spectrometry (SILAC-IP). We find multiple tandem bromodomain proteins including members of the BET family of transcriptional regulator proteins highly enriched in H2A.Z chromatin compared to H2A. We demonstrate genome-wide that H2A.Z co-localizes with Brd2 at the promoter of active genes. In contrast, PRC1-mediated H2A.Zub, which is necessary to maintain repression of bivalent genes through recruitment of PRC2, prevents the recruitment of Brd2. Loss of H2A.Zub by mutation of the PRC1 target lysines leads to an increase in Brd2 recruitment, whereas RNAi-mediated knockdown of Brd2 or small-molecule inhibition restores silencing in H2A.Zub mutants by promoting recruitment of PRC2. Notably, depletion of another BET family member Brd4 known to be critical for maintenance of ESC identity does not rescue this effect, suggesting that Brd2 and Brd4 play distinct roles in ESCs. Collectively, these data reveal how H2A.Z post-translation modifications coordinate specific activating and repression systems to regulate transcriptional output in ESCs.

Results

Identification of H2A.Z-specific interacting proteins in ESCs

We designed an approach to quantify H2A.Z-specific protein interactions in mouse ESCs. Using SILAC (Stable Isotope Labeling of Amino acids in Cell culture) (Ong et al., 2002) in combination with immunoprecipitation (IP) followed by mass spectrometry (SILAC-IP) we identified differentially enriched H2A.Z-associated proteins compared to H2A (Figure 1A). ESC lines harboring either a doxycycline inducible H2A.Z-YFP or H2A-YFP transgene were cultured in heavy or light isotope media to label proteins for analysis (Figures S1A and S1B). YFP-tagged histones are incorporated normally and do not affect ESC state or differentiation capacity (Sarcinella et al., 2007; Subramanian et al., 2013). SILAC-IP was performed on paired H2A.Z-YFP and H2A-YFP samples (e.g. H2A.Z-YFP heavy and H2A-YFP light), along with biological replicates using isotope swaps for the two ESC lines (Figure S1C). In total, we identified 1557 candidate H2A.Z and H2A interacting proteins by requiring the identification of multiple peptides in both replicates (Supplemental Table 1). We then ranked differential enrichment based on a modified T statistic for significance of reproducibility and differential interaction (Mertins et al., 2013; Sancak et al., 2013). We focused further analysis on the subset of 99 proteins that were differentially enriched (p-value <0.1, Figure 1B). Specifically 71 and 28 proteins are enriched or depleted in H2A.Z chromatin, respectively, compared to H2A (Supplemental Table 1).

Figure 1

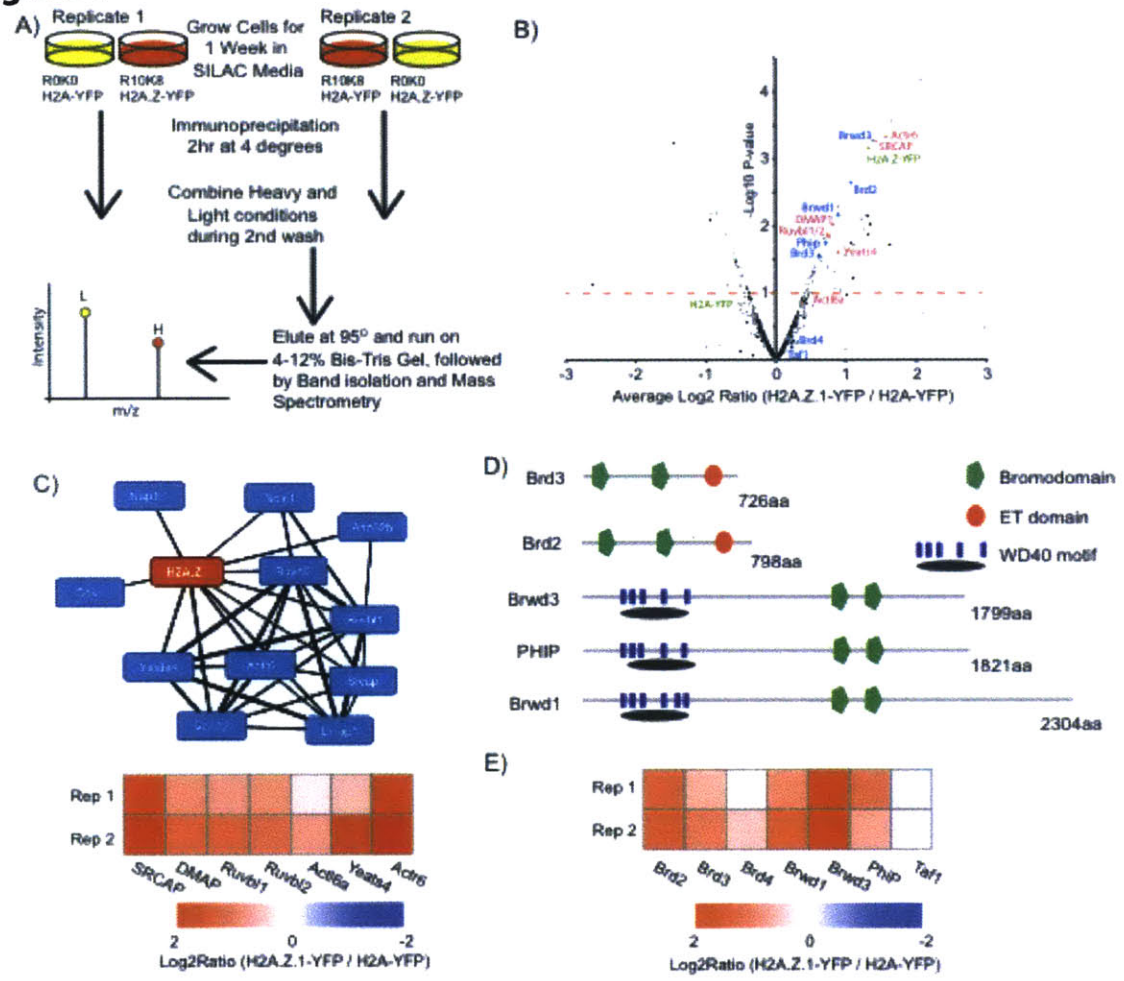

Figure 1: Tandem Bromodomain Proteins enriched in H2A.Z chromatin. (A) Schematic for SILAC-IP approach. Cells were grown for 1 week in either light or heavy (labeled arginine and lysines) media, resulting in 93% efficiency of metabolic labeling. Quantification is performed by mass spec on purified proteins from paired samples. (B) Volcano plot comparing average ratio of enrichment of biological replicates based on SILAC intensity for H2A.Z-YFP over H2A-YFP against p-value for significance of reproducibility. Proteins in the upper right are significantly enriched in H2A.Z-containing chromatin. (C) 11 proteins identified as enriched in H2A.Z containing chromatin have previously annotated interactions with H2A.Z as determined by STRING, including components of SRCAP, the H2A.Z specific deposition complex. Heatmap represents SILAC Log2 ratio of H2A.Z-YFP IP over H2A-YFP IP. (D) Five proteins in the tandem bromodomain family of proteins were identified as enriched in H2A.Z chromatin, including members of the BET family (Brd2 and Brd3). (E) Heatmap of SILAC Log2 ratio of all identified tandem bromodomain proteins, showing reproducible enrichment of five of the seven.

We identified numerous proteins known to interact with H2A.Z including seven members of the H2A.Z-specific ATP-dependent deposition complex SRCAP, validating our approach (Figure 1C) (Ruhl et al., 2006). Using the PFAM Domain search within STRING (Franceschini et al., 2013), a database of annotated protein interactions, we identified the bromodomain as the most highly enriched motif (p-value < 2e-3) among H2A.Z-interacting proteins. While there exist over 40 bromodomain-containing proteins in the mouse genome, only 8 of these proteins harbor a tandem bromodomain motif (Filippakopoulos et al., 2012). Remarkably, 5 out of the 7 of these proteins are expressed in ESCs and are enriched in H2A.Z chromatin, two BET proteins (Brd2 and Brd3) and three proteins that harbor a WD40 motif in addition to the tandem bromodomain (Brwd2, Brwd3, and Phip) (Figures 1D, 1E and S1D). BET proteins have Important roles in development and can regulate polymerase recruitment and pause release (Belkina and Denis, 2012; Filippakopoulos and Knapp, 2014) and bind to acetylated lysines often on histones (Denis et al., 2010; Zeng and Zhou, 2002). Our data suggest that H2A.Z specifically interacts with tandem bromodomain containing proteins including BET proteins in ESCs.

H2A.Z and Brd2 show similar distribution patterns at active gene promoters

We focused further analysis on the functional relationship between H2A.Z and Brd2 given its high enrichment by SILAC-ChIP and high expression in ESCs (Figures 1D and S1D). While H2A.Z recruits Brd2 upon stimulus to two target loci in LNCaP cells (Draker et al., 2012), whether they co-localize genome-wide and the role of Brd2 in ESCs has not been determined. Similar to H2A.Z, Brd2 shows a bimodal distribution at transcription start sites (TSSs) as determined by ChIP-Seq (Figure 2A). Specifically, Brd2 is enriched at 11,536 regions including 6,856 genes that significantly overlap H2A.Z enriched genes (84% of Brd2 enriched genes overlap H2A.Z peaks) (Figure 2B). Brd2 enriched promoters show high levels of H2A.Z as well as acH2A.Z and RNA polymerase II (RNAPII), consistent with the high ratio of acetylated H2A.Z to total H2A.Z at active genes (Figure 2C) (Ku et al., 2012). Moreover, Brd2 occupancy most closely correlated with active chromatin marks in ESCs including H3K27ac and H3K4me3 (Figure 2D), suggesting a role for Brd2 with H2A.Z at active genes. Conversely, Brd2 is largely

absent from poised, bivalent genes, marked by H3K27me3 (Figures 2D and 2E). Our data show that Brd2 co-occupies active promoters with H2A.Z in ESCs.

Figure 2

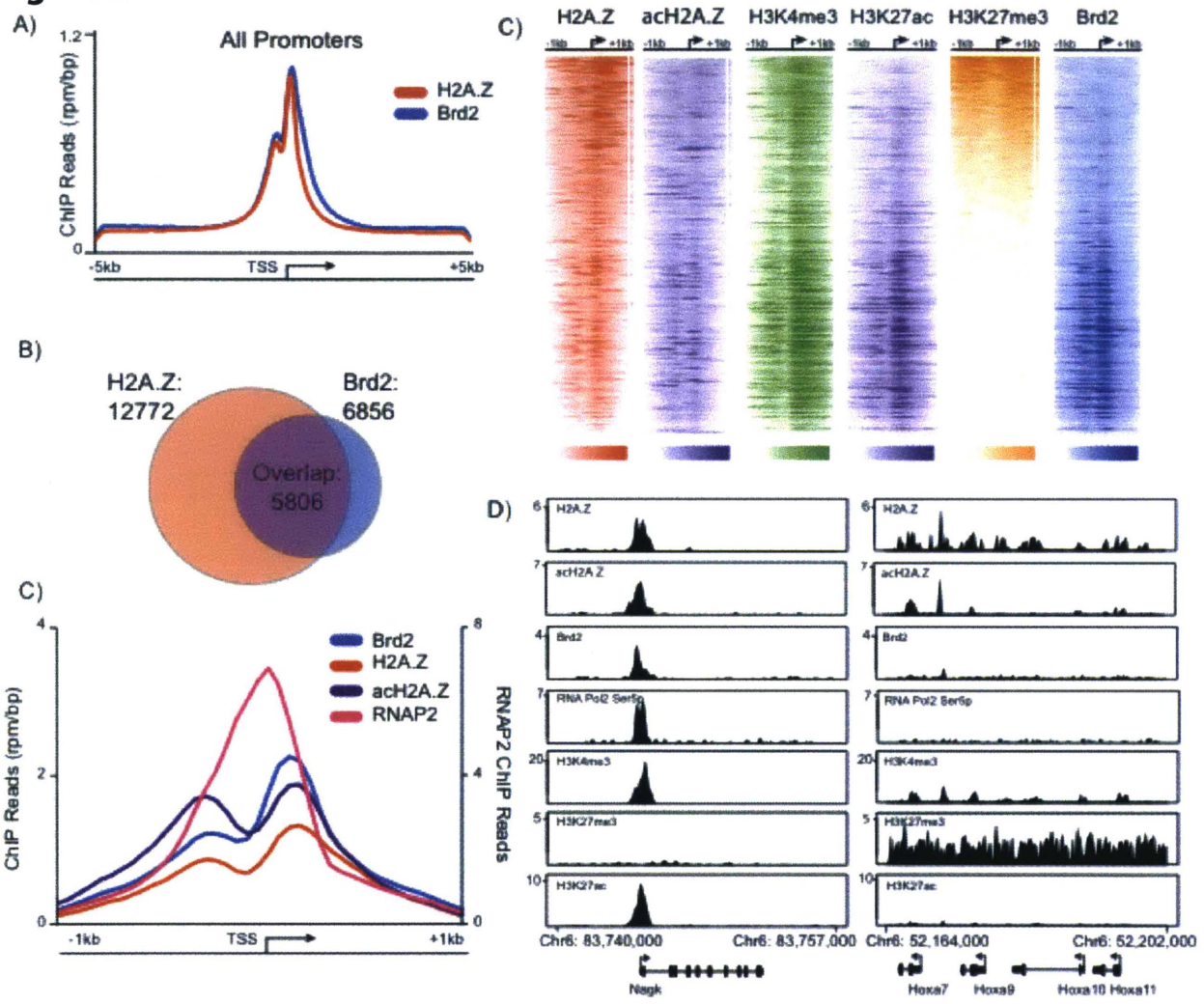

Figure 2: Brd2 is localized to active promoters co-incident with H2A.Z. (A) Average signal of ChIP-seq reads across the TSS of all genes (+/- 5kb) for Brd2 compared to H2AZ. (B) Overlap of Brd2 and H2A.Z enriched promoters (within 2kb), as defined by GREAT. (C) Average signal of ChIP-seq reads for Brd2, H2A.Z, acH2A.Z and RNAP2 across the TSS of Brd2 enriched genes (+/- 1kb) as defined by GREAT. RNAP2 plotted on the secondary axis. (D) Heatmaps of ChIP-seq reads for H2A.Z, acH2A.Z, H3K4me3, H3K27me3, H3K27ac and Brd2 across the TSS (+/- 1kb) of all genes sorted by H3K27me3 levels. Brd2 is most closely correlated with acH2A.Z and H3K27ac levels and anti-correlated with H3K27me3 levels. (D) Genome tracks of representative active H2A.Z target gene Nagk, enriched for H3K27ac and RNAPol2. HoxA cluster represents poised, bivalent H2A.Z target genes co-enriched for H3K27me3 and low Brd2 levels. Reads are normalized to reads per million per base pair.

H2A.Z ubiquitylation inhibits Brd2 recruitment to bivalent genes

PRC1-mediated H2A.Z ubiquitylation is enriched at poised, bivalent promoters in ESCs and has critical roles in regulating developmental gene expression patterns (Surface et al., Submitted). H2A.Z nucleosomes can be dually modified by both acetylation and ubiqutylation (Ku et al., 2012), and the presence of H2A.Z at promoters is necessary for gene activation in ESCs (Creyghton et al., 2008). To test whether H2A.Zub inhibits recruitment of Brd2 to poised genes, we analyzed Brd2 localization by ChIP-seq in ESCs that express either an inducible wild-type H2A.Z ($H2A.Z^{WT}$) or H2A.Z where the three PRC1-target lysines are mutated to arginine ($H2A.Z^{K3R3}$) (Figure S2A), in a background where endogenous H2A.Z is depleted by shRNA. Consistent with our previous studies, $H2A.Z^{K3R3}$ is incorporated at promoters in ESCs similar to wild-type H2A.Z (Figure S2B) (Surface et al., Submitted). Notably, we observed a significant increase of Brd2 at poised, bivalent genes in $H2A.Z^{K3R3}$ ESCs compared $H2A.Z^{WT}$ controls (Figure 3A, median fold change 1.50, Wilcox test $p<2.4e-118$, compared to all genes). For example, Brd2 shows increased occupancy at the bivalent gene Mesp1 as well as the HoxA gene cluster in $H2A.Z^{K3R3}$ ESCs (Figure 3B), whereas active genes including Hira and Cul1 showed no difference in Brd2 levels (Figures 3A and 3C). Brd2 transcript and proteins levels as well as H2A.Z incorporation are similar in $H2A.Z^{WT}$ and $H2A.Z^{K3R3}$ ESCs, suggesting that the change in localization is due to its re-distribution to these promoters and not to an increase in overall Brd2 protein levels (Figures S2B, S2C and S2D).

Figure 3

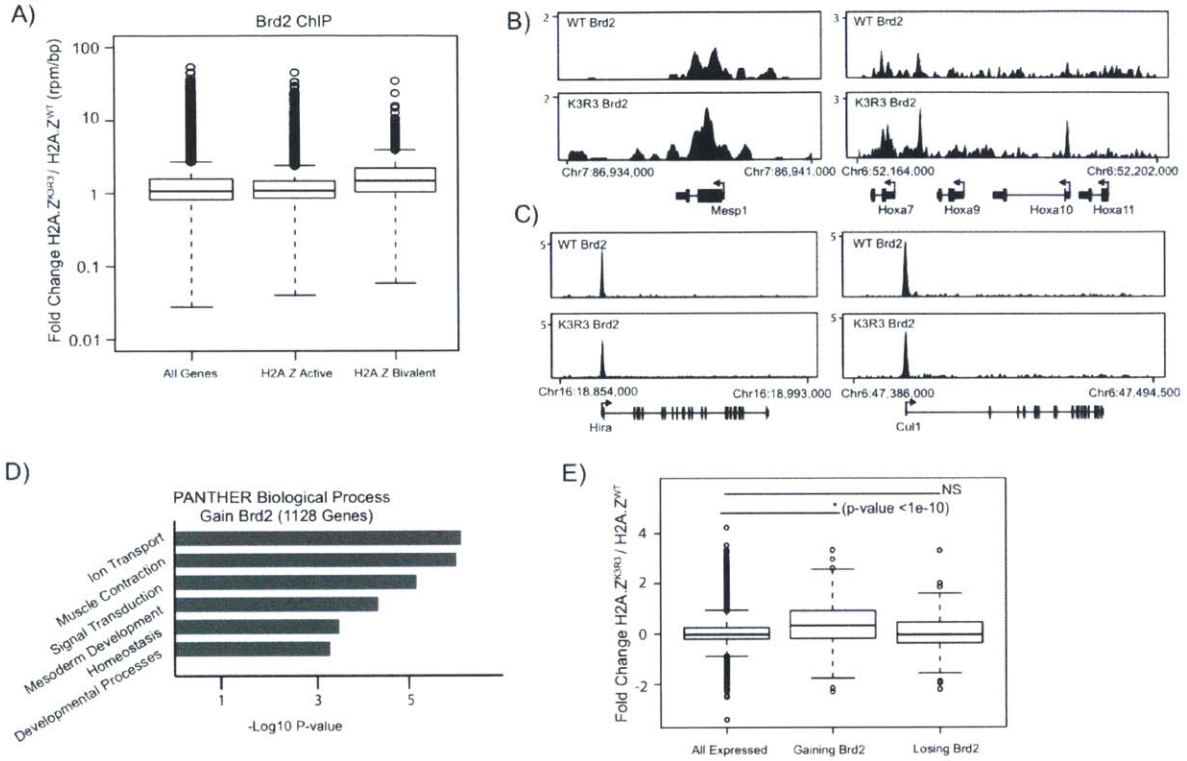

Figure 3: H2A.Z ubiquitin inhibits the recruitment of Brd2 to the promoter of bivalent genes. (A) Boxplot representing median fold change in Brd2 ChIP-seq reads across the promoter regions (TSS +/- 300bp) of all genes, H2A.Z enriched active genes and H2A.Z enriched bivalent genes. H2A.Z bivalent target genes show a significant gain in Brd2 levels (Wilcox test p<2.4e-118). (B) Representative gene tracks Brd2 of the bivalent gene Mesp1 and genes of the HoxA cluster that show gains in Brd2 at their promoter regions. Reads are normalized to reads per million per base pair. (C) Gene tracks of representative active genes Hira and Cul1, which show no change in the levels of Brd2 at their TSS. (D) GO analysis for Panther Biological Processes of genes gaining Brd2 at the TSS as determined by DAVID (Huang et al., 2009a; 2009b) (E) Boxplot of the median fold change in expression of genes gaining Brd2 greater than 2-fold at the TSS, which are significantly upregulated (p-value 6.3e-11, un-paired t test). Genes losing Brd2 do not show a significant change as a population.

In total, we found that 1128 genes exhibited a 2-fold or greater change in Brd2 enrichment in both biological replicates. GO analysis revealed that genes gaining Brd2 have roles in Signal Transduction, Ion Transport and Developmental Processes, including Mesoderm and Skeletal (Figure 3D), consistent with a de-repression of developmental regulators observed in H2A.Z^{K3R3} ESCs (Surface et al., Submitted). Moreover, genes that displayed >2-fold enrichment

of Brd2 in the H2A.Z^{K3R3} ESCs showed a significant increase in expression compared to all genes (median change 1.3 Fold, Wilcox test p<9.2e-17) (Figure 3E). In contrast, the expression of genes that displayed >2-fold depletion of Brd2 at their promoter regions was largely unaffected. H2A.Z^{K3R3} ESCs do not display significant differences in the levels of either H4 acetylation or H2A.Z acetylation at bivalent genes (Figures S3A and S3B) suggesting a change in acetylation levels in H2A.Z^{K3R3} is not the primary driver of this recruitment. These data support a specific functional role for H2A.Zub in the recruitment of Brd2. As an independent validation, we performed SILAC-IP in H2A.Z^{K3R3} ESCs and compared enriched proteins with those associated with H2A.Z^{WT} (Figure S4A; Supplemental Table 2). We find that Brd2 shows higher enrichment in H2A.Z^{K3R3} chromatin whereas the SRCAP subunits as well as non-BET tandem bromodomain proteins were similarly enriched suggesting specific coordination between H2A.Zub and Brd2 (Figures S4B). Our results show that PRC1-mediated H2A.Zub acts to restrict the recruitment of Brd2 to the promoters of developmental genes.

Brd2 and not Brd4 recruitment to bivalent promoters is necessary for gene activation. Brd4, another member of the BET family of dual bromodomain proteins, plays a key role in regulating gene expression and cell identity in ESCs and in many cancers (Asangani et al., 2014; Di Micco et al., 2014; Liu et al., 2014; Wu et al., 2015a). Our SILAC-IP data showed Brd4 is equally enriched in H2A.Z and H2A chromatin (Figure 1D; Supplemental Table 1), suggesting independent roles for these BET family members in regulating gene expression in ESCs. To test this idea, we compared Brd4 and Brd2 localization patterns by ChIP-seq. Similar to Brd2, Brd4 is enriched at promoter regions although its distribution pattern differs from Brd2 (Figure 4A). Brd4 is highly enriched at enhancers and most notably at super enhancer clusters where H2A.Z is largely absent (Figure 4B) (Anand et al., 2013; Brown et al., 2014; Hnisz et al., 2013). In contrast, Brd2 and H2A.Z are most highly enriched at promoters (Figures 2A and 4B), consistent with our SILAC-IP showing that Brd2 is specifically enriched in H2A.Z chromatin whereas Brd4 is equally enriched in H2A.Z and H2A chromatin (Figure 1D; Supplemental Table 1). We next determined whether Brd4 is recruited to bivalent promoters in H2A.Z^{K3R3} ESCs. We find Brd4 shows a more modest gain at bivalent promoters compared to Brd2 in H2A.Z^{K3R3} ESCs (Figure 4C, median fold change 1.29). These data are in agreement with our

SILAC-IP where Brd2 is more highly enriched in H2A.Z^{K3R3} chromatin compared to H2A.Z^{WT} whereas Brd4 shows a minimal change (Figure S3C; Supplemental Table 2).

Figure 4

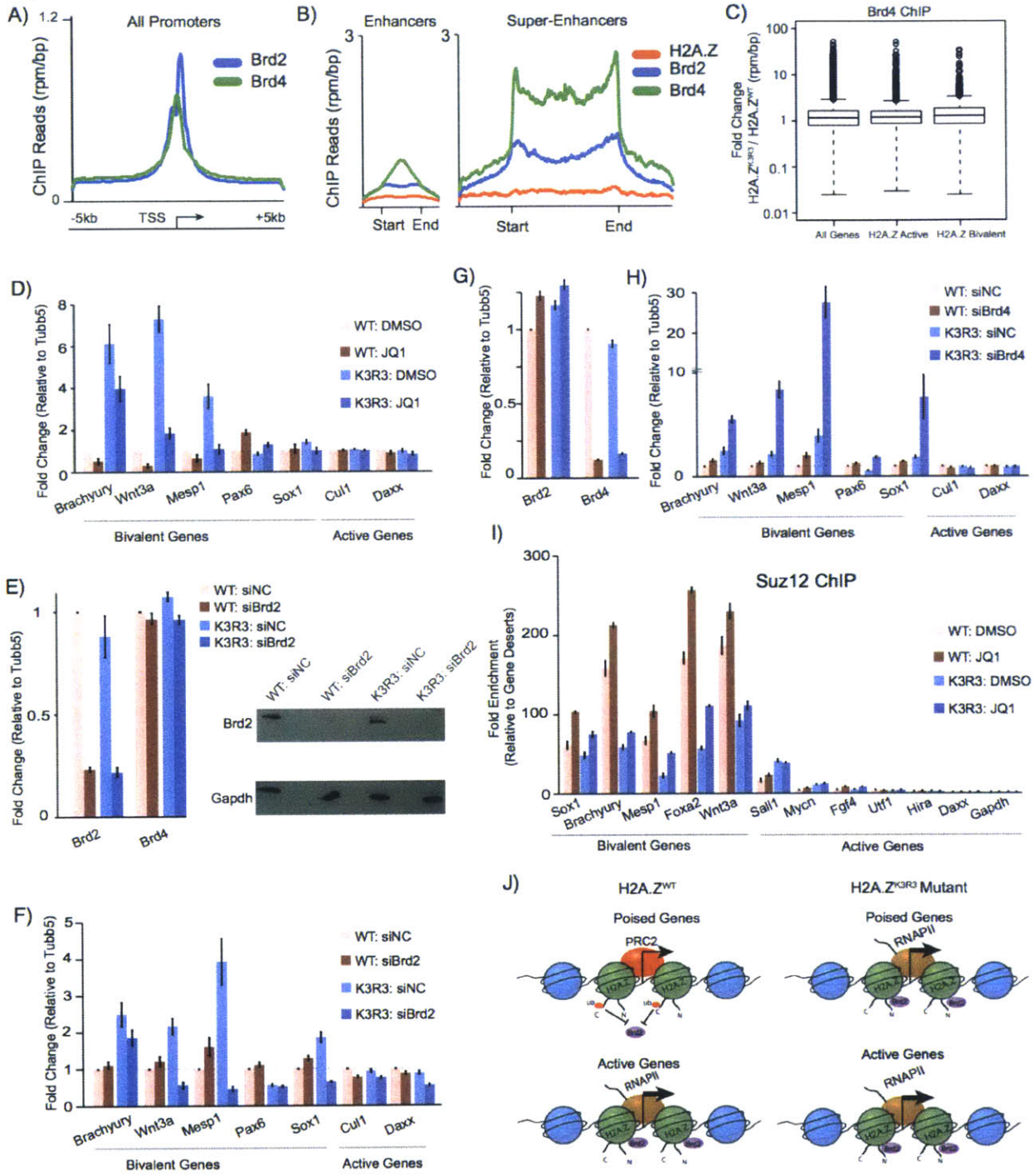

Figure 4: Brd2 and not Brd4 recruitment to bivalent promoters is necessary for gene activation. (A) Average signal for ChIP-seq reads of Brd2 and Brd4 acroos all promoters in mESCs (Brd2 represents the same as Figure 2A). Brd2 and Brd4 are both enriched across the TSS. Reads are normalized to reads per million per base pair. (B) Average signal for ChIP-seq of H2A.Z, Brd2 and Brd4 for 8000 traditional enhancers in mESCs and 216 super-enhancers as defined in (Hnisz et al., 2013). (C) Boxplot representing median fold change in Brd4 ChIP-seq reads across the promoter regions (TSS +/- 300bp) of gene subsets. H2A.Z bivalent target genes show a slight gain in Brd4 levels (Wilcox test $p<1.9e-17$). (D) mRNA expression upon treatment of $H2A.Z^{WT}$ and $H2A.Z^{K3R3}$ mutant ESCs with pan-BET inhibitor JQ1 at 100nM or DMSO control for 24hrs. Expression is determined by qRT-PCR and calculated using 2^{-ddCp} as normalized to Tubb5 and compared to $H2A.Z^{WT}$ DMSO treated. Error bars represent standard error. (E) mRNA expression and protein levels after 48hrs of RNAi mediated knock-down in both $H2A.Z^{WT}$ and $H2A.Z^{K3R3}$ cells. Expression levels are calculated as above, normalized to a non-targeting siRNA in $H2A.Z^{WT}$ ESCs. Immunoblots for Brd2 and Gapdh performed on whole cell lysates following siRNA treatment. (F) mRNA expression for H2A.Z bivalent and active target genes after treatment with either a negative control siRNA or siRNA to Brd2. Expression is calculated as above (G) mRNA expression levels after RNAi mediated knock-down of Brd4 in both $H2A.Z^{WT}$ and $H2A.Z^{K3R3}$ cells for 48hrs. (H) mRNA expression for H2A.Z bivalent and active target genes after treatment with either a negative control siRNA or siRNA to Brd4. (I) Suz12 ChIP followed by qPCR after treatment of $H2A.Z^{WT}$ or $H2A.Z^{K3R3}$ mutant ESCs with either 100nM JQ1 or DMSO. Enrichment values are normalized to two gene desert regions. Error bars represent standard error. (J) Model for Brd2 recruitment to bivalent genes. In $H2A.Z^{WT}$ ESCs, Brd2 is localized to active promoters in a bimodal distribution at the TSS, whereas it shows low enrichment at H2A.Zub-marked (indicated by red circles) bivalent genes. Loss of H2A.Zub in $H2A.Z^{K3R3}$ mutant ESCs allows recruitment of Brd2 at promoters of developmental genes and to gene activation.

Our results suggest that specific recruitment of Brd2 to promoters in ESCs is necessary for gene activation. To test this idea, we treated cells with the pan-BET inhibitor JQ1 at a low concentration to inhibit BET proteins without inducing dramatic change in cell state or cell death as has been seen with high concentrations of JQ1 (Liu et al., 2014; Wu et al., 2015a). After 24hr of treatment with 100nM JQ1, repression of bivalent gene expression in $H2A.Z^{K3R3}$ ESCs was restored to near wild-type levels whereas the expression of active genes was unaffected (Figure 4D). We next tested whether bivalent gene repression can be rescued by Brd2 inhibition in $H2A.Z^{K3R3}$ ESCs. Depletion of Brd2 by siRNA resulted in a significant decrease in both transcript and protein levels of Brd2 with no affect on Brd4 levels (Figure 4E). $H2A.Z^{K3R3}$ ESCs depleted of Brd2 showed a significant decrease in the expression of bivalent genes

whose levels were restored upon JQ1 treatment whereas loss of Brd2 had little effect on active genes or on bivalent genes in H2A.Z^{WT} ESCs (Figure 4F). In contrast, depletion of Brd4 led to a slight increase in bivalent gene expression in H2A.Z^{WT} cells and to a dramatic increase in the expression of developmental genes in H2A.Z^{K3R3} ESCs (Figures 4G and 4H), consistent with a role for Brd4 in the maintenance of cell identity (Di Micco et al., 2014; Liu et al., 2014; Wu et al., 2015b). Thus, Brd2 and Brd4 play distinct roles in regulating gene expression patterns in ESCs.

H2A.Zub is critical for the maintenance of PRC2 at bivalent genes (Surface et al., Submitted), leading us to ask whether Brd2 antagonizes PRC2 to allow for gene activation. To test this idea, we analyzed Suz12 occupancy, a PRC2 component, by ChIP-qPCR upon JQ1 treatment of H2A.Z^{WT} and H2A.Z^{K3R3} ESCs. Upon BET inhibition, we observed an increase in Suz12 binding in both H2A.Z^{WT} and H2A.Z^{K3R3} ESCs specifically at bivalent genes consistent with our results showing that Brd2 depletion restores gene silencing at developmental genes (Figure 4I). Together, our study supports a model where H2A.Z coordinates recruitment of PRC2 and Brd2 to regulate transcriptional output and developmental programs in ESCs.

Discussion

The incorporation of histone variants is a key mechanism used to regulate cell identity and to respond to developmental cues. We demonstrate how H2A.Z post-translational modifications can influence downstream effector binding to modulate gene expression states. While H2A.Z was shown to recruit Brd2 to two loci upon hormone stimulation in LNCaP cells (Draker et al., 2012), H2A.Z is enriched prior to induction raising the question of how Brd2 is selectively recruited upon induction. We show that in ESCs, H2A.Z post-translational modification at promoters acts as a molecular switch for gene control whereby H2A.Zub poises genes for activation through maintenance of PRC2 and by inhibiting Brd2 recruitment in the absence of a stimulus (Figure 4J). In contrast, Brd4 is critical for the establishment and maintenance of pluripotency, possibly through activation of super-enhancers associated with ESC identity genes (Di Micco et al., 2014; Hnisz et al., 2013). Consistent with this model, Brd4-null mice die early in embryogenesis due to defects associated with the inner cell mass (Houzelstein et al.,

2002). On the other hand, Brd2-null embryos, while also embryonic lethal, survive longer but are smaller and display many developmental abnormalities (Gyuris et al., 2009; Shang et al., 2009).

Loss of H2A.Zub results in a significant de-repression of bivalent genes and decrease in PRC2 binding and H3K27me3 (Surface et al., Submitted). Loss of H3K27me3 alone is not sufficient to confer transcriptional activation (Marks et al., 2012; Tee and Reinberg, 2014), suggesting it must be coupled to an activating mechanism. Our data suggest that Brd2 recruitment to developmental genes is key for activation. However, how Brd2 drives transcription remains unclear, as it lacks the p-TEFb interacting motif of Brd4 (Jang et al., 2005). *In vitro* Brd2 promotes transcription through a chromatinized template by directly acting a chromatin remodeler (Leroy et al., 2008), suggesting a possible direct role for Brd2 in promoting transcription at bivalent genes. Additionally Brd2 can bind both TBP and Mediator, which are normally absent from bivalent promoters (Denis et al., 2006; Ku et al., 2012; Peng et al., 2006; Pinz et al., 2015). Given the low level of transcription that occurs at bivalent genes when ESCs are cultured in serum (Bernstein et al., 2006; Marks et al., 2012), H2A.Zub may block RNAPII engagement, while the recruitment of Brd2 may enhance productive elongation at bivalent genes. While we were unable to detect an increase in either RNA Pol2 or Mediator at bivalent genes in H2A.Z^{K3R3} due to the overall low levels of RNAPII at bivalent genes, further studies are needed to test this model.

H2A.Z homologs in both yeast and *C. elegans* coordinate with BET proteins to regulate gene expression states (Shibata et al., 2014; Zhang et al., 2005), suggesting that coordination between H2A.Z and Brd2 is an evolutionarily conserved mechanism for regulating inducible gene expression. Although we focused on Brd2 due to its high expression in mESCs, our data opens the door for future investigations of the roles of the broader class of dual bromodomain proteins. Both Phip and Brwd3 were recently shown to be associated with H3K27ac and H3K4me3 in a proteomic screen in mESCs (Ji et al., 2015), and the *Drosophila* homolog of Brwd3 regulates H3.3 deposition (Chen et al., 2015), which often co-localizes with H2A.Z at regions of high chromatin flux (Jin et al., 2009). Thus, our H2A.Z proteomics data

provide a valuable resource to dissect gene regulation in ESCs. H2A.Z has also been implicated in the process of tumorigenesis including in prostate and breast cancers (Dryhurst et al., 2012; Svotelis et al., 2010; Valdés-Mora et al., 2012). H2A.Z occupies the promoters of genes that become activated upon cancer progression and H2A.Z de-ubiquitination is required for the activation of specific PSA responsive genes (Draker et al., 2011). Thus, our investigations into how H2A.Z and other epigenetic modifiers crosstalk reveal important insights into the control mechanisms that regulate development and how faulty regulation can contribute to cancer.

Experimental Procedures

Growth of mouse embryonic stem cells (mESCs)

V6.5 (129SvJae and C57BL/6; male) ESCs were plated on irradiated murine embryonic fibroblasts (MEFs) and grown under typical ES cell conditions on gelatinized tissue culture plates. Cells were grown in Knockout DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), leukemia inhibitory factor (LIF), non-essential amino acids (Invitrogen), L-glutamine (Invitrogen), and penicillin/streptomycin (Invitrogen) as previously described (Boyer et al., 2006) (Subramanian et al., 2013). To harvest cells for experiments, ESCs were pre-plated to remove MEFs and plated without MEFs for the final passage. Cells for K3R3 and WT rescue experiments were grown on blast resistant feeders cells with 5ug/ml blastocidin. Doxcycline (Sigma, D9891) induction at 1ug/ml was performed for a minimum of four days prior to experiments. For JQ1 experiments cells were treated for 24hrs at 100nM. Control cells were treated with equal volume of DMSO.

SILAC Based Immunoprecipitation

Cells were grown for one week in SILAC medium containing DMEM supplemented with 15% FBS, Pen/Strep, NEAA, Glucose, M_0, P_0 and either R_0K_0 or $R_{10}K_8$. One day prior to IP, antibodies are coupled to Protein A beads as previously described (Cheeseman et al., 2004). Briefly 100ul Protein A beads (Bio-rad 156-0006) are equilibrated in PBST (PBS + 0.1% Tween-20), 3-4 times, then incubated for 1 hr. at RT with 55ug of GFP antibody, then washed 3 more times. Beads and antibody are washed 3 times with 0.2 M Sodium Borate pH 9.0 and cross-linked for 30min at RT with 20mM dimethylpimelimidate (Sigma D8388), and then washed two times with 0.2M ethanolamine, 0.2M NaCl pH 8.5 to inactivate crosslinker, and stored overnight at 4°.

10^9 cells were collected for each condition for immunoprecipiation as modified from (Umlauf et al., 2004). Cells were resuspended in 10ml cell suspension buffer (CSB) (20mM HEPES pH 7.5, 15mM NaCl, 60mM KCl, 0.32M Sucrose, 0.15mM spermine, 0.5mM spermidine, 10mM DTT, 1mM EDTA, 0.1mM EGTA, 1x protease inhibitor (Roche 05056489001)), and then lysed by the addition of 10ml of lysis buffer (CSB + 0.5% NP-40), and incubated for 11 minutes at 4°. The NP-40 is diluted by the addition of 20ml Reaction buffer (CSB without EDTA/EGTA), and

washed twice with reaction buffer, spins at 1000g for 10min. Resuspend in 1ml of reaction buffer for MNase treatment. Split the cells into two 500ul aliquots and add 500ul reaction buffer with 2mM $CaCl_2$ and 5ul MNase (NEB M0247S). Incubate at 37 for 4.5 minutes. Terminate reaction by adding 1ml STOP buffer (10mM EDTA, 0.5mM EGTA, 10mM Tris HCl pH 8, 100mM NaCl, 0.1% Na-Deoxycholate, 0.5% N-lauroyl sarcosine, 1% Triton X-100). Spin for 10min at 15000rpm, and combine supernatant with beads, which have been pre-eluted by 3x washes with 1ml Glycine pH 2.5 and 3x washes with 1x Wash Buffer (50mM HEPES pH 7.4, 1mM EGTA, 1mM $MgCl_2$, 75mM KCl, 10% Glycerol, 0.5% NP-40, 1mM PMSF, 1x Protease Inhibitor (Roche 05056489001)). Beads and Lysate are incubated for 2hrs at 4°. IPs were washed twice with wash buffer (with 15mM KCl), on the second wash, the paired samples (heavy and light) are combined. Samples are twice eluted off the beads in 50ul 1x LDS Sample Buffer (Life Technologies NP0007) with 10mM DTT for 5min at 95° and combined.

In-gel digestion, mass spectrometry, and data analysis

In-gel digestion and extraction was completed essentially as described in (Sancak et al., 2013). Briefly, eluted proteins were loaded onto a NuPAGE Novex Bis-Tris 4-12% gel and separated for approximately 1 hour at 130V. The resulting gel was stained overnight with Coomassie G-250 (Invitrogen). Each lane was manually cut into 6 gel bands such that the entire molecular weight range was utilized for the experiment. Each band were destained with 500 ul of 1:1 acetonitrile:100 mM ammonium bicarbonate pH 8.0 solution followed by dehydration with acetonitrile and swelling with 100 µL of 10 mM DTT for one hour while shaking. The DTT solution was removed and 100 µL of 55 mM iodoacetamide was added to the gel bands for 45 minutes in the dark, removed, and bands were dehydrated with acetonitrile. For digestion, ~10-15 µL of 20 ng/µL Sequencing Grade Trypsin (Promega) was added to each gel band for overnight incubation with shaking at room temperature. Prior to peptide extraction, excess trypsin solution was removed and discarded. Gel bands were incubated with 20 µL of 60% acetonitrile/0.1% TFA for 10 min, after which the extraction solution was removed and collected. The extraction procedure was repeated twice followed by a final extraction with 100% acetonitrile. Extracted peptides were completely dried by vacuum centrifugation and reconstituted in 0.1% formic acid (FA). Peptides were desalted using C18 StageTips packed

with C18 Empore™ high performance extraction disks (3M) exactly as previously described (Rappsilber et al., 2007) (Sancak et al., 2013).

Samples were analyzed by nanoflow-HCD-MS/MS using a Q Exactive mass spectrometer (Thermo Fisher Scientific) coupled to a Proxeon Easy-nLC 1000 esentially as previously described (Sancak et al., 2013). Samples were resuspended in Solvent A (3% MeCN/1% FA) and injected onto a microcapillary column (360 µm o.d X 75 µm i.d) equipped with an integrated electrospray emitter tip (10 µm), packed to ~24 cm with ReproSil-Pur C18-AQ 1.9 µm beads (Dr. Maisch GmbH), The column was heated to 50°C for online separations. Peptides were eluted into the mass spectrometer at a flow rate of 200 nL/min using the HPLC gradient previously described (Sancak et al., 2013). The Q Exactive instrument was operated such that after each MS1 scan (R=70,000), HCD MS/MS scans (R=17,500) were recorded on the 12 most abundant ions. Ion targets $3x10^6$ ions $5x10^4$ ions were used for MS1 and MS2 acquisitions, respectively. MS2 scans were recorded using a 120 ms maximum ion time, an HCD normalized collision energy of 25, and a dynamic exclusion time of 20 s.

MS data were analyzed using MaxQuant v1.2.2.5 (Cox and Mann, 2008; Cox et al., 2011). Data were searched against the mouse Uniprot database containing the exact amino acid sequence of the H2A-YFP and H2A.Z-YFP or H2A.Z^{WT}-YFP and H2A.Z^{K3R3}-YFP constructs and 248 common laboratory contaminants provided by the MaxQuant program. Trypsin was used as the specified enzyme for searching considering a maximum of 2 missed cleavages. The precursor mass tolerances for the first search and the main search were set to 50 ppm and 6 ppm, respectively. Carbamidomethylation of cysteine was searched as a fixed modification and oxidation of methionine and acetylation of protein N-termini were searched as variable modifications. Peptide and protein false discovery rates (FDR) were set to 1% and the minimum peptide length was set to 6. Proteins identified by ≥2 unique peptides and ≥2 ratio counts in both biological replicates were considered for this dataset. Normalized protein ratios provided by MaxQuant were subjected to a moderated T-test to assess statistical significance (Smyth, 2004). The nominal p-values arising from the moderated t-statistic were corrected for multiple testing by controlling the false discovery rate as proposed by Benjamini

and Hochberg (Benjamini and Hochberg, 1995). Proteins with an FDR adjusted p-value of less than 0.1 were deemed to be reproducibly enriched for further analysis.

Chromatin Immunoprecipitation (ChIP and ChIP-seq)

ChIPs were preformed as previously described (Rahl et al., 2010; Wamstad et al., 2012), with the modifications that $30x10^6$ cells were sonicated in 50mM Tris-HCl pH 7.5, 140mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 0.1% Na-Deoycholate, 0.1% SDS, on a Diagenode Bioruptor (UCD-200) for 35 cycles of 30s on, 30s off. $15x10^6$ cells were used per ChIP. ChIPs were washed 4 times, 1 each: low salt (150mM NaCl, 2mM EDTA, 20mM Tris-HCl pH 8, 1% Triton X-100, 0.1% SDS), high salt (500mM NaCl, 2mM EDTA, 20mM Tris-HCl pH 8, 1% Triton X-100, 0.1% SDS), LiCl (250mM LiCl, 1mM EDTA, 10mM Tris-HCl pH 8, 1% NP-40, 1% Na-Deoxycholate), TE + NaCl (10mM Tris-HCl, 1mM EDTA, 50mM NaCl). Samples were eluted 2x 15 minutes at 65° with shaking in elution buffer (10mM EDTA, 50mM Tris-HCl pH 8, 1% SDS). Samples were reverse cross-linked at 65° for 6 hours, followed by 2hr RNase, and 2hr Proteinase K. Phenol:Chloroform extraction was performed and samples were precipitated overnight at -80°. Final samples were resuspended in 40ul water. Antibodies used were Brd2 (Bethyl A302-583A), Brd4 (Bethyl A301-985A50), Suz12 (Cell Signaling 3737). After precipitation ChIPs were resuspended in 40ul of pure water. Previously published datasets are available on GEO, H2A.Z-YFP and H3K27me3 (GSE40063), acetylH2A.Z (GSE39237), H3K27ac (GSE24164), H3K4me3 (GSE11724), RNAPol2 (GSE20485), Brd4 (GSE36561).

ChIP libraries were prepared by SPRI-works Fragment Library System I (Beckman Coulter) for each library and sequenced on an Illumina Hi-Seq. ChIP-reads were aligned to mm9 using Bowtie 1.0.1, and enriched regions called with Macs 1.4.2 with a p-value of 1e-09. Enriched genes were defined by GREAT 2.0.2 as the single nearest gene within 2kb (McLean et al., 2010). Genomic location of enriched regions were determined using CEAS. Gene tracks and metagene figures were generated as described in (Brown et al., 2014) (Lovén et al., 2013), and represent reads per million per base. Fold change for promoter regions was calculated by adding a pseudo-count of 0.01 rpm/bp over the region +/- 300bp of the TSS of respective

genes and comparing H2A.Z^{K3R3} to H2A.Z^{WT}. Heatmaps were generated in R using the pheatmap function.

ChIP-qPCR reactions using SYBR green (KAPA biosystems) and specific primers (Supplementary Table 3), were performed on ChIP and whole cell extract DNA. Reactions were done in triplicate on Roche LightCycler 480. Fold enrichment was calculated by first % Input = $2^{\wedge}(Cp_{WCE} - Cp_{ChIP})$ and then normalizing to the average of two gene desert regions (Chr 7 and Chr 17).

RNA isolation and quantitative real-time PCR

RNA was extracted from ESCs using Isol-RNA (5Prime, 2302700). RNA was then reverse transcribed to cDNA with M-MLV reverse transcriptase (Invitrogen, 28025-013) and random hexamers according to manufacturer protocols. qRT-PCR was performed with SYBR Green Master Mix (KAPA Biosystems). Primer sequences are listed in Supplementary Table 3. Quantification was performed using Roche Lightcycler 480 Software Version 1.5 normalized to Tubb5.

RNA-seq

RNA-seq for H2A.Z^{WT} and H2A.Z^{K3R3} was analyzed as in (Surface et al., submitted) and is available from GSE53208. Analysis was restricted to transcripts showing greater than 5 reads in each replicate.

siRNA Transfections

2.5 x 10^5 mESCs were plated off MEFs 24hrs prior to transfection. siRNAs to Brd2 and Brd4 (Origene, SR419051 and SR422790) were transfected overnight at 25nM with 10ul DharmaFect 1 (GE Healthcare) in mESC media. A non-targeting siRNA was used as a negative control (Origene SR30004). Expression was assayed 48hrs after the start of siRNA transfection.

Accession Numbers

All sequencing data reported in this chapter have been deposited under the GEO accession ID GSE67944.

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Figure S1, Related to Figure 1: Doxycycline inducible transgenes of H2A.Z-YFP and H2A-YFP provide a system to examine the H2A.Z interactome in mESCs. (A) Live cell images showing inducible expression of H2A.Z-YFP and H2A-YFP in mESCs. Doxycycline was induced for 72hrs at 1ug/ml. Scale bar indications 100uM (B) Immunoblot analysis of whole cell lysate for H2A.Z-YFP and H2A-YFP transgenic lines with or without doxycycline induction. Transgene is expressed in both cell lines to a comparable level as determined by GFP antibody (reacts with YFP). (C) Silver stain gel of isolated proteins from SILAC-IP of H2A.Z-YFP and H2A-YFP. Isolated bands were subjected to tandem mass spec. (D) RNA-seq data for tandem bromodomain proteins in wild-type mESCs, values represent RPKM for each transcript. Seven out of the eight are expressed in mESCs except for Brdt (testis specific).

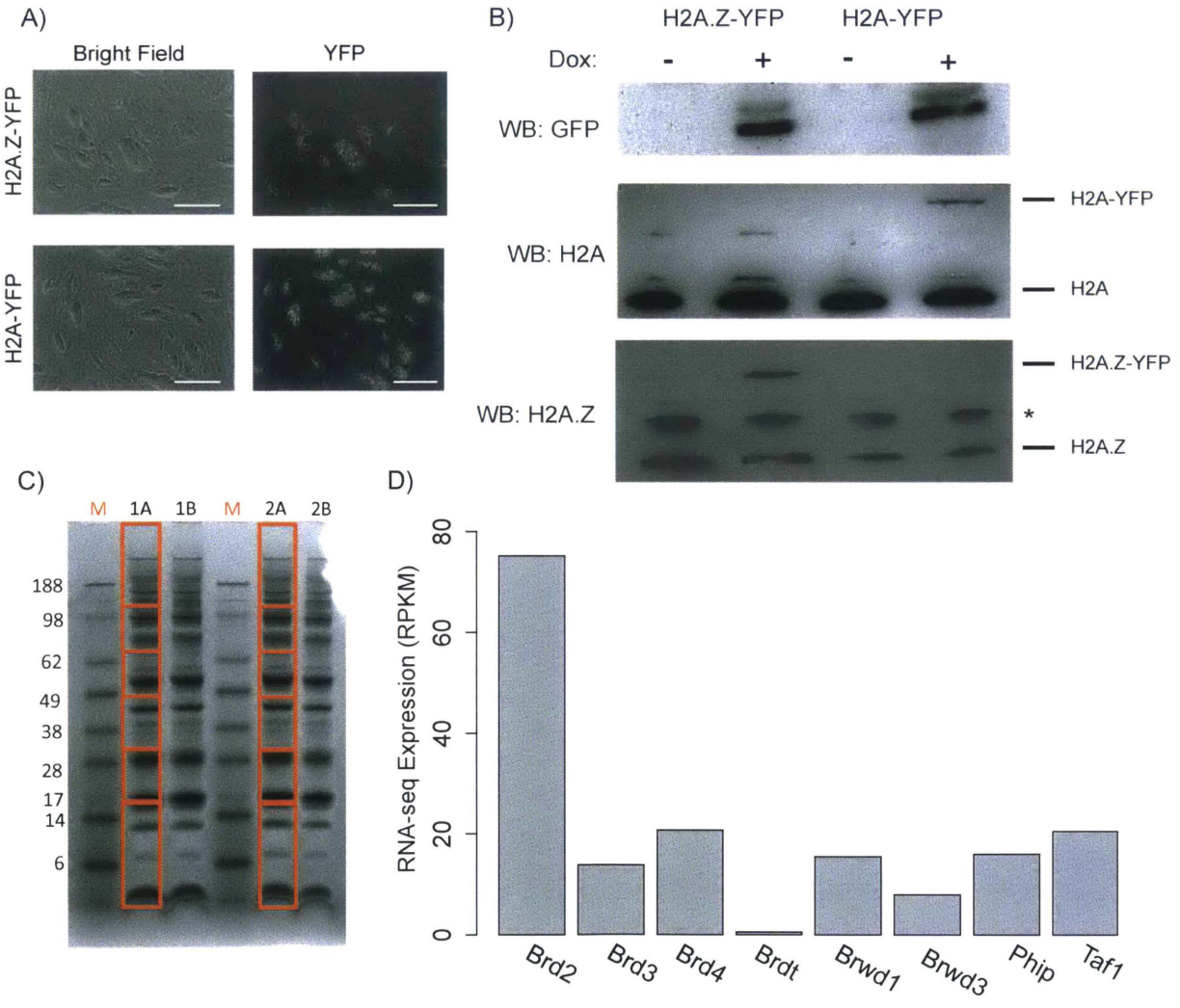


Figure S2, Related to Figure 3: H2A.Z^{WT} and H2A.Z^{K3R3} mutant ESCs are expressed similarly and don't influence BET protein levels. (A) Immunoblot for transgene in H2A.Z^{WT}-YFP and H2A.Z^{K3R3}-YFP, as detected by GFP antibody, after induction for 72 hrs. H3 is used as a load control. H2A.Zub band is absent in the H2A.Z^{K3R3}-YFP mutant ESCs. (B) ChIP-qPCR for GFP ChIP in H2A.Z^{WT}-YFP and H2A.Z^{K3R3}-YFP expressing lines. Transgenic proteins are incorporated to comparable levels. Enrichment values are normalized to two gene desert regions. Error bars represent standard error. (C) mRNA expression for transcripts of Brd2 and Brd4 in H2A.Z^{WT} and H2A.Z^{K3R3} ESCs normalized to Tubb5. Error bars represent standard error. (D) Immunoblot of whole cell lysate for levels of Brd2 and Brd4 protein in H2A.Z^{WT} and H2A.Z^{K3R3} ESCs. Gapdh was used as load control.

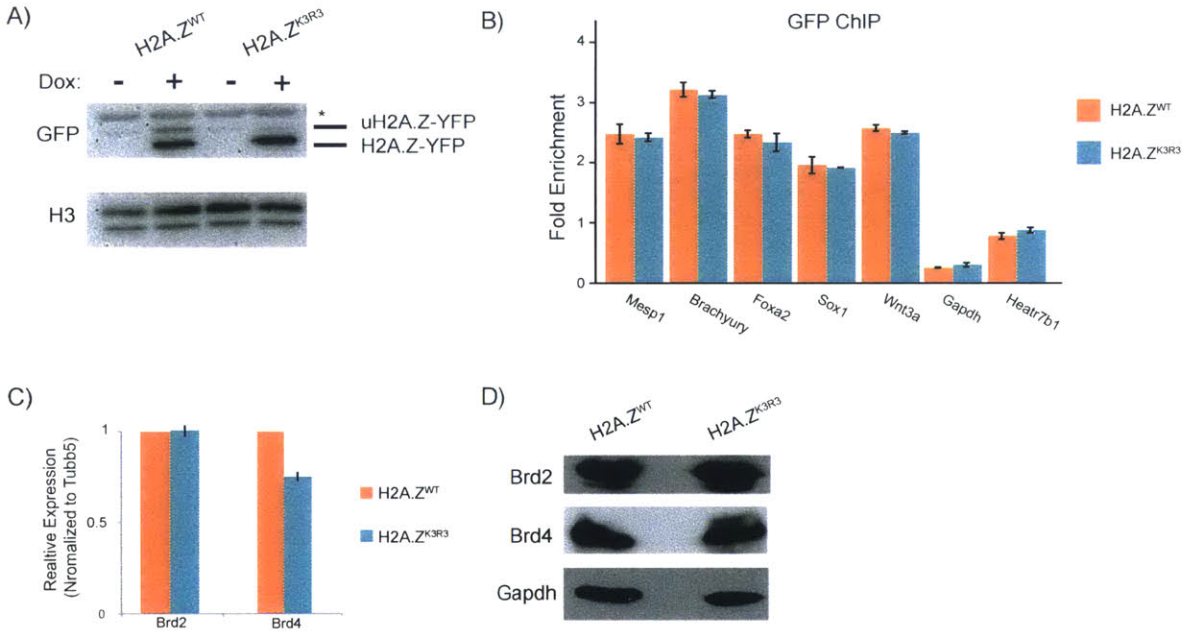


Figure S3, Related to Figure 3: acetyl-H2A.Z and acetyl-H4 levels are not altered in the H2A.Z^{K3R3} mutant ESCs. qPCR following (A) acetyl-H2A.Z ChIP and (B) acetyl-H4 in H2A.Z^{WT} and H2A.Z^{K3R3} cells. Target genes represent H2A.Z active and bivalent targets and non-H2A.Z target genes. Active genes are H3K4me3+,H3K27me3-, bivalent genes are H3K4me3+, H3K27me3+, Negatives are non-H2A.Z enriched genes. Enrichment values are normalized to two gene desert regions. Error bars represent standard error.

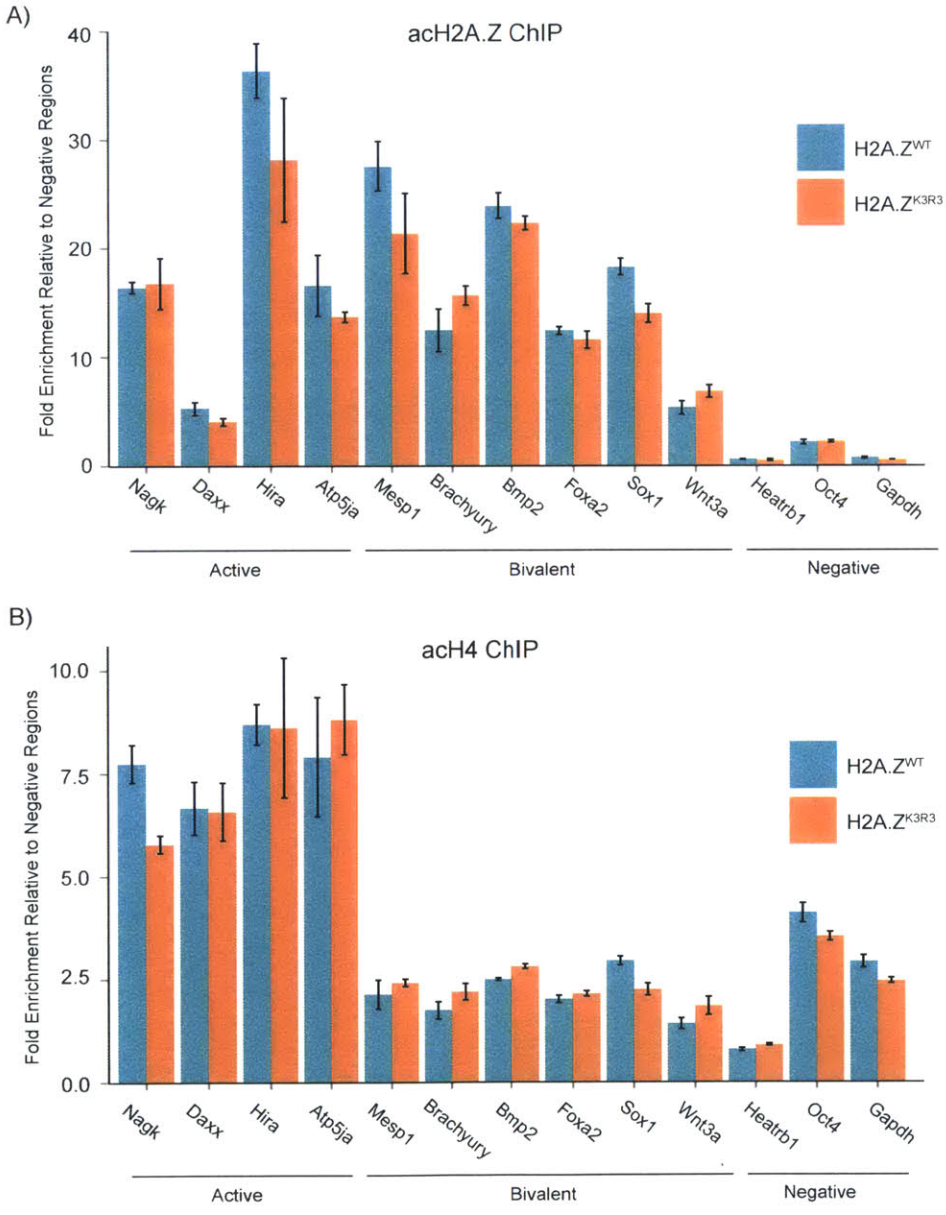
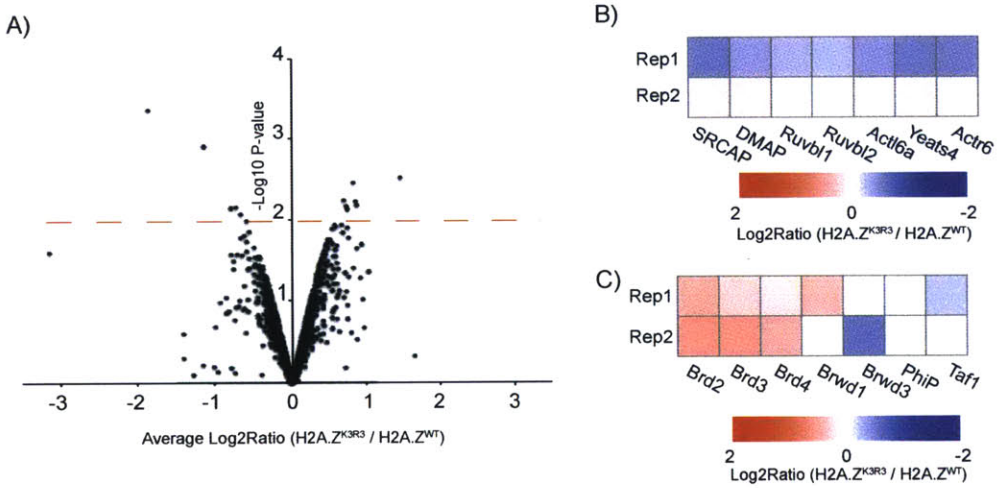


Figure S4, Related to Figure 3: BET proteins enriched in H2A.Z^{K3R3} chromatin compared to H2A.Z^{WT} by SILAC-IP. (A) Volcano plot of H2A.Z^{K3R3}-YFP vs. H2A.Z^{WT}-YFP immunoprecipitated proteins. Plot represents average ratio of enrichment of biological replicates based on SILAC intensity for H2A.Z^{K3R3}-YFP vs. H2A.Z^{WT}-YFP against p-value for significance of reproducibility. (B) Heatmap of Log2 ratio of H2A.Z^{K3R3}-YFP vs. H2A.Z^{WT}-YFP intensity for SRCAP complex proteins from SILAC-IP. (C) Heatmap of Log2 ratio of H2A.Z^{K3R3}-YFP vs. H2A.Z^{WT}-YFP intensity for tandem bromodomain proteins from SILAC-IP. BET proteins are specifically enriched in H2A.Z^{K3R3} chromatin, while other tandem bromodomain proteins are unaffected.



Supplemental Table 1: Proteins differentially associated with H2A.Z-YFP compared to H2A-YFP. Sorted based on moderated-t p-value.

Table S1:

Protein Names	Log2 Ratio YFP H2Az/YFP H2A (H/L) Rep01	Log2 Ratio YFP H2Az/YFP H2A (L/H) Rep02	Moderated t pvalue
L-threonine 3-dehydrogenase	2.10	2.00	0.00
Protein YL-1; VPS72	2.39	2.00	0.00
Serine hydroxymethyltransferase	1.55	1.91	0.00
B-CK;Creatine kinase B chain;Creatine kinase B-type	1.51	1.77	0.00
Protein Srcap	1.53	1.52	0.00
Actin-related protein 6	1.77	1.36	0.00
Bromodomain and WD repeat-containing protein 3	1.40	1.38	0.00
DNA topoisomerase 2-beta	-1.63	-1.29	0.00
Histone aminotransferase 1	1.06	1.89	0.00
Bromodomain containing 2	1.09	1.00	0.00
Aspartate aminotransferaseprotein	0.92	2.28	0.00
Damage-specific DNA-binding protein 1	0.88	0.88	0.01
Microtubule-associated protein 4	-0.93	-0.78	0.01
Echinoderm microtubule-associated protein-like 4	-1.16	-0.70	0.01
14-3-3 protein epsilon	0.68	1.43	0.01
Bromodomain and WD repeat-containing protein 1	1.05	0.71	0.01
Heat shock 86 kDa	0.72	1.83	0.01
2-phospho-D-glycerate hydro-lyase	0.73	1.89	0.01
Heat shock 84 kDa	0.68	1.74	0.01
Uncharacterized protein	-1.25	-0.65	0.01
Pyruvate dehydrogenase E1 component subunit beta	0.68	2.02	0.01
Estrogen related receptor, beta 2	2.00	0.67	0.01
DNA methyltransferase 1-associated protein 1	0.97	0.65	0.01
Malate dehydrogenase	0.62	1.79	0.01
Triosephosphate isomerase	0.62	1.87	0.01
ATP synthase subunit beta	0.58	2.04	0.01
RuvB-like protein 2	0.86	0.61	0.01
RuvB-like 1	0.94	0.57	0.01
Nervous system Polycomb-1;Polycomb group	0.69	0.68	0.02

RING finger protein 1			
Acidic leucine-rich nuclear phosphoprotein 32 family member B	0.48	1.65	0.02
IRS-1 PH domain-binding protein	0.53	0.88	0.02
ATP-dependent RNA helicase eIF4A-1	0.46	1.75	0.02
AHD-M1	0.55	2.68	0.02
C-terminal-binding protein 2	0.78	0.55	0.02
Suppressor of variegation 3-9 homolog 1 (Drosophila)	-0.64	-0.63	0.02
YEATS domain-containing protein 4	1.37	0.39	0.02
DNA methyltransferase 3B	-0.59	-0.62	0.03
Cyclophilin A	0.37	1.59	0.03
Myelodysplasia-myeloid leukemia factor 2	0.51	0.69	0.03
Aldehyde reductase	0.36	1.61	0.03
CCT-theta	0.36	1.30	0.03
14-3-3 protein zeta/delta	0.35	1.49	0.03
Bromodomain containing 3	0.79	0.45	0.03
Transformation related protein 53 binding protein 1	-0.46	-0.72	0.03
17 kDa myosin light chain	0.39	0.89	0.03
40S ribosomal protein S27a	0.38	0.91	0.03
DNA methyltransferase 3B	-0.48	-0.66	0.03
ATP synthase subunit alpha	0.30	1.70	0.03
Myosin, heavy polypeptide 10, non-muscle	0.30	1.39	0.04
Histone-lysine N-methyltransferase SUV420H2	0.49	0.62	0.04
Elongation factor 1-alpha 1	0.28	1.67	0.04
Rcc1 protein	-0.41	-0.73	0.04
SET domain-containing protein 5	0.27	1.39	0.04
Actin-RPV	-0.46	-0.62	0.04
Cell division control protein 2 homolog (CDK1)	0.65	0.44	0.04
Nucleosome assembly protein 1-like 1	0.29	1.16	0.04
ATP-dependent helicase ATRX	-0.44	-0.63	0.04
Cingulin-like protein 1	0.64	0.41	0.05
High mobility group-like nuclear protein 2 homolog 1	0.38	0.67	0.05
Nanog homeobox	1.45	0.22	0.05
Actin-related protein 2/3 complex subunit 1A	0.35	0.74	0.05
Hyaluronan mediated motility receptor	-0.46	-0.55	0.05
C-Jun-amino-terminal kinase-interacting protein 4	-0.43	-0.57	0.05
Jumonji domain-containing protein 1C	0.69	0.35	0.05
Protein FAM83D	-0.45	-0.53	0.05

Bromodomain adjacent to zinc finger domain protein 1A	-0.66	-0.36	0.06
Destrin	0.60	0.39	0.06
Gprasp1 protein	0.48	0.46	0.06
Pyruvate kinase isozymes M1/M2	0.11	2.07	0.06
Microspherule protein 1	0.78	0.26	0.07
Proteasome subunit alpha type-7	-5.35	0.13	0.07
Ladinin-1	-0.12	-1.28	0.07
ELKL motif serine/threonine-protein kinase 3	-0.83	-0.21	0.08
ATPase WRNIP1	-0.39	-0.48	0.08
Leucine-rich repeat-containing protein 59	-0.66	-0.27	0.08
Cellular myosin heavy chain, type A	0.12	1.12	0.08
Melanoma antigen, family D, 1	0.21	0.77	0.08
Methyl-CpG-binding domain protein 1 (MBD1)	-0.41	-0.43	0.08
Elongation factor 2	0.03	1.65	0.09
BRCA1/BRCA2-containing complex subunit 3	0.60	0.28	0.09
Zinc finger protein 598	-0.89	-0.16	0.09
Dynein cytoplasmic 1 intermediate chain 2	-0.25	-0.65	0.09
B-cell receptor-associated protein 32	0.21	0.74	0.09
Nuclear pore complex protein Nup85	-0.01	1.84	0.09
Uncharacterized protein C12orf41 homolog	0.72	0.21	0.09
Breast cancer type 2 susceptibility protein homolog	0.50	0.34	0.09
Nucleolar protein family A, member 2	0.22	0.70	0.09
H/ACA ribonucleoprotein complex subunit 1	0.27	0.59	0.09
RAS related protein 1b	0.33	0.48	0.09
ATP-dependent RNA helicase p54	-0.39	-0.41	0.10
Nucleolar protein Nop52	0.14	0.85	0.10
Cisplatin resistance-associated-overexpressed protein	-0.75	-0.18	0.10

Supplemental Table 2: Proteins differentially associated with H2A.Z^{K3R3}-YFP compared to H2A.Z^{WT}-YFP. Sorted based on moderated-t p-value.

Table S2:

Protein Names	Log2 Ratio (YFP H2Az3KR/YFP H2Az) H/L Rep01	Log2 Ratio (YFP H2Az3KR/YFP H2Az) L/H Rep02	Moderated t pvalue
Histone-lysine N-methyltransferase SUV420H2	1.75	1.18	0.00
ATP-dependent DNA helicase VIII	0.79	0.89	0.00
JmjC domain-containing histone demethylation protein 3A	-1.11	-1.14	0.00
Cofilin, non-muscle isoform	-1.72	-1.99	0.00
Histone H3-K9 methyltransferase 2	1.01	0.71	0.01
CMP-N-acetylneuraminic acid synthase	0.94	0.56	0.01
Zinc finger protein 57	0.75	0.67	0.01
Chromosome segregation 1-like (S. cerevisiae)	0.71	1.05	0.01
Caprin-1	0.64	0.88	0.01
PAI1 RNA-binding protein 1	0.64	0.84	0.01
Arginine--tRNA ligase	0.54	0.82	0.01
Protein cordon-bleu	0.53	0.64	0.01
Cellular myosin heavy chain, type A	0.53	0.56	0.01
Deubiquitinating enzyme 10	0.52	0.67	0.01
IGF-II mRNA-binding protein 3	0.52	0.87	0.01
Retinoic acid-induced protein 1	-0.49	-0.60	0.01
Putative uncharacterized protein	-0.59	-0.73	0.01
Death effector domain-containing, isoform CRA_a	-0.60	-0.57	0.01
Nestin	-0.63	-0.81	0.01
Transformation related protein 53 binding protein 1	-0.65	-0.91	0.01
G2/mitotic-specific cyclin-B1	0.74	0.41	0.02
Cytokeratin endo A	0.59	1.30	0.02
Ladinin-1	0.57	0.40	0.02
Bromodomain containing 2	0.56	0.44	0.02
Lysyl-tRNA synthetase	0.54	1.22	0.02
Fragile X mental retardation syndrome-related protein 1	0.53	1.00	0.02
ATP-dependent RNA helicase DDX42	0.51	0.41	0.02
Cell division control protein 2 homolog	0.48	0.50	0.02
H1 VAR.2	0.47	0.46	0.02
Leucyl-tRNA synthetase	0.45	0.48	0.02
ATP-dependent helicase RENT1	0.45	0.58	0.02
Cytoskeleton-associated protein 5	0.44	0.49	0.02

Cleavage stimulation factor, 3 pre-RNA, subunit 3	0.44	0.44	0.02
Major vault protein	0.44	0.54	0.02
Bifunctional aminoacyl-tRNA synthetase	0.44	0.79	0.02
BCL-6 corepressor-like protein 1	0.43	0.67	0.02
Ubiquitin-associated protein 2-like	0.42	0.78	0.02
Cavin-1	-0.44	-0.71	0.02
Protein YL-1	-0.48	-0.83	0.02
NOC2-like protein	-0.57	-0.41	0.02
Cingulin-like protein 1	-0.76	-0.48	0.02
Poly (ADP-ribose) polymerase family, member 1	0.81	0.39	0.03
Alpha-actinin-4	0.54	0.38	0.03
PDZ and LIM domain 7	0.51	0.34	0.03
Lamina-associated polypeptide 2, isoforms alpha/zeta	0.44	0.41	0.03
DnaJ homolog subfamily A member 3, mitochondrial	0.42	0.40	0.03
Polo-like kinase 1	0.42	0.38	0.03
Nuclear factor erythroid 2-related factor 2	0.41	0.38	0.03
Adaptor protein complex AP-1, gamma 1 subunit	0.38	0.50	0.03
ATP-dependent RNA helicase p54	0.37	0.87	0.03
Guanine nucleotide-binding protein subunit beta-2-like 1	0.37	0.50	0.03
Putative uncharacterized protein Mov10	0.34	0.62	0.03
IGF-II mRNA-binding protein 2	0.33	0.57	0.03
[Histone-H3]-lysine-36 demethylase 1B	0.33	0.53	0.03
U1 small nuclear ribonucleoprotein A	0.33	0.57	0.03
Female-lethal(2)D homolog	-0.37	-0.51	0.03
Vimentin	-0.37	-0.73	0.03
Putative uncharacterized protein	-0.40	-0.45	0.03
Caveolin-1	-0.41	-0.88	0.03
Collagen alpha-1(XII) chain	-0.43	-1.07	0.03
Collagen alpha-2(V) chain	-0.44	-0.96	0.03
Putative uncharacterized protein Nop16	-0.48	-0.38	0.03
H/ACA ribonucleoprotein complex subunit 3	-0.58	-0.36	0.03
60S ribosomal protein L7-like 1	-1.10	-0.47	0.03
ATP-binding cassette sub-family F member 1	1.60	0.48	0.04
Polypyrimidine tract binding protein 1	0.74	0.34	0.04
Regulator of differentiation 1	0.61	0.32	0.04
H1 VAR.5	0.58	0.30	0.04
Putative uncharacterized protein	0.43	1.24	0.04
Ewing sarcoma breakpoint region 1	0.40	0.36	0.04
100 kDa coactivator	0.39	0.38	0.04
Borealin	0.36	0.38	0.04

Lamina-associated polypeptide 2, isoforms beta/delta/epsilon/gamma	0.36	0.38	0.04
NACHT, LRR and PYD domains-containing protein 4F	0.35	0.42	0.04
Outer mitochondrial membrane protein porin 2	0.34	0.38	0.04
CDC-like kinase 3	0.33	0.41	0.04
Ataxin-2	0.33	0.80	0.04
MCG120108, isoform CRA_a	-0.32	-0.66	0.04
E3 SUMO-protein ligase RanBP2	-0.32	-0.43	0.04
Collagen, type V, alpha 1	-0.33	-0.61	0.04
182 kDa tankyrase-1-binding protein	-0.33	-0.77	0.04
Protein virilizer homolog	-0.35	-0.39	0.04
Cavin-2	-0.39	-1.13	0.04
Coronin, actin binding protein 1C	-0.39	-0.37	0.04
Zinc finger protein 318	-0.46	-0.32	0.04
Nucleolar protein family A, member 2	-0.48	-0.30	0.04
High mobility group protein 20A	-0.50	-0.29	0.04
rRNA-processing protein FCF1 homolog	-0.52	-0.31	0.04
U3 small nucleolar ribonucleoprotein protein IMP3	-0.52	-0.32	0.04
MAP7 domain-containing protein 1	-0.81	-0.33	0.04
Adrenal hypoplasia congenita-like protein	0.85	0.30	0.05
Actin-binding protein anillin	0.75	0.28	0.05
Binding factor for early enhancer	0.58	0.29	0.05
Putative uncharacterized protein	0.56	0.27	0.05
Kinesin-like protein KIF18B	0.49	0.28	0.05
Cold shock domain-containing protein A	0.40	1.44	0.05
Putative uncharacterized protein	0.39	0.30	0.05
Zinc finger protein X-linked	0.39	0.32	0.05
Protein regulator of cytokinesis 1	0.37	0.30	0.05
37 kDa laminin receptor precursor	0.36	0.33	0.05
GAP SH3 domain-binding protein 2	0.36	1.07	0.05
La ribonucleoprotein domain family member 1	0.35	1.17	0.05
MCG127945, isoform CRA_a	0.34	0.34	0.05
ATP-dependent helicase ATRX	0.32	0.38	0.05
40S ribosomal protein S2	0.30	0.42	0.05
Abnormal spindle-like microcephaly-associated protein homolog	0.28	0.81	0.05
DEAH box protein 36	0.26	0.57	0.05
Dedicator of cytokinesis protein 6	-0.26	-0.57	0.05
Sal-like protein 2	-0.27	-0.60	0.05
Putative uncharacterized protein Dpf2	-0.28	-0.57	0.05
Transformer-2 protein homolog A	-0.29	-0.44	0.05
Heterogeneous nuclear ribonucleoprotein G	-0.31	-0.35	0.05

CCAAT/enhancer-binding protein zeta	-0.32	-0.35	0.05
Arginine/serine-rich-splicing factor 14	-0.33	-0.35	0.05
Coilin	-0.34	-0.32	0.05
Ran GTPase-activating protein 1	-0.42	-0.28	0.05
Heat shock 86 kDa	-0.70	-0.29	0.05
Liver receptor homolog 1	0.95	0.27	0.06
Bromodomain containing 3	0.55	0.24	0.06
ADNP homeobox protein 2	0.48	0.24	0.06
ATPase family AAA domain-containing protein 2	0.43	0.26	0.06
RecQ-mediated genome instability protein 1	0.41	0.27	0.06
Rap1-interacting factor 1 homolog	0.34	0.30	0.06
La ribonucleoprotein domain family member 4	0.26	0.88	0.06
Small nuclear ribonucleoprotein 70 (U1)	0.26	0.45	0.06
NSD1-interacting zinc finger protein 1	0.25	0.51	0.06
Protein lin-28 homolog A	0.25	0.50	0.06
DEAH box protein 30	0.24	0.68	0.06
Pre-rRNA-processing protein TSR1 homolog	0.24	0.61	0.06
Methyl-CpG-binding domain protein 3	-0.24	-0.60	0.06
Splicing factor, arginine/serine-rich 9	-0.25	-0.73	0.06
Small ubiquitin-related modifier 1	-0.29	-0.35	0.06
CDC-like kinase 1	-0.31	-0.30	0.06
Coiled-coil domain-containing protein 137	-0.38	-0.28	0.06
Zinc finger protein 326	-0.39	-0.26	0.06
Nucleolar and spindle-associated protein 1	-0.40	-0.26	0.06
Periodic tryptophan protein 1 homolog	-0.62	-0.24	0.06
B4 integrin interactor	-0.97	-0.26	0.06
60 kDa chaperonin	-5.19	-1.09	0.06
60S acidic ribosomal protein P0	0.76	0.24	0.07
Serine/threonine-protein kinase VRK2	0.39	0.25	0.07
Zinc finger protein 15	0.36	0.24	0.07
Inner centromere protein	0.33	0.27	0.07
Cyclin-dependent kinase 9 (CDC2-related kinase)	0.24	0.42	0.07
ATP-dependent RNA helicase DDX1	0.24	0.43	0.07
60 kDa BRG-1/Brm-associated factor subunit A	-0.22	-0.50	0.07
Adenine nucleotide translocator 1	-0.24	-0.42	0.07
Nucleolar protein EMG1 homolog	-0.30	-0.29	0.07
60S ribosomal protein L27	-0.33	-0.26	0.07
H/ACA ribonucleoprotein complex subunit 1	-0.46	-0.24	0.07
DNA (cytosine-5)-methyltransferase 3A	-0.46	-0.22	0.07
60 kDa BRG-1/Brm-associated factor subunit B	-0.53	-0.23	0.07
Fertilization antigen 1	-0.74	-0.21	0.07

Ras-responsive element-binding protein 1	1.04	0.24	0.08
Protein RCC2	1.03	0.21	0.08
Basic transcription element-binding protein 2	0.55	0.20	0.08
Putative uncharacterized protein	0.40	0.22	0.08
Nervous system Polycomb-1	0.36	0.23	0.08
Metal regulatory transcription factor 2	0.34	0.25	0.08
Myc-induced nuclear antigen	0.33	0.24	0.08
CCR4-associated factor 1	0.29	0.27	0.08
Eukaryotic translation initiation factor 4 gamma 1	0.25	1.22	0.08
Lamin A	0.22	0.41	0.08
Coding region determinant-binding protein	0.22	0.87	0.08
BRG1-associated factor 57	-0.19	-0.51	0.08
Transcription elongation factor SPT6	-0.19	-0.49	0.08
Putative uncharacterized protein	-0.20	-0.59	0.08
Dehydrogenase/reductase SDR family member 4	-0.22	-0.37	0.08
Putative uncharacterized protein	-0.22	-0.38	0.08
Casitas B-lineage lymphoma-transforming sequence-like protein 1	-0.23	-0.35	0.08
Putative uncharacterized protein	-0.23	-0.35	0.08
Putative uncharacterized protein	-0.23	-0.37	0.08
DNA methyltransferase 3B	-0.31	-0.25	0.08
DNA methyltransferase 3B	-0.36	-0.24	0.08
Down-regulated in metastasis protein	-0.38	-0.23	0.08
ATP-dependent helicase CHD1	-0.40	-0.23	0.08
Cyclophilin B	1.04	0.19	0.09
H1 VAR.3	0.69	0.17	0.09
Methyl-CpG-binding domain protein 4	0.68	0.19	0.09
33 kDa VAMP-associated protein	0.47	0.19	0.09
Origin recognition complex subunit 1	0.38	0.20	0.09
Histone H1	0.34	0.22	0.09
Kinetochore-associated protein KNL-2 homolog	0.28	0.26	0.09
Novel protein	0.25	0.28	0.09
Heterogeneous nuclear ribonucleoprotein U	0.24	0.31	0.09
YTH domain family protein 3	0.23	0.32	0.09
MAP1 light chain LC1	0.21	1.19	0.09
DnaJ homolog subfamily A member 2	0.21	0.33	0.09
Putative uncharacterized protein	0.18	0.45	0.09
Aspartate--tRNA ligase	0.17	0.57	0.09
Nuclear receptor corepressor 2	-0.18	-0.51	0.09
Glutamate-rich WD repeat-containing protein 1	-0.21	-0.38	0.09
Silica-induced gene 41 protein	-0.21	-0.36	0.09
E3 ubiquitin-protein ligase RING1	-0.23	-0.31	0.09

PHD finger protein 14	-0.27	-0.27	0.09
rRNA-processing protein UTP23 homolog	-0.29	-0.24	0.09
Coronin, actin binding protein 1B	-0.34	-0.22	0.09
Cytoplasmic dynein 1 heavy chain 1	-0.40	-0.20	0.09
MRT4, mRNA turnover 4, homolog (S. cerevisiae)	-0.72	-0.18	0.09
Putative uncharacterized protein ENSMUSP00000103235	-1.45	-0.23	0.09
C-terminal-binding protein 2	0.39	0.19	0.10
Nuclear poly(A)-binding protein 1	0.38	0.19	0.10
75 kDa glucose-regulated protein	0.30	0.23	0.10
40S ribosomal protein S3	0.27	0.24	0.10
Isoleucine--tRNA ligase	0.23	1.68	0.10
Putative uncharacterized protein Rbm42	0.18	0.43	0.10
Zinc finger and BTB domain containing 34	0.17	0.49	0.10
Putative uncharacterized protein	0.17	0.44	0.10
Molecule associated with JAK3 N-terminus	0.16	0.70	0.10
Protein lin-41 homolog	0.16	0.72	0.10
ES cell-associated protein 11	0.16	0.63	0.10
U11/U12 small nuclear ribonucleoprotein 48 kDa protein	-0.17	-0.46	0.10
Cofactor required for Sp1 transcriptional activation subunit 6	-0.25	-0.27	0.10
Nucleoplasmin-3	-0.28	-0.24	0.10
RNA 3-terminal phosphate cyclase-like protein	-0.53	-0.17	0.10
Ribosomal RNA-processing protein 15	-0.95	-0.17	0.10

Supplemental Table 3, Related to Experimental Procedures: Primers used for mRNA expression qPCR and ChIP-qPCR analysis

Table S3:

Gene Name	Forward	Reverse	Experiment
Tubb5	AAC CAA CTC AGC TCC CTC TG	GCT GGA CCG AAT CTC TGT GT	mRNA RT-PCR
Brachyury	gctctaaggaaccaccggtcatc	atgggactgcagcatggacag	mRNA RT-PCR
Wnt3a	CCCTTTCCAGTCCTGGTGTA	CTTGAAGAAGGGGTGCAGAG	mRNA RT-PCR
Mesp1	CGC CTG CCT ACC CTA GAC C	AGG TTT CTA GAA GAG CCA GCA	mRNA RT-PCR
Pax6	GAC CGG AAG CTG GGG CAC AC	TGG CAG CCA TCT TGC GTG GG	mRNA RT-PCR
Sox1	CCT GAA AAT GAT GCT GCT GA	GGA GTA GCT GTG GGT GTG GT	mRNA RT-PCR
Cul1	GAT GGC AAT CCT GCT TCA GT	GAT GGC AAT CCT GCT TCA GT	mRNA RT-PCR
Daxx	TCA GTC TCT CCG GCA GTG TA	GGG AGG AGC TCT GTC CAT CT	mRNA RT-PCR
Brd2	GCA TCC TGA AGG AAC TGC TC	ATC TTC CGC TTG ACA GTG CT	mRNA RT-PCR
Brd4	TAC AAG CCT GTG GAT GTG GA	TGG AGA ACA TCA ATC GGA CA	mRNA RT-PCR
Mesp1	AAGGGAGATCCTGAGTGCAG	TGTACGCAGAAACAGCATCC	ChIP-qPCR
Brahyury	CTGCGCTTCAAGGAGCTAAC	CTCAGCGGGAAGAAACAAAG	ChIP-qPCR
Foxa2	CTAGCCCATCTCCTGCTGTC	GTGGCTTGATCCCAGGTCT	ChIP-qPCR
Sox1	AGACTTCGAGCCGACAAGAG	TCACTCAGGGCTGAACTGTG	ChIP-qPCR
Wnt3a	CTGTCCAGCCTCTCCAAGAC	CGCTGTCGCCTAGCTTCTAC	ChIP-qPCR
Gapdh	ACTCCCCTTCCCAGTTTCC	CTCTCTGCTCCTCCCTGTTC	ChIP-qPCR
Heatr7b1	TCCAAAGGTGCATTGGCTGA	AGAGACTGTCCGGGAACCTT	ChIP-qPCR
Sall1	CCCCATTACTCAGCCGAAGTTT	CCCCATTACTCAGCCGAAGTTT	ChIP-qPCR
Mycn	TCACTCCTAATCCGGTCATCCT	TGGCCGTGCTGTAGTTTTTCGT	ChIP-qPCR
Fgf4	TTG CTC TCG CTA CTT AGG TCT G	AGC AGC GTC CCT GTG GTC	ChIP-qPCR
Utf1	ACG TCT GAT GCC TTC GCT AC	AGC GTG CCC AGA AGT AGC TC	ChIP-qPCR
Hira	TACTAAGCTGGACTCGGAGC	GATACTCCGCTGCCACTGAT	ChIP-qPCR
Daxx	GGAGGGAACTATGCGAGGAT	CCTTCAGGGCTAGCAAGGTA	ChIP-qPCR
Oct4	ACACCAGTGATGCGTGAAAA	CCAGTCACACCCAACCTCTT	ChIP-qPCR
Atp5ja	GGA CAC CAG GAC TTC AAG ATG	GTT CCT TCT CAC CCC ACT GT	ChIP-qPCR
Nagk	ACAGGGCTCTTGTGTCAACC	CCGGAGCACCAAGATAAGAC	ChIP-qPCR
Chr17 GD	AAC CTC ACA CAC AAC AAG CTG	TGT GAT AGG GAG AAT GCT TGC	ChIP-qPCR
Chr7 GD	CACAACCCGAGGAAGTGAGT	CACACATTTGCACTCTGAAGC	ChIP-qPCR

Chapter 3

Discussion

Summary and Significance

The work presented in this thesis focuses on dissecting the mechanistic role of H2A.Z in chromatin and how it acts in a context dependent manner to regulate gene expression. H2A.Z is a non-allelic variant of the canonical histone H2A, and is necessary for the development of all multi-cellular organisms by regulating gene expression programs in response to diverse cellular cues (Biterge and Schneider, 2014). H2A.Z has specific divergent regions from H2A, including the N- and C-terminal tails (Suto et al., 2000). We hypothesized that these divergent regions may have important roles in facilitating specific protein interactions and that in conjunction with H2A.Z post-translational modifications may regulate transcriptional output. To address this question we developed a quantitative chromatin immunprecipitation coupled to mass spectrometry approach (SILAC-IP). We focused our efforts on understanding the role of H2A.Z in mESCs because these cells provide a good model for investigating factors that mediate developmental decisions. We identified specific proteins enriched in H2A.Z chromatin compared to H2A, including a class of tandem bromodomain containing proteins, which included Brd2, a BET (bromodomain and extra-terminal) protein. Further, we found that Brd2 co-localizes with H2A.Z at active promoters in mESCs. Notably, PRC1-mediated H2A.Z monoubiquitination (H2A.Zub) is necessary for modulating this interaction, as loss of H2A.Zub results in a recruitment of Brd2 to silent genes and transcriptional up-regulation. Moreover, we find distinct roles for Brd2 and Brd4, another BET family member, in the regulation of ESC transcriptional networks. In summary, our work reveals novel insights into how H2A.Z can act in a context dependent manner to regulate the balance between active and silent genes in ESCs.

The work presented in Chapter 2, based on our SILAC-IP data, focused on the role of H2A.Z in the regulation of Brd2 recruitment and transcriptional regulation. More broadly, our SILAC-IP data identifying H2A.Z enriched proteins compared to H2A, along with our work presented in Appendix A studying the effect of H2A.Z mutations on altering proteins interactions, suggest that H2A.Z modulates a wide range of chromatin-associated proteins interactions. These differentially enriched proteins have functions in many DNA-mediated process, including transcriptional activation, DNA damage repair and DNA replication, demonstrating important

roles for H2A.Z in chromatin biology. Thus the data presented here provides a valuable resource for both identifying candidate H2A.Z interacting proteins and for understanding how H2A.Z interacts with downstream effectors to modulate chromatin-regulated processes. Future work will be necessary to validate and characterize the functional role of these proteins in ESC biology and in other systems, but the data presented here lays the groundwork for specific hypotheses for how H2A.Z functions.

Brd2 and H2A.Zub in transcriptional regulation

Our work demonstrates an important functional connection between H2A.Z and Brd2 in the regulation of transcriptional states in ESCs. We find that in wild-type ESCs, H2A.Z and Brd2 co-occupy active gene promoters, but that Brd2 is excluded from poised, bivalent genes. Using a transgeneic system to characterize the function of H2A.Zub, where we express a non-ubiquitylatable form of H2A.Z, we find that upon loss of H2A.Zub, Brd2 is re-distributed to bivalent gene promoters, and leads to an increase in gene expression. Depletion of Brd2 by either small molecule inhibition or RNAi demonstrated that Brd2 is necessary to mediate this transcriptional upregulation. However, future work will be necessary to dissect out the question of both how H2A.Zub represses transcription and how Brd2 recruitment leads to transcriptional upregulation at bivalent genes.

One hypothesis is that the presence of H2A.Zub either blocks RNA Polymerase II (RNAPII) initiation or elongation. Whether RNA Polymerase II is present in significant levels at bivalent promoters in ESCs is unclear and different studies have reported contrasting results (Min et al., 2011; Stock et al., 2007). ChIP-seq studies using antibodies to total RNAPII or RNAPII modified by serine 5 phosphorylation, a mark of initiation, have shown very low levels of RNAPII at promoter regions of bivalent genes (Min et al., 2011; Rahl et al., 2010; Williams et al., 2015). However, these genes are known to have low levels of transcription and are susceptible to transcriptional inhibition (Bernstein et al., 2006; Stock et al., 2007), thus some RNAPII must become engaged and transcriptionally competent. Little work has specifically addressed the mechanistic role of H2A.Zub at promoters, although multiples studies have addressed the role of H2Aub at these genes (Brookes et al., 2012; Stock et al., 2007). Recent

work suggests that H2Aub acts to restrain RNAPII in a poised state at the promoter regions (Stock et al., 2007), however due to biochemical similarity and cross-reaction of the antibodies, H2A.Zub might be playing a role as well. Additionally, the form of RNAPII that H2Aub restrains is specific to one RNAPII antibody (Stock et al., 2007), which makes the data difficult to interpret in the broader model of how H2Aub and/or H2A.Zub acts on RNAPII. We were unable to show an increase in RNAPII binding at bivalent gene promoters upon loss of H2A.Zub, however this does not rule out the possibility that H2A.Z works to alter RNAPII binding dynamics or activity. The small total levels of RNAPII may make subtle changes difficult to detect. Future *in vitro* studies using either chromatinized templates with reconstituted H2A.Zub compared to H2A.Z or through direct nucleosome binding assays may determine whether H2A.Zub can directly inhibit RNAPII binding or whether it restrains RNAPII in a poised DNA bound state. Moreover, recent advances using single molecule imaging may also allow the direct visualization of how H2A.Zub alters the kinetics of RNAPII recruitment and activation using reporter promoters (Hodges et al., 2009; Jin et al., 2010). Given the conserved role of H2A.Z in regulating inducible promoters, whether it influences RNAPII kinetics is an important unanswered question with implications for a broad range of responsive cell types.

H2A.Zub appears to inhibit Brd2 recruitment to maintain a poised, repressed state. During cell state transitions, H2A.Z must be de-ubiquitinated to facilitate gene activation. Whether this process is critical for linage commitment during ESC differentiation is not known. USP10 was specifically shown to de-ubiquinate H2A.Z in a model of prostate cancer and was necessary for gene activation (Draker et al., 2011). Moreover, multiple H2A-type de-ubiquinases have been identified, including USP16, which has a role in maintaining the ESC state and regulating differentiation (Nakagawa et al., 2008; Yang et al., 2014; Zhu et al., 2007), however whether they also act on H2A.Z is unknown. Thus, further investigations into the role of H2A.Z-specific de-ubquitination in ESCs will be necessary to fully understand how these cells regulate transcriptional output during developmental decisions. Given the documented role for H2A.Z in cancer progression, H2A.Z de-ubiquinases may be potential targets for therapeutic intervention.

Our work suggests that the recruitment of Brd2 to developmental promoters is a key step in the activation of these genes, in agreement with a role for Brd2 in transcriptional activation (Leroy et al., 2008). Like H2A.Zub, how Brd2 mechanistically acts on transcriptional machinery *in vivo* remains unclear. Specifically, Brd2 lacks the p-TEFb interacting domain that appears critical to facilitate transcriptional activation and pause release by Brd4 (Jang et al., 2005). Early work demonstrated that Brd2 could directly facilitate the progression of RNAPII through a chromatinized template *in vitro* by acting as a histone chaperone (Leroy et al., 2008), however whether this occurs *in vivo* has not been tested. Consistent with this idea, a short form of Brd4 that lacks the p-TEFb interacting domain associates with elongating RNAPII through gene bodies enhancing transcription (Kanno et al., 2014). Together these studies demonstrate an important role for BET proteins in transcriptional elongation. However, whether these proteins act as histone chaperones *in vivo* to facilitate elongation remains to be tested. Our work suggests that H2A.Z nucleosomes may create a preferential binding substrate for Brd2 compared to H2A. An alternative mechanism for how Brd2 facilitates transcriptional activation could be through the recruitment of downstream activators, including mediator and TBP (Denis et al., 2006; Peng et al., 2006). Bivalent gene promoters are largely devoid of these proteins in ESCs (Ku et al., 2012), however whether Brd2 can recruit these proteins to target genes is unknown. Recent advances using the Crispr-Cas9 system have fused a catalytically inactive version of Cas9 with transcriptional activators to target genes for activation (Mali et al., 2013; Perez-Pinera et al., 2013). Fusing Brd2 to catalytically inactive Cas9 could provide better insights into how Brd2 drives transcriptional activation. Furthermore, this system could dissect out whether Brd2 can up-regulate transcription in the presence of endogenous H2A.Zub. Further work to dissect the mechanism of Brd2 in transcriptional regulation will be important for understanding its role in development and disease and how it coordinates with other BET proteins.

More broadly our work suggests a general model for how Brd2 and H2A.Z act to regulate developmental transitions. Recent work examining Brd2 in a model of hematologic malignancy demonstrated that Brd2 acts with STAT5 to regulate transcriptional elongation,

downstream of RNAPII recruitment (Liu et al., 2014). In a follow-up study, histone deacetylase inhibitors (Tricostatin A) restrict Brd2-mediated gene activation by triggering a re-distribution of Brd2 and TBP away from its target loci to newly hyper-acetylated sites (Pinz et al., 2015). Interestingly, histone acetylation increases rapidly during ESC differentiation and Tricostatin A can induce similar gene expression changes (Karantzali et al., 2008; McCool et al., 2007; Saraiva et al., 2010). Together, these data suggest a potential model where both increased histone acetylation and decreased H2A.Zub are necessary for the recruitment of Brd2 and full transcriptional activation of developmental genes. Testing whether Brd2 is re-distributed in ESCs treated with TSA, and during normal development will be important for understanding how Brd2 acts downstream of H2A.Z to drive developmental gene expression.

H2A.Z and tandem bromodomain proteins

Our work demonstrates an important role for H2A.Z in the regulation of Brd2. However we also identified four other tandem bromodomain proteins enriched in H2A.Z containing chromatin. While there are over 40 bromodomain containing proteins in the mouse proteome, only eight have this motif (Filippakopoulos et al., 2012), suggesting a biologically significant function for the interaction between H2A.Z and tandem bromodomain proteins. Brd3, like Brd2, is a member of the BET family of proteins and can directly facilitate transcriptional activation, although its effects are less robust than Brd2 (Leroy et al., 2008). Interestingly, BET proteins can heterodimerize at genomic locations (Garcia-Gutierrez et al., 2012), indicating that Brd2 and Brd3 may be cooperating in gene activation, and could facilitate chromatin looping. Knockout studies in mice have shown that both Brd2 and Brd4 are embryonic lethal (Houzelstein et al., 2002; Shang et al., 2009), however they have distinct phenotypes, with Brd4 null mice exhibiting early embryonic lethality, while Brd2 null mice survive longer. Consistent with these data, our knockdown experiments in ESCs suggests independent roles for Brd2 and Brd4. A role for Brd3 in mammalian development remains to be identified. It is possible that Brd3 is not required for normal development, or that it is redundant with other BET proteins, however given that our data suggests distinct roles for Brd2 and Brd4 it will be important to dissect out the role of each of the individual proteins. If Brd3 null mice develop normally, they could still be more susceptible to other genomic

stresses, which could identify novel functions for BET proteins. Overall understanding the individual roles of each of the BET proteins will be necessary to understand the consequences of inhibition of this class of proteins by recently emerging small molecule drugs (Filippakopoulos et al., 2010; Winter et al., 2015), and to properly target them in disease models.

In addition to BET proteins, we identified three proteins, Brwd1, Brwd3 and Phip, that contain a WD40 motif on the N-terminal half of the protein, with a tandem bromodomain on the C-terminal enriched in H2A.Z containing chromatin. This class of proteins has been poorly studied and do not have known roles in development. Determining their loss of function phenotypes and functional roles will be critical to expanding the understanding of H2A.Z downstream effectors. Both Phip and Brwd3 were identified in an unbiased ChIP-Mass Spec screen as being associated with H3K27ac and H3K4me3 in ESCs, suggesting they may have roles in transcriptional activation (Ji et al., 2015). Moreover, Brwd3 in *drosophila* can regulate H3.3 deposition (Chen et al., 2015) which often co-localizes with H2A.Z at active regulatory regions of the genome (Chen et al., 2014; Thakar et al., 2009). The WD40 motif is one of the most abundant motifs in the mammalian proteome, and often acts as a platform for protein interactions (Xu and Min, 2011), thus these proteins could be acting as intermediates between H2A.Z and other downstream effectors. Both Brwd3 and Phip are also up-regulated in certain cancers (De Semir et al., 2012; Suh et al., 2012), although a mechanistic role remains to be elucidated. Notably, unlike Brd2, these proteins were unaffected by H2A.Z^{K3R3} suggesting that these proteins could be regulated differently than BET proteins. Thus, dissecting the role for these proteins in coordinating with H2A.Z could provide broader context for how H2A.Z regulates DNA-mediated processes and how these interactions could act in development and disease states.

H2A.Z post-translational modifications in mediating protein interactions

The work in this thesis describes the first evidence that post-translational modification (PTM) of histone variants is critical for regulating downstream protein interactions. H2A.Z is unique in that it is one of only two histone variants that is essential for mammalian development

(Maze et al., 2014). Understanding the role of H2A.Z PTMs in regulating gene expression and in mediating developmental transitions will be important to fully elucidate how H2A.Z acts as a molecular rheostat to sense and respond to cellular cues. We demonstrate that proteomic approaches can identify key regulators of downstream action. In addition to monoubiquitination, H2A.Z can be N-terminally acetylated and methylated (Sevilla and Binda, 2014). Thus, dissecting the function of these H2A.Z PTMs and in particular H2A.Z acetylation will have important implications for how H2A.Z functions, especially given that bromodomains specifically recognize acetylated lysines.

H2A.Z acetylation can occur on the five N-terminal lysines, and H2A.Zac localizes to active gene promoters (Hu et al., 2013; Ku et al., 2012; Millar et al., 2006; Valdés-Mora et al., 2012). During gene activation, the ratio of acetylated H2A.Z to total H2A.Z increases at target promoters (Valdés-Mora et al., 2012), suggesting that H2A.Zac may recruit activators. Currently, downstream effectors that specifically bind to acetylated H2A.Z have not been identified, thus future proteomic approaches using both acetyl-mutant and acetyl-mimic versions of H2A.Z may yield new insights into the role of this modification. Our data suggests that Brd2 is recruited to H2A.Z target loci upon induction. Whether this recruitment depends on H2A.Zac remains to be tested. While *in vitro*, acetylated H2A.Z peptides failed to compete with the interaction between Brd2 and H2A.Z-containing nucleosomes, these studies did not specifically address the role of H2A.Zac as these nucleosomes also harbored high levels of H4 acetylation. One hypothesis is that during gene induction H2A.Z acetylation is necessary for the initial recruitment of Brd2 and subsequent acetylation of H4 by activating factors. It was recently shown that an acetyl mutant version of H2A.Z led to impaired gene activation (Law and Cheung, 2015), possibly through a failure to recruit activators. This result was particularly striking as the mutant version was expressed at a fraction the level of endogenous H2A.Z, suggesting a dominant negative role. Thus, the incorporation of even a small amount of non-acetylatable H2A.Z may prevent nucleosome turnover and gene activation. Exploring the function of H2A.Z acetylation in ESCs and during development, may lend new insights into how H2A.Z acts to activate gene promoters.

In addition to acetylation, H2A.Z can also be methylated on the N-terminal tail (Binda et al., 2013). In contrast to acetylation, methylation is a repressive mark and loss of this modification impairs stem cell proliferation. Unlike H2A.Zub, H2A.Zme increases upon differentiation, suggesting that while both marks are repressive in ESCs, they may have different roles (Binda et al., 2013; Surface, 2014). The H2A.Z methyltransferase SETD6 has been shown to be a co-activator with ERα in breast cancer models, thus alterations in H2A.Zme may contribute to the role of H2A.Z mediating gene expression changes in cancer (O'Neill et al., 2014). Together, these results suggest that further investigations into the role of H2A.Z PTMs promises to improve our understanding of the functions of H2A.Z in normal development and cancer. Overall, our results demonstrate the utility of proteomics-based approaches to dissect the role of H2A.Z post-translational modifications.

H2A.Z in the regulation of higher order chromatin structure

Beyond its role in regulating local chromatin structure at promoter regions, H2A.Z can also influence interactions between promoter and enhancers of CCND1 in a breast cancer cell line (Dalvai et al., 2012). Recent advances in chromatin capture technology have allowed for more high throughput analysis of interactions between loci, either genome-wide or at specific promoters and enhancers (Dekker et al., 2013). Higher order chromatin structure is often dramatically altered during gene activation and in particular during ESC differentiation (Kagey et al., 2010; Phillips-Cremins et al., 2013). Whether H2A.Z directly influences higher order chromatin structure in embryonic stem cells will be of particular interest in further understanding how it regulates gene expression changes. Given that loss of H2A.Z in ESCs causes both an initial de-repression and an inability to fully activate genes when signaled to do so (Creyghton et al., 2008; Subramanian et al., 2013), suggests broader roles in regulating the activity of chromatin domains, similar to its role at the CCND1 locus. For example, the presence of H2A.Z could be necessary for establishing proper genomic conformations upon activation. Moreover, the cross-talk between H2A.Z and H3.3 at promoters and enhancers influences the activation of target loci in ESCs upon retinoic acid stimulation, suggesting a possible mechanisms for facilitating the physical association between promoter and enhancer during gene activation (Chen et al., 2013). Further studies using 4-C or higher

throughput methods upon depletion of H2A.Z with or without H3.3 will be necessary for a more complete understanding of how H2A.Z regulates dynamic higher order chromatin organization.

H2A.Z in the DNA Damage response

While the work in this thesis has focused on the role of H2A.Z in transcriptional regulation, H2A.Z has roles in a wide range of DNA-mediated processes (Zlatanova and Thakar, 2008). In particular, our proteomic approach using SILAC-IP has identified proteins involved in the DNA damage response. While, few DNA damage proteins were specifically enriched in H2A.Z containing chromatin compared to H2A, these proteins were more dramatically affected in the H2A.Z mutants. H2A.Z is recruited to sites of double stranded breaks and is necessary for both acetylation and ubiquitination of other proteins as well as the recruitment of Ku70 (Xu et al., 2012). Loss of H2A.Z does not influence the recruitment of 53BP, a mediator of the DNA damage response (Xu et al., 2012), however H2A.Z^{K3R3} shows an impaired association with 53BP. These results suggest that there may be both H2A.Z specific roles at sites of DNA damage, but also roles for post-translational modifications. While some of these features may be rescued by the presence of H2A, H2A.Z mutants may also act in a dominant-negative fashion for other interactions. Given that both acetylation and ubiquitination of histones are features at DSBs, future work dissecting the specific role for H2A.Z PTMs will facilitate an understanding of the role of chromatin structure in the response to DSBs and in their repair pathways.

H2A.Z as molecular rheostat for gene expression

Collectively the results presented in this thesis demonstrate an important role for H2A.Z in acting as a molecular gatekeeper of developmental gene expression programs, in particular in the transition from silent to active expression. H2A.Z integrates upstream signals to regulate downstream transcriptional responses. The work presented here identified a previously unknown pathway for H2A.Z in the regulation of silent and active genes in ESCs. However as described in this chapter many aspects of H2A.Z biology remain unknown. Expanding upon this work, future studies will be needed to address how H2A.Zub specifically

restrains gene activation, and how this mark is removed during differentiation. Moreover, it remains to be tested whether Brd2 is directly recruited in an H2A.Z-dependent manner upon gene induction during normal differentiation. More broadly understanding how H2A.Z influences the combinatorial network of epigenetic modifications, and how specific epigenetic modifications can influence downstream responses will allow a better understanding of its precise mechanisms of action. Specifically, development of new mechanistic tools including small molecule inhibitors and Crispr-Cas9, will allow for dissecting how epigenetic changes lead to changes in gene expression. Thus, we fully expect that continued investigations into how H2A.Z functions to integrate upstream signaling into downstream transcriptional outputs, will contribute new insights into its role in development and disease and may facilitate the design of novel therapeutic approaches to treat congenital diseases and cancer.

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Appendix A

Mutation of conserved regions of H2A.Z alters chromatin-associated protein interactions

Author Contributions for Appendix A:

Experiments designed by Paul Fields and Laurie Boyer. Experiments and analysis performed by Paul Fields. Mass spectrometry performed by Namrata Udeshi and Jake Jaffe. Cell lines generated by Lauren Surface and Vidya Subramanian.

Summary

Chromatin regulation is a key mechanism for cells to regulate transcriptional activity and other DNA-mediated processes. Understanding how the multiple levels of chromatin regulation cross-talk with each other to mediate context specific outcomes is a major open question in the field. H2A.Z, a highly conserved variant of the canonical histone H2A is involved in many chromatin-mediated processes, including transcriptional regulation. We have adapted the approach described in Chapter 2 to interrogate the protein interaction landscape of two specific mutant versions of H2A.Z, previously described by our lab (Subramanian et al., 2013; Surface, 2014). Here we describe that the conserved acidic patch of H2A.Z is important for mediating interactions with chromatin remodelers, as well as proteins involved in the DNA damage response. Moreover, we expand on our findings in Chapter 2 that H2A.Z ubiquitination is important both for mediating interactions with transcriptional activators and also DNA damage response proteins and histone modifying enzymes. Together these results suggest important roles for the conserved, divergent regions of H2A.Z as well as its post-translation modifications in mediating chromatin-protein interactions. Future work exploring the specific regulation behind individual interactions, as well as expanding this approach to other conserved regions of H2A.Z will lend further insight into the mechanism of action of H2A.Z in both development and disease.

Introduction

Chromatin regulation is a key mechanism allowing cells to respond to external stimuli and to translate them into a transcriptional response. Upon stimulus a multitude of changes occur at target loci in the genome, often initiated by the binding of transcription factors (Tee and Reinberg, 2014). How these proteins are targeted to the genome and how they execute their downstream programs often depends on the initial epigenetic landscape, and subsequent protein interactions between chromatin and downstream regulators. Incorporation of non-allelic histone variants is one mechanism cells use to create specialized chromatin domains that confer unique responses to cellular stimuli (Banaszynski et al., 2010; Biterge and Schneider, 2014). As discussed in Chapter 2 we have developed a novel approach to quantitatively assess protein interactions between the histone variant H2A.Z and the canonical histone H2A. H2A.Z has multiple functionally distinct, divergent regions from H2A that likely play critical roles in the interaction with chromatin-associated proteins (Suto et al., 2000; Thatcher and Gorovsky, 1994). We have utilized this methodology to interrogate the interactions between two H2A.Z mutants compared to wild type H2A.Z to gain new insights into how they may be functioning. We address the role of the conserved H2A.Z extended acidic patch and H2A.Z monoubiquitination in facilitating chromatin-protein interactions.

We have previously shown that mutation of the three divergent acidic patch residues of H2A.Z (H2A.Z^{AP3}) back to their corresponding H2A residues results in a hyper-dynamic nucleosome (Subramanian et al., 2013). Moreover, this mutant results in abnormal gene expression patterns, demonstrating that the extended acidic patch is necessary for proper gene regulation of H2A.Z target genes in embryonic stem cells (Subramanian et al., 2013). This result is consistent with previous work in *drosophila* where the extended acidic patch of H2A.Z is essential for development and cannot be rescued by the corresponding residues of H2A (Clarkson et al., 1999). Examination of the protein interactions of the acidic patch may shed new insight into how this protein mediates chromatin dynamics and is necessary to regulate developmental gene expression patterns.

We have shown that PRC-1 mediated post-translational ubiquitination of H2A.Z (H2A.Zub) is necessary for proper multi-lineage differentiation and execution of gene expression programs (Surface, 2014). Loss of H2A.Zub by mutation of the three C-terminal lysines to arginines ($H2A.Z^{K3R3}$) results in an initial de-repression of bivalent genes and in particular alters Wnt signaling in mESCs (Surface, 2014). As described in Chapter 2, loss of H2A.Zub results in an increased interaction with Brd2, however a comprehensive investigation of the altered protein interactions in the $H2A.Z^{K3R3}$ mutant may shed insight into the broader functioning of H2A.Zub. In particular, protein interactions lost in $H2A.Z^{K3R3}$ ESCs may represent key downstream effectors of H2A.Zub, and proteins interactions gained in $H2A.Z^{K3R3}$ may normally be inhibited by H2A.Zub. Interestingly, while both $H2A.Z^{K3R3}$ and $H2A.Z^{AP3}$ mutants lead to de-repression of H2A.Z target genes, they have distinct cellular phenotypes, thus investigating the downstream proteins altered in these H2A.Z mutants will provide insight into the individual roles of H2A.Z protein domains.

H2A.Z acidic patch is preferentially associated with proteins involved in DNA damage

We utilized the SILAC-IP method described in Chapter 2 to investigate the differential protein interactome between wild-type H2A.Z and the extended acidic patch mutant (termed $H2A.Z^{AP3}$), where the three divergent resides of H2A.Z (Gly92, Asp97, Ser98) are mutated back to their corresponding H2A residues (Asn89, Asn94, Lys95). We expressed either wild-type H2A.Z or $H2A.Z^{AP3}$ tagged with YFP under a doxycline-inducible promoter in the presence of an shRNA directed against the endogenous transcript (Subramanian et al., 2013). Using SILAC as described previously (Ong et al., 2002), chromatin immunopurification of nucleosomes containing $H2A.Z^{WT}$ and $H2A.Z^{AP3}$ resulted in the identification of 1276 proteins that were reproducibly present with at least 2 peptides. 95 of the identified proteins had a p-value of less 0.1 for differential enrichment and reproducibility between samples (Figure 1; Table 1). Among the most differentially enriched proteins we identified multiple components involved in DNA damage, including Rad52 and Reql1 as enriched in $H2A.Z^{AP3}$ chromatin. Rad52 is the mammalian homolog of the yeast Rad52 protein, which together with Rad51 are involved in ssDNA binding and double stranded break repair. While the yeast protein Rad52 has a direct role in mediating DNA double stranded break repair through strand invasion and recruitment

of Rad51, the mammalian homolog does not appear to have the same role (Lok and Powell, 2012). Loss of mammalian Rad52 is synthetic with BRCA1 and other repair enzymes in increasing susceptibility to DNA damage, suggesting that Rad52 is important for feeding into the Rad51 pathway and regulating the response to DNA damage (Lok and Powell, 2012). However, the exact mechanism of Rad52 function in mammalian double strand break repair remains unknown and could be assisting in translating upstream signals to effector proteins. Interestingly, H2A.Z gets recruited to sites of DNA damage both in yeast and mammalian systems (Kalocsay et al., 2009; Xu et al., 2012). It is possible that the H2A.Z extended acidic patch contributes to regulation of DNA damage proteins. It also remains possible that due to the hyper dynamic nature of H2A.Z^{AP3}, that it is incorporated at a higher rate at sites of DNA damage compared to H2A.Z^{WT}. Alternatively, DNA is more prone to DNA damage in the presence of H2A.Z^{AP3}. Dissecting a role for the extended H2A.Z acidic patch in mediating the DNA damage is an exciting future direction and consistent with previously described roles for H2A.Z.

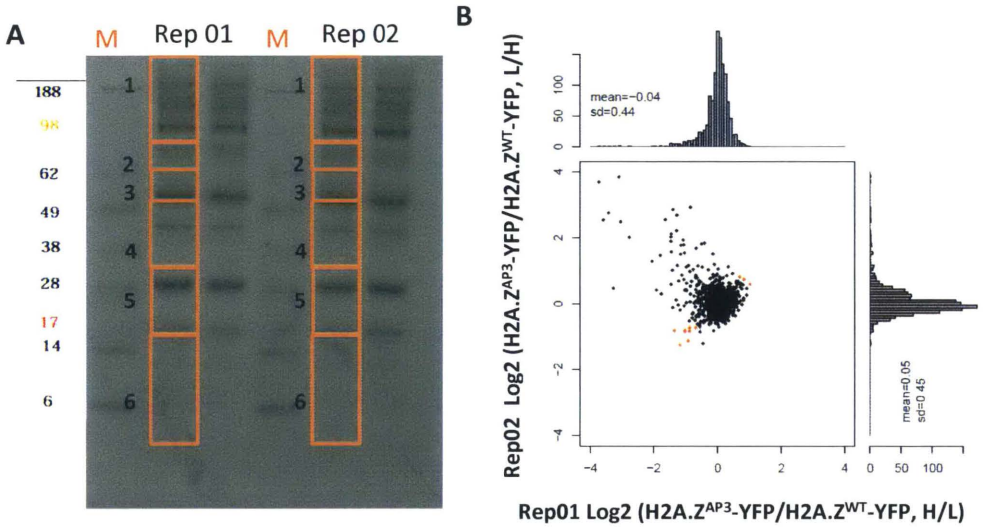

Figure 1: SILAC-based Immunoprecipitation followed by Mass Spec for H2A.Z^{AP3} compared to H2A.Z^{WT}. A) Biological replicates using isotope swap of heavy and light were gel separated and band isolated prior to mass spec. B) Comparison of biological replicates, highly differentially enriched proteins fall in the first and third quadrant. Ratios were normalized to bait ratios to account for difference in mean intensity.

In addition to Rad52, Recql1 was enriched with H2A.Z^{AP3}. Recql1 is a member of the Recql family of ATP-dependent DNA helicases that have key roles in maintaining genome integrity in response to stresses (Veith and Mangerich, 2014). While loss of Recql1 is not associated

with a phenotype in metazoans, in contrast to other members of the family, it is the most highly expressed, and loss of Recql1 predisposes cells to genome instability including aneuploidy and chromosomal breakage (Croteau et al., 2014). Analysis of the domain structure of Recql1 shows a high degree of similarity to INO80 (Figure 2), the chromatin-remodeling complex responsible for removing H2A.Z from chromatin in yeast (Papamichos-Chronakis et al., 2011), including two conserved helicase domains. Moreover, yeast INO80 has roles in mediating both DNA damage repair and DNA replication through fork progression (van Attikum et al., 2007; Watanabe and Peterson, 2010). Similarly, mammalian Recql1 has roles in both DNA repair through an interaction with Rad51 and in regulating fork progression (Croteau et al., 2014; Sharma and Brosh, 2007). These results suggest that there could be a conserved role for H2A.Z in mediating DNA synthesis and fork progression in mammalian cells through interactions with Recql1 and other family members, including Rad52. In yeast, H2A.Z deletion results in a slow growth defect, in part through delays of origin firing (Dhillon et al., 2006), whether this is conserved in higher eukaryotes remains unknown. While, H2A.Z^{AP3} does not alter cell cycle dynamics (Subramanian et al., 2013), the increase in the H2A.Z^{AP3} interaction could be through preferential localization of H2A.Z^{AP3} at sites of DNA replication. Alternatively, given that the hyper-dynamic nature of H2A.Z^{AP3} is transcription dependent, in H2A.Z^{AP3} ESCs a higher fraction of chromatin-associated H2A.Z^{AP3} may be associated with DNA damage, fork progression and other non-transcription dependent roles. These results suggest that the acidic patch of H2A.Z may have a key role in mediating the response to DNA damage through specific downstream effectors, and provide potential targets for future study.

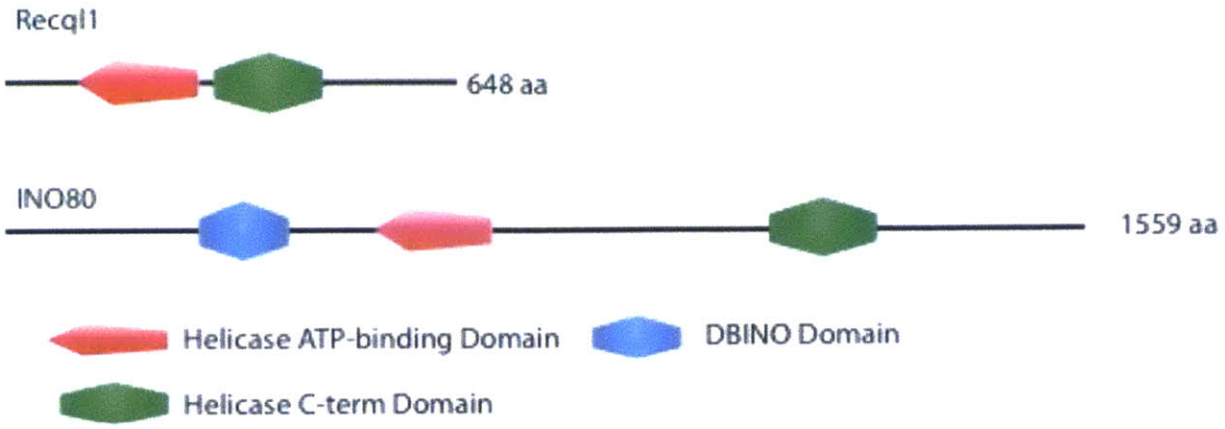

Figure 2: Conserved Protein Motifs shared between Recql1 and INO80. Both Recql1 and INO80 share two conserved protein motifs, with a Helicase ATP-binding Domain followed by a Helicase C-term Domain, suggestive of possible similar protein interactions or functions. Motif analysis from ExPASy Prosite.

The H2A.Z^{AP3} mutant also led to decreased interactions with many proteins. Decreased interaction between H2A.Z^{AP3} and chromatin-associated proteins compared H2A.Z^{WT} could be interactions facilitated directly by the acidic patch. The extended acidic patch of H2A.Z is located at the nucleosome docking domain which is the major binding site for nucleosome binding proteins (Kato et al., 2011; Suto et al., 2000). However, the caveat to these results is that due to the hyper-dynamic nature of H2A.Z^{AP3}, interactions lost in H2A.Z^{AP3} ESCs could be due to the lower fraction of H2A.Z localizing to certain genomic regions. Among the most the most depleted proteins in H2A.Z^{AP3} chromatin compared to H2A.Z^{WT} are multiple members of the tandem bromodomain family of proteins, including Brd2, Brd3, Brwd3 and Phip. This is of particular interested because while both H2A.Z^{AP3} ESCs and H2A.Z^{K3R3} ESCs show a de-repression of bivalent genes, we have shown in Chapter 2 that H2A.Z^{K3R3} de-repression is mediated through the recruitment of BET proteins. In contrast, this suggests that H2A.Z^{AP3} utilizes an alternate method to mediate de-repression, potentially through directly de-stabilizing the +1 nucleosome, normally a barrier to transcriptional initiation (Weber et al., 2014). *In vitro* transcription assays will allow for the further dissection of how H2A.Z^{AP3} mediates increased transcription. Furthermore biochemical studies will also be necessary to validate whether candidate interactions are lost due to the changed residues of the H2A.Z acidic patch or due to the hyper-dynamic mutant nucleosome. These results, though, are

consistent with the extended acidic patch of H2A.Z being necessary to mediate proper protein interactions and provide an exciting direction for future studies on the functional role of the H2A.Z acidic patch.

H2A.Zub is necessary for facilitating proteins interaction with DNA methylation machinery and transcriptional regulators

H2A.Z is post-translationally monoubiquitinated by the Polycomb Repressive Complex (PRC)-1 complex. This modification occurs on both the transcriptionally silent X chromosome and at bivalent genes along with H3K27me3 (Ku et al., 2012; Sarcinella et al., 2007; Surface, 2014). Monoubiquitination of histones is known to mediate specific interactions (Hunter, 2007), thus we hypothesized that mutation of the three C-terminal lysines targeted by PRC-1 of H2A.Z may results in changes in the protein interaction landscape of H2A.Z. Specifically proteins depleted in the H2A.Z^{K3R3} mutant are interactions normally facilitated by the ubiquitin moiety. Protein interactions gained in the H2A.Z^{K3R3} mutant are suggestive of interactions inhibited by the presence of H2A.Zub. As described in Chapter 2, we performed SILAC-IP on H2A.Z^{K3R3} compared to H2A.Z^{WT}. We showed that the BET family of bromodomain containing proteins and specifically Brd2 are enriched in H2A.Z^{K3R3} chromatin, while H2A.Z deposition machinery is unaffected (Chapter 2). In addition, multiple other proteins interactions were altered in H2A.Z^{K3R3} ESCs. We identified 1299 proteins that were represented in both replicates with greater than 2 peptides in both. Of these 229 were differentially enriched with a p-value of <0.1, with approximately equal gained and lost (Figure 3; Chapter 2 Supplemental Table 2). Many of the differentially bound proteins had known roles in chromatin-mediated processes including transcriptional activity and DNA damage.

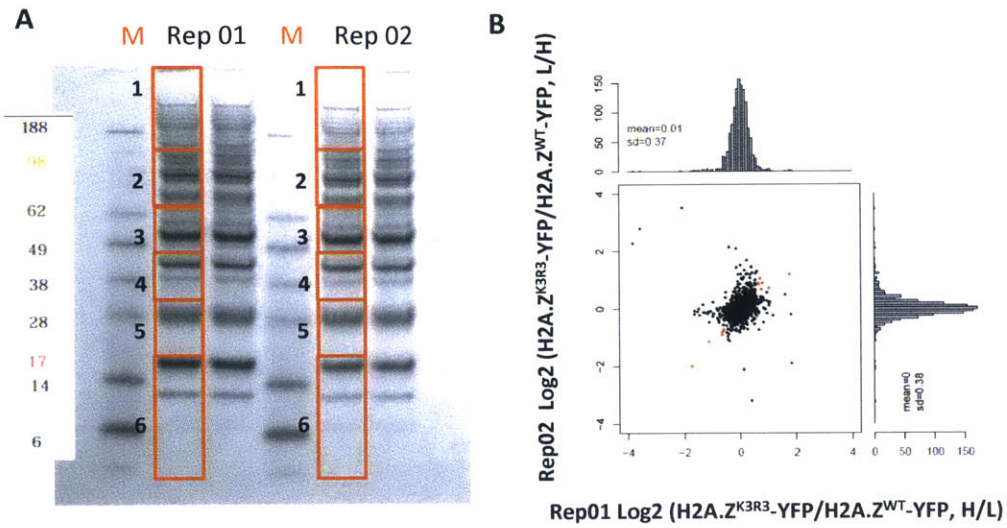

Figure 3: SILAC-based Immunoprecipitation followed by Mass Spec for H2A.Z^{K3R3} compared to H2A.Z^{WT}. A) Biological replicates using isotope swap of heavy and light were gel separated and band isolated prior to mass spec. B) Comparison of biological replicates, highly differentially enriched proteins fall in the first and third quadrant.

Among the proteins depleted in H2A.Z^{K3R3} were members of the *de novo* DNA methyltransferases DNMT3a and DNMT3b. H2A.Z is anti-correlated with DNA methylation and this is conserved across evolution (Conerly et al., 2010; Zilberman et al., 2008). Moreover, DNA methylation plays a key role in gene regulation during development (Smith and Meissner, 2013). Together these results are suggestive of a role for H2A.Zub in mediating DNA methylation. H2A.Zub is known to localize to repressed regions and bivalent genes in mESCs (Surface, 2014), thus one hypothesis is that H2A.Zub may function in the initiation of DNAme at bivalent gene promoters that get permanently repressed during differentiation (Voigt et al., 2013). Preliminary work suggests that H2A.Z^{K3R3} mutant ESCs have lower global levels of 5-meC as well as 5-hmC (Surface, 2014). Future work will be necessary to understand how H2A.Zub regulates DNA methylation during dynamic cell state changes, in particular whether bivalent genes that gain DNA methylation are dependent on H2A.Zub, as well as whether those regions that exhibit altered DNA methylation have changes in gene expression in H2A.Z^{K3R3} ESCs.

DNA damage response proteins were also found depleted in H2A.Z^{K3R3} chromatin. Both 53BP1 and JMJD2A, which have known roles in mediating the DNA damage response (Mallette et al.,

2012), were depleted in H2A.Z^{K3R3} chromatin. Under normal conditions both 53BP1 and JMJD2A have affinity for H4K20me, but during DNA damage JMJD2A is degrading allowing 53BP1 to bind (Mallette et al., 2012). The decreased association of both 53BP1 and JMJD2A suggests that H2A.Z^{K3R3} may have defects in DNA repair and that normally ubiquitination of H2A.Z may facilitate this process. This is consistent with the above-described role for H2A.Z in double stranded break repair (Kalocsay et al., 2009; Xu et al., 2012). It was shown that loss of H2A.Z results in decreased levels of ubiquitin foci at sites of DSB (Xu et al., 2012), however the presence of H2A.Zub in this process is as yet unknown. Bmi1 ubiquinates H2A at K119 during double stranded breaks (Ginjala et al., 2011), however due to cross-reactivity of the antibody for H2Aub, it is possible that this is also recognizing H2A.Zub. Further work will be necessary to mechanistically dissect out the roles of H2A.Z and H2A.Zub in mediating DNA damage response. H2A.Z^{WT}-YFP and H2A.Z^{K3R3}-YFP will provide useful tools in this investigation as they allow for distinguishing between H2A.Zub vs. H2Aub, which cannot be done with endogenous antibodies alone.

In addition to the transcriptional activators including Brd2 and Brd3, other proteins enriched in H2A.Z^{K3R3} chromatin were surprisingly Suv39h2 and Suv420h2, which are methyltransferases for the repressive marks H3K9me3 and H4K20me3 respectively (Bulut-Karslioglu et al., 2014; Del Rizzo and Trievel, 2014). This result was unexpected as H2A.Z^{K3R3} mutant cells exhibit de-repression of target genes (Surface, 2014). H3K9me3 and H4K20me3 are marks often associated with silent and imprinted loci (Delaval et al., 2007). Recently it has been shown that H2A.Z and H3K9me3 are co-localized at retrotransposons in the mouse genome (Rangasamy, 2013). Our preliminary work suggests that the H2A.Z^{K3R3} mutant results in increased levels of H3K9me3 and H4K20me3 at both repetitive elements and imprinted loci (Surface, 2014). However overall there were very low levels of H2A.Z and H2A.Zub at these sites, so it is unknown how H2A.Zub directly facilitates this process, thus future work will be necessary to determine the role of H2A.Zub at both imprinted loci and repetitive regions. It is possible that our results are due to the complex nature of these loci (either many repetitive copies or heterogenicity for imprinted sites) making it technically difficult to preform ChIP on these regions. One hypothesis is that these regions require the presence of low levels of

H2A.Zub to restrict the activity of the silent heterochromatin marks and create a repressed but poised state to allow them to be activated if necessary. Collectively, these results suggest that H2A.Zub regulates the interaction of both activating and repressive complexes with H2A.Z nucleosomes in a context dependent manner and is necessary for regulating many DNA-mediated processes.

Concluding Remarks

Along with Chapter 2, the results described here suggest that H2A.Z acts as an important mediator of downstream chromatin regulators and that both its conserved extended acidic patch and post-translational monoubiquitination influence these interactions. These specific H2A.Z mutations affect the interaction of both epigenetic writers and readers with the nucleosome and thus likely have important impacts on transcriptional activity. These results are consistent with a role for the conserved regions of H2A.Z in regulating gene expression and cellular physiology (Creyghton et al., 2008; Subramanian et al., 2013; Suto et al., 2000)(Surface et al., 2015). Furthermore our results suggest that H2A.Z-facilitated proteins interactions are likely involved in mediating other chromatin-mediated functions including the DNA damage response. This is consistent with existing literature where H2A.Z plays a key role in mediating the recruitment of specific proteins, including Ku70 and RPA, to sites of double stranded breaks (Kalocsay et al., 2009; Xu et al., 2012).

We have focused our analysis on two specific H2A.Z mutants, the extended acidic patch and the sites of C-terminal ubiquitination, each of which shows a distinct subset of altered protein interactions. Future work utilizing this methodology to examine other mutants of H2A.Z, including the role of N-terminal acetylation, as well as other histone variants, will be important for further defining our understanding of the combinatorial nature of chromatin protein interactions, and how specific conserved regions and post-translational modifications influence this process. Additionally these different interactions will likely have both temporal and location dependence and thus further work using reporter assays for gene activation and DNA damage and focusing on specific interactions will allow for new mechanistic insight into how H2A.Z functions in a context dependent manner.

Material and Methods

As Described in Chapter 2.

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Table 1: Proteins differentially associated with H2A.Z^{AP3}-YFP compared to H2A.Z^{WT}-YFP. Sorted based on moderated-t p-value.

Protein Names	Log2 Ratio H/L (YFP H2AzAP3/YFP H2Az) Normalized by Bait Rep01	Log2 Ratio L/H (YFP H2AzAP3/YFP H2Az) Normalized by Bait Rep02	Moderated t pvalue
Protein YL-1	-1.18	-1.24	0.00
MCG11326, isoform CRA_a	-0.92	-1.12	0.00
IRS-1 PH domain-binding protein	-1.03	-0.83	0.00
Centromere protein V	-1.35	-0.81	0.00
Serine/threonine-protein kinase VRK3	-1.03	-0.80	0.00
Bromodomain and WD repeat-containing protein 3	-0.89	-0.82	0.00
Protein FAM111A	-0.88	-0.72	0.01
DNA repair protein RAD52 homolog	0.81	0.75	0.01
ATP-dependent DNA helicase Q1 (Recql1)	0.69	0.82	0.01
Uncharacterized protein KIAA2022	1.00	0.60	0.01
Apoptosis-enhancing nuclease	-0.70	-0.72	0.01
Microtubule-associated protein 2	-0.56	-0.86	0.01
Clathrin interactor 1	0.71	0.61	0.01
Epidermal growth factor receptor pathway substrate 15-related sequence	0.64	0.68	0.01
DNA (Cytosine-5-)-methyltransferase 3-like, isoform CRA_b	0.79	0.57	0.01
Putative transposase element L1Md-A101/L1Md-A102/L1Md-A2	-0.89	-0.53	0.01
DNA (cytosine-5)-methyltransferase 3A	0.72	0.57	0.01
Bromodomain containing 2	-0.64	-0.62	0.01
Replication protein A1	0.58	0.67	0.02
Putative uncharacterized protein	-0.56	-0.71	0.02
Damage-specific DNA-binding protein 1	-0.57	-0.66	0.02
ATP-dependent helicase CHD9	0.59	0.62	0.02
66 kDa neurofilament protein	-0.46	-1.21	0.02
Zinc finger protein 568	0.90	0.46	0.02
Embryonal carcinoma differentiation regulated protein	0.46	0.91	0.02
Dedicator of cytokinesis protein 6	-0.72	-0.51	0.02
Jumonji domain-containing protein 1C	-0.56	-0.60	0.02
Rbmxrt protein	0.71	0.50	0.02
40S ribosomal protein S27a	-0.80	-0.44	0.02
Itsn2 protein	0.76	0.44	0.02

Sal-like protein 1	0.58	0.51	0.03
Ataxin-10	0.32	1.12	0.03
Bromodomain containing 3	-0.45	-0.57	0.03
Nuclear protein 220	0.55	0.46	0.03
Adrenal hypoplasia congenita-like protein	-0.69	-0.39	0.03
DnaJ homolog subfamily A member 3, mitochondrial	-0.67	-0.39	0.03
2(3)-polynucleotidase	-0.44	-0.57	0.03
Neighbor of Brca1 gene 1	0.50	0.50	0.04
Zinc finger protein 318	0.39	0.61	0.04
Nuclear receptor co-repressor 1	-0.60	-0.39	0.04
Novel KRAB box domain containing protein	-0.80	-0.32	0.04
Methyl-CpG-binding domain protein 1	-0.43	-0.53	0.04
Cofactor required for Sp1 transcriptional activation subunit 6	0.63	0.37	0.04
Zinc finger protein of the cerebellum 3	-0.43	-0.52	0.04
Zinc finger and BTB domain containing 39	0.35	0.62	0.05
Leucine zipper putative tumor suppressor 2	-0.64	-0.34	0.05
Myc-induced nuclear antigen	-0.59	-0.36	0.05
100 kDa coated vesicle protein A	0.28	0.85	0.05
Histone-lysine N-methyltransferase SUV420H2	-0.77	-0.28	0.05
Collagen alpha-1(XII) chain	0.90	0.26	0.05
Histone deacetylase complex subunit Sin3b	0.39	0.52	0.05
Apolipoprotein B mRNA-editing complex 3	-0.38	-0.52	0.05
UAP56-interacting factor	0.48	0.41	0.05
Zinc finger protein 7, isoform CRA_a	0.41	0.47	0.05
Adaptor-related protein complex 2, beta 1 subunit	0.37	0.52	0.05
Putative uncharacterized protein Samd1	0.57	0.33	0.06
DNA polymerase delta catalytic subunit	-1.17	-0.20	0.06
tRNA-dihydrouridine synthase 3-like	-0.60	-0.31	0.06
Coiled-coil domain containing 102A	0.48	0.37	0.06
SEC16 homolog A (S. cerevisiae)	0.49	0.36	0.06
Eukaryotic translation initiation factor 2, subunit 3, structural gene X-linked	-0.55	-0.31	0.06
Nucleolar protein 1	0.39	0.44	0.06
Nuclear protein NHN1	0.57	0.30	0.07
Pericentriolar material 1 protein	0.38	0.43	0.07
Chromodomain protein, Y chromosome-like 2	0.32	0.51	0.07
Caveolin-1	0.38	0.43	0.07
Zfp617 protein	0.38	0.43	0.07
Suppressor of hairy wing homolog 4	-0.41	-0.38	0.07
Novel KRAB box domain containing protein	-0.48	-0.33	0.07
RNA pseudouridylate synthase domain-containing	-1.40	-0.12	0.07

protein 4			
Zinc finger protein 75, isoform CRA_a	0.60	0.26	0.07
Cytoskeleton-associated protein 2	0.57	0.27	0.07
Cleavage and polyadenylation specificity factor subunit 6	0.40	0.37	0.08
ATP-dependent RNA helicase A	0.35	0.43	0.08
30000 Mr metaphase complex	-0.47	-0.30	0.08
Phosphoribosyl pyrophosphate amidotransferase	0.49	0.29	0.08
Lysyl-tRNA synthetase	0.17	0.78	0.09
Collagen alpha-2(V) chain	0.81	0.16	0.09
Beta-catenin	0.41	0.33	0.09
Heterogeneous nuclear ribonucleoprotein G	-0.22	-0.60	0.09
Zinc finger protein 512	-0.23	-0.56	0.09
Transcription initiation factor TFIID 100 kDa subunit	-0.60	-0.20	0.09
100 kDa coactivator	0.36	0.35	0.09
Nucleoporin 98	-0.28	-0.45	0.10
Cell division cycle protein 27 homolog	-0.13	-0.82	0.10
Lmo7 protein	0.39	0.32	0.10