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H2AZ Is Enriched at Polycomb Complex Target Genes in ES Cells and Is Necessary for Lineage Commitment

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SUMMARY

Elucidating how chromatin influences gene expression patterns and ultimately cell fate is fundamental to understanding development and disease. The histone variant H2AZ has emerged as a key regulator of chromatin function and plays an essential but unknown role during mammalian development. Here, genome-wide analysis reveals that H2AZ occupies the promoters of developmentally important genes in a manner that is remarkably similar to that of the Polycomb group (PcG) protein Suz12. By using RNAi, we demonstrate a role for H2AZ in regulating target gene expression, find that H2AZ and PcG protein occupancy is interdependent at promoters, and further show that H2AZ is necessary for ES cell differentiation. Notably, H2AZ occupies a different subset of genes in lineage-committed cells, suggesting that its dynamic redistribution is necessary for cell fate transitions. Thus, H2AZ, together with PcG proteins, may establish specialized chromatin states in ES cells necessary for the proper execution of developmental gene expression programs.

INTRODUCTION

A major challenge in biology is to understand how pluripotent cells in the mammalian embryo and their in vitro derivatives, namely embryonic stem (ES) cells, execute the diverse gene expression programs that lead to cellular specification. The regulation of chromatin structure facilitates the establishment and maintenance of heritable gene expression patterns during development. ES cells are a valuable model system for the study of changes in chromatin as a function of cell state because of their distinctive ability to differentiate into multiple lineages (Keller, 2005; Jaenisch and Young, 2008). Recent studies have begun to reveal unique chromatin states in pluripotent and lineage-committed cells (Meshorer and Misteli, 2006; Mendenhall and Bernstein, 2008). Therefore, knowledge of how chromatin influences gene expression patterns in ES cells is expected to provide key insights into the process of cell fate specification and for understanding of the progression from normal to disease states.

Chromatin structure is highly regulated by a variety of complex processes that are not well understood. These include nucleosome remodeling and posttranslational modification of histone proteins (Dunn and Kingston, 2007; Kouzarides, 2007; Workman, 2006). An additional regulatory mechanism is the replacement of conventional histones with specific variants. Histone variants are structural components of chromatin and play important roles in all eukaryotes by influencing a wide range of DNA-mediated processes such as genome integrity, X inactivation, DNA repair, and gene regulation (Henikoff and Ahmad, 2005; Guillemette and Gaudreau, 2006; Hake and Allis, 2006; Li et al., 2007; Raisner and Madhani, 2006). The genes that code for them are evolutionarily conserved, nonallelic variants of the major histone genes. Their expression, however, is not linked to the cell cycle, and incorporation into chromatin is independent of DNA replication. Variants also differ from canonical histones in their primary sequence, and their incorporation can have functional consequences on the biophysical properties of the nucleosome core particle. These data indicate that histone variants perform specialized functions and suggest an important role for histone replacement in the regulation of chromatin states.

The histone H2A variant H2AZ is of particular interest because it is essential in multicellular organisms (Faast et al., 2001; Liu et al., 1996; Ridgway et al., 2004; van Daal and Elgin, 1992). H2AZ has been implicated from yeast to human in many DNA-mediated processes, including gene regulation. Interestingly, H2AZ has been linked to both gene activation and repression. Genome-wide studies in a range of organisms have shown that the distribution of H2AZ across the genome appears to be largely confined to small regions flanking transcription start sites, although enrichment has also been reported at larger regions proximal to telomeres or centric heterochromatin (Albert et al., 2007; Barski et al., 2007; Guillemette et al., 2005; Li et al., 2005; Mavrich et al., 2008; Meneghini et al., 2003; Raisner et al., 2005; Rangasamy et al., 2003; Zhang et al., 2005). Moreover, studies have shown that H2AZ incorporation can affect local histone modification patterns, the activity of chromatin

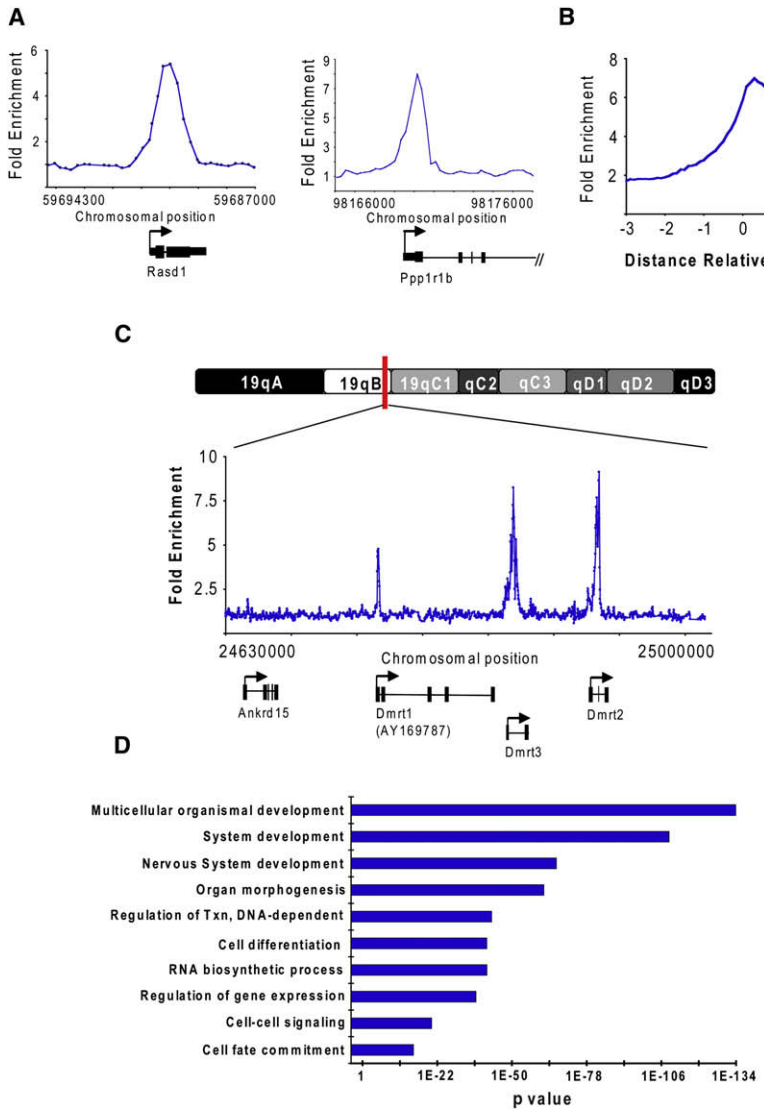

Figure 1. H2AZ Occupies Promoter Regions in ES Cells

(A) Representative examples of DNA sequences occupied by H2AZ isolated with chromatin immunoprecipitation (ChIP) and promoter microarrays. ChIP with core histone H3 was hybridized together with H2AZ to control for nucleosome density. Note that hybridization of H2AZ ChIP-DNA with bulk chromatin as input yielded highly similar results in a replicate set of experiments. The plots show unprocessed enrichment ratios (blue) for all probes within a genomic region. Chromosomal positions are from NCBI build 34 (mm6) of the mouse genome. Genes are shown to scale below plots. The start and direction of transcription are both indicated by an arrow.

(B) Distribution of the distance between bound probes and the closest transcription start site (TSS). Data are the average unprocessed enrichment ratios for each oligonucleotide probe within the −4 kb to +4 kb genomic region for all enriched genes.

(C) Representative example of DNA sequences occupied by H2AZ identified by ChIP and tiled chromosome 19 microarrays. The plots show unprocessed enrichment ratios (blue) for all probes within a genomic region (HZAZ versus histone H3). Genes are shown to scale below plots as in (A).

(D) Gene ontology analysis for biological process of H2AZ-enriched genes. Ontology terms are represented on the y axis, and the p value for enrichment of bound genes relative to all genes represented on the microarray is shown for each category on the x axis.

remodeling enzymes, and chromatin conformation (Fan et al., 2002; Li et al., 2005; Millar et al., 2006; Raisner et al., 2005; Swaminathan et al., 2005; Zhang et al., 2005). Thus, the function of H2AZ appears to be highly context-dependent in a manner that influences transcriptional output.

To investigate the essential role of H2AZ during mammalian development, we have generated genome-wide maps in ES cells, and we found that H2AZ is enriched at a large set of silent developmental genes in a manner that is highly similar to that of the Polycomb group (PcG) protein Suz12 (Boyer et al., 2006; Lee et al., 2006). PcG proteins are transcriptional repressors that play important roles in regulating developmental gene expression patterns by epigenetic modification of chromatin structure (Ringrose and Paro, 2007; Schuettengruber et al., 2007; Schwartz and Pirrotta, 2007). We also show that H2AZ is required for the regulation of target gene expression in ES cells and further demonstrate an important role for H2AZ in mediating cell fate transitions upon induction of differentiation. Conversely, H2AZ enrichment was detected at active genes in multipotent neural precursors, suggesting that its dynamic redistribution is necessary for lineage specification. Together, these findings establish a critical role for H2AZ in regulating developmental gene expression programs in ES cells and provide important insights into the essential nature of this variant during mammalian development.

RESULTS

H2AZ Occupies Promoter Regions in ES Cells

Because the localization of H2AZ might reveal its essential role during early mammalian development, we mapped this histone variant throughout the genome in murine ES cells. For this purpose, DNA sequences occupied by H2AZ were identified by chromatin immunoprecipitation (ChIP) with specific antibodies followed by hybridization to DNA microarrays (chip) as previously described (Boyer et al., 2005, 2006). DNA microarrays were designed to contain 60 base pair (bp) oligonucleotide probes that span the region from −4 kilobase pair (kb) to +4 kb relative to the transcription start sites (TSSs) of ~20,000 annotated mouse genes (see the Supplemental Data available online). The genomic sites occupied by H2AZ were identified as peaks of ChIP-enriched DNA with a previously validated algorithm (Figure 1A; Table S1). Notably, the

majority (92%) of enriched regions were detected within 1 kb of a TSS (Figure 1B; Table S2). Control ChIP experiments with antibodies against histone H3 or IgG did not yield similar enrichment (data not shown). Thus, our genome-wide analysis found that H2AZ occupied nucleosomes at the promoter regions of 1655 (8%) protein-coding genes in ES cells with high confidence.

Because promoter arrays were used to interrogate enrichment, it was possible that H2AZ occupied other genomic sequences in ES cells. To test this, DNA microarrays were designed to contain 60 bp oligonucleotide probes that span the entire nonrepeat portion of chromosome 19 in the mouse genome (see the Supplemental Data). H2AZ ChIP-enriched DNA was hybridized to chromosome arrays, and binding events were identified with the above criteria (Table S3). Similar to the promoter array data, H2AZ occupied genomic regions in close proximity to known or predicted TSSs, and additional sites of enrichment were not observed along the chromosome (Figure 1C). These data indicate that H2AZ predominantly occupies promoter regions at a defined set of genes in ES cells.

H2AZ Is Enriched at Key Developmental Regulators in ES Cells

To gain insights into the biological roles of the genes occupied by H2AZ in ES cells, we determined which gene ontology (GO) terms were overrepresented in this set relative to all genes with representative probes on the microarray. This analysis revealed a striking enrichment for genes connected to the developmental and transcription hierarchies, including those with roles in organ and system development, cell differentiation, and cell fate commitment, as well as in the regulation of gene expression (Figure 1D; Table S4). Further analysis indicated that many of these genes are transcription factors with important roles in a variety of developmental processes.

H2AZ was enriched at the majority of homeodomain genes, including members of the *Pou*, *Pax*, *Six*, *Dlx*, *Irx*, and *Lhx* gene families and all of the homeotic genes found in *Hox* gene clusters (Tables S2 and S4). Homeodomain-containing transcription factors are evolutionarily conserved regulators that specify cell fate during embryonic development through transcriptional control of other developmental regulators (Deschamps, 2007; Pearson et al., 2005). H2AZ also occupied promoters of members of the *Fox*, *Sox*, *Gata*, and *Tbx* transcription factor families that have important roles in development and disease (Burch, 2005; Lehmann et al., 2003; Schepers et al., 2002; Showell et al., 2004). Interestingly, the targets were also enriched for genes that encode components of signaling pathways such as members of the *Wnt* family (Clevers, 2006). These data suggest that H2AZ predominantly occupies the promoters of genes in ES cells that, when expressed, would promote developmental progression and differentiation.

H2AZ and Suz12 Occupy a Highly Similar Set of Genes in ES Cells

It was particularly intriguing that H2AZ occupied the promoter regions of developmental genes in ES cells, including many that code for transcription factors and signaling components. Previous studies in both human and mouse ES cells have revealed that Polycomb group (PcG) proteins occupy the promoters of developmental genes (Boyer et al., 2006; Lee et al., 2006). This led us to compare the set of H2AZ-enriched genes with PcG protein-binding targets. In this study, Suz12 was used as a proxy for PcG protein occupancy because core components of PRC2 (Suz12 and Eed, as well as the modification catalyzed by PRC2 and H3K27me3) and PRC1 (Rnf2 and Phc1) were found previously to significantly overlap in ES cells (Boyer et al., 2006) and because Suz12 has been shown to be critical for the function of the PRC2 complex (Cao and Zhang, 2004; Pasini et al., 2004). To control for potential differences in the probes used in the prior genome-wide analysis, we generated binding data for Suz12 (Table S5) on the same promoter microarray design platform as that described above. As expected, the Suz12 target genes were highly similar to those previously reported (Table S6) (Boyer et al., 2006). Further analysis found that most (93%) of the H2AZ-enriched regions were also occupied by Suz12 (Figure 2A). PcG proteins have been shown to globally maintain their target genes in a silent state (Boyer et al., 2006; Bracken et al., 2006; Lee et al., 2006). Therefore, H2AZ occupies the promoter regions of a defined set of genes in ES cells that are targets of Polycomb-mediated repression.

H2AZ and Suz12 Display Highly Similar Spatial Patterning in the ES Cell Genome

Genome-wide studies in yeast, *Drosophila*, and human T cells have shown that H2AZ maps to a limited number of nucleosomes around TSSs (Albert et al., 2007; Barski et al., 2007; Guillemette et al., 2005; Mavrich et al., 2008; Raisner et al., 2005). In agreement with these studies, ~75% of H2AZ-enriched regions in ES cells are localized within discrete intervals of less than 2 kb around the TSS (Figure 2B). Surprisingly, ~25% of the promoter regions showed extended regions of H2AZ occupancy of greater than 2 kb. Analysis of Suz12 revealed a similar length distribution in ES cells. These data indicate that although the majority of enriched regions are found within small domains at promoters in ES cells, H2AZ also extends across larger regions.

A previously observed feature of Suz12 binding in ES cells at the subset of genes encoding developmental transcription factors was the extensive span over which the regulator occupied the locus. For example, Suz12 occupancy was found to encompass large domains extending from the promoter into the gene (Lee et al., 2006). A similar genomic distribution has also been observed for H3K27me3, the histone modification catalyzed by PRC2 (Boyer et al., 2006; Mikkelsen et al., 2007). Strikingly, we found that the distribution pattern at target genes were comparable between H2AZ and Suz12 (Figure 2C). Thus, H2AZ also exhibits an unusual tendency to occupy extended regions at development genes.

An additional unique feature of PcG protein occupancy was the finding that Suz12 encompassed multiple contiguous genes that extended across the entire *HoxA*, *HoxB*, *HoxC*, and *HoxD* clusters but that it did not occupy adjacent genomic sequences (Lee et al., 2006). This led us to compare H2AZ and Suz12 enrichment across the *HoxA* gene cluster as well as flanking genomic regions (Tables S1 and S5). Surprisingly, H2AZ extended over contiguous genes that encompassed the *HoxA* gene cluster, yielding a highly defined spatial pattern nearly identical to that of Suz12 (Figure 2D). Because a similar spatial patterning of H2AZ and Suz12 suggests an important functional interaction

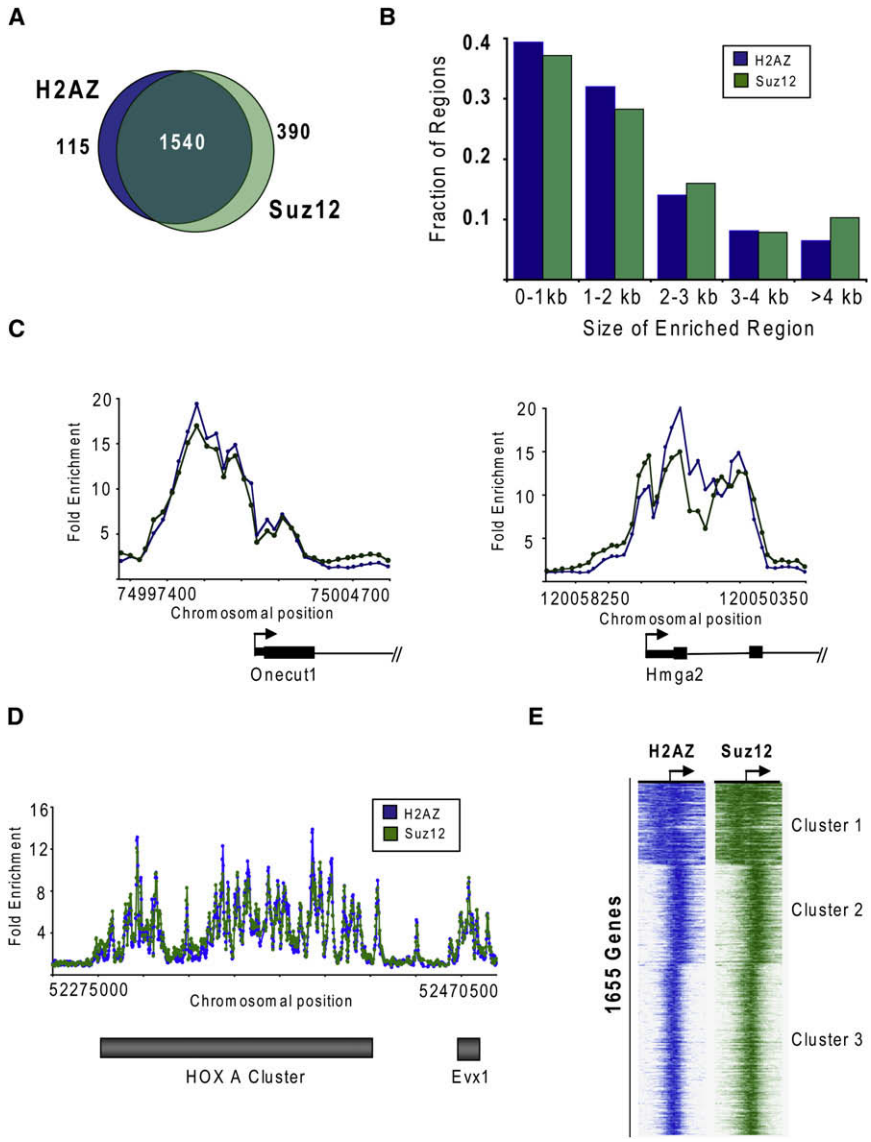

Figure 2. H2AZ and Suz12 Occupy a Highly Similar Set of Genes in ES Cells

(A) Venn diagram showing the overlap of genes enriched with H2AZ and occupied by Suz12 at high confidence in ES cells as determined by ChIP combined with promoter arrays. Ninety-three percent of H2AZ-enriched genes were also occupied by Suz12 when high-confidence threshold criteria were used.

(B) H2AZ- and Suz12-enriched regions were binned according to length of the bound region as determined by promoter microarrays. The majority of bound regions are less then 2 kb, whereas a small fraction (~25%) exhibit an extended binding domain of more than 2 kb.

(C) H2AZ and Suz12 display the same spatial patterning at target genes. The plots show unprocessed enrichment ratios for H2AZ (blue) and Suz12 (green) for all probes within a genomic region as in Figure 1A.

(D) H2AZ and Suz12 occupy large domains (>100 kb) that span multiple contiguous genes encompassing the HOX gene clusters. Data are derived from the promoter microarrays that were also designed to contain probes tiling the entire HoxA locus. Unprocessed enrichment ratios for H2AZ (blue) and Suz12 (green) for all probes within the region are shown. The gray bars represent the approximate location of the gene cluster.

(E) H2AZ and Suz12 display a highly similar defined spatial pattering across target genes in ES cells. K-means clustering was performed with the set of 1655 H2AZ-enriched genes using the Cluster algorithm (http://rana.lbl.gov/EisenSoftware.htm). Each horizontal line represents an individual gene, and the enrichment values for H2AZ (blue) and Suz12 (green) for each probe within the region −3.5 kb to +3.5 kb relative to the TSS are represented by color intensity (darker color represents higher enrichment ratio).

between these two chromatin regulatory pathways in ES cells, their genomic distribution was examined at all target loci. To this end, the pattern of H2AZ and Suz12 enrichment was compared for the set of 1655 H2AZ occupied regions. The probe enrichment scores across a 7 kb region centered at the TSS were clustered with K-means clustering, and three nodes were selected (Figure 2E; Table S8 lists genes in each cluster). Higher node numbers were tested, but additional nodes showed similar binding behavior on the basis of composite profiles (data not shown). These data reveal that the genomic distribution of H2AZ in ES cells is remarkably similar to that of Suz12.

H2AZ and H3K27me3 Are Enriched at a Distinct Set of Genes in Neural Precursors

The process of lineage commitment is characterized by a dramatic reorganization of chromatin structure, and recent evidence suggests that every cell type may harbor a unique chromatin signature (Mendenhall and Bernstein, 2008). Given the unexpected similarity between H2AZ and Suz12 enrichment in ES cells, we next asked whether this pattern was maintained in differentiated cells. H2AZ occupancy was examined in neural precursors (NPs) because these cells can be directly derived from ES cells and because many of the target genes have known roles in neural development (see Figure 1D). Similarly to its distribution in ES cells, H2AZ was significantly enriched at the promoter regions of a large set of genes (1206) in NPs, and 94% of the bound regions were within 1 kb of a TSS (Figure 3A; Tables S9 and S10). In contrast, the set of H2AZ-enriched genes was remarkably different in NPs as compared to ES cells (Table S10). Moreover, analysis of the spatial distribution in NPs found that H2AZ localized primarily to discrete intervals of less than 2 kb and that the more extended regions of enrichment were unique to ES cells (Figure 3B). These data indicate that H2AZ occupies the promoters of a different set of genes in ES cells and neural precursors.

Given that H2AZ and Suz12 co-occupy target genes that code for developmental regulators that are silent in ES cells, we

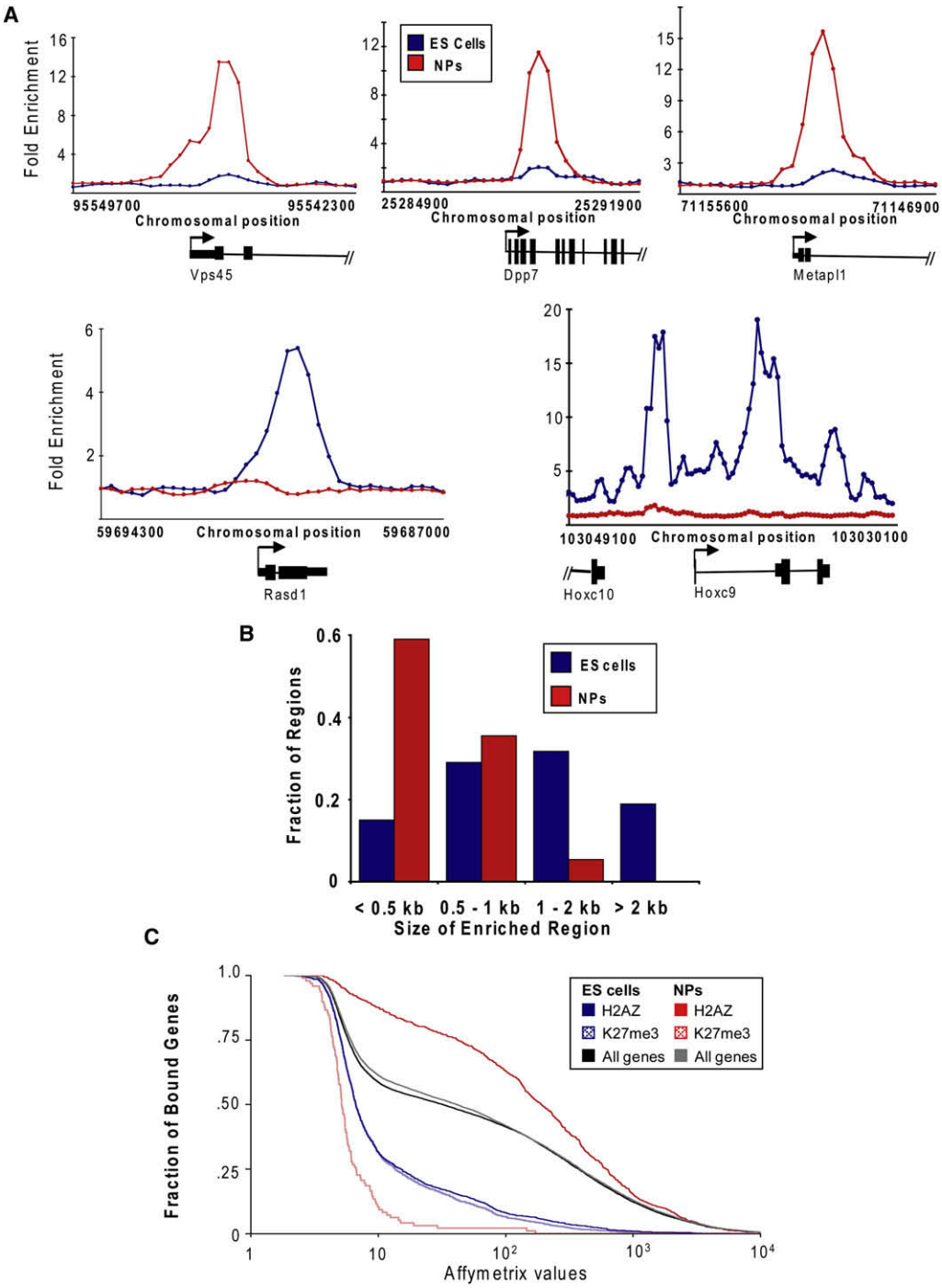

Figure 3. H2AZ Is Enriched at a Distinct Set of Genes in Neural Precursors

(A) Representative examples of DNA sequences occupied by H2AZ from a replicate set of ChIP experiments combined with DNA microarrays. ChIP with core histone H3 was hybridized together with H2AZ to control for nucleosome density. The plots show unprocessed enrichment ratios for H2AZ in ES cells (blue) and NPs (red) for all probes within a genomic region as shown in Figure 1A.

(B) The spatial distribution of H2AZ differs in NPs as compared to ES cells. H2AZ-enriched regions in ES cells (blue) and NPs (red) were binned according to length of the bound region on the basis of data from the promoter microarrays.

(C) H2AZ is enriched at active genes in NPs. A cumulative distribution plot of the expression of genes associated with H2AZ or H3K27me3 enrichment in ES cells and NPs is shown. Distribution of all genes in ES cells (black) and NPs (gray) is shown for reference. Affymetrix expression and H3K27me3 enrichment data were derived from Mikkelsen et al. (2007).

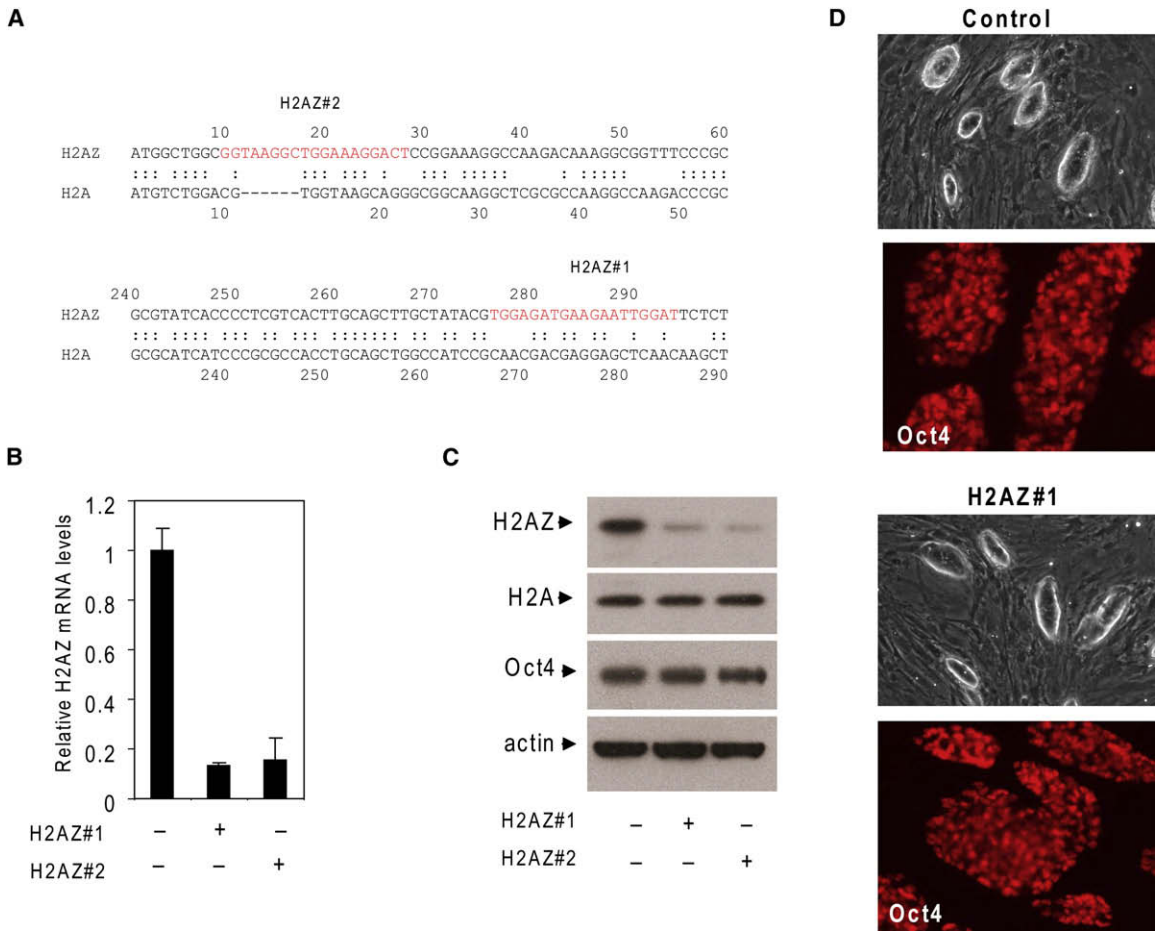

Figure 4. RNAi-Mediated H2AZ Depletion in ES Cells

(A) Schematic representation of H2AZ hairpin targeting sequences (red) and the homology between H2A and H2AZ.

(B) mRNA levels were significantly reduced upon RNAi-mediated suppression of H2AZ as measured by quantitative real-time PCR in H2AZ-depleted ES cell lines relative to the control ES cell line. Data were normalized to Gapdh, and error bars represent two standard deviations.

(C) H2AZ protein abundance as determined by immunoblot analysis is specifically reduced in depleted ES cell lines, whereas no reduction in histone H2A or Oct4 levels is observed in the control cell line. Actin serves as a loading control.

(D) ES cell colony morphology and Oct4 levels are similar between control and H2AZ-deficient ES cell lines.

performed a similar comparison in NPs. To this end, genome-wide H3K27me3 binding and expression data were analyzed in both cell types (Mikkelsen et al., 2007; data are from the same genetic background as that used in this study). H3K27me3 enrichment has been shown to strongly correlate with Suz12 in ES cells (Boyer et al., 2006; Lee et al., 2006). As expected, H2AZ and H3K27me3 were enriched at genes in ES cells with lower expression levels compared to all genes (Figure 3C). Conversely, although H3K27me3 enrichment at promoters correlated with genes that exhibit lower expression in NPs (consistent with its role as a mark of silent chromatin), H2AZ predominantly occupied genes that displayed high expression levels compared to all genes in NPs. Further analysis of the set of H2AZ-occupied genes in NPs revealed a significant enrichment for GO terms associated with a wide range of metabolic processes (Figure S1; Table S10). H2AZ enrichment at active gene promoters in NPs is similar to prior studies in other differentiated cell types in mammals (Barski et al., 2007; Schones et al., 2008). These data suggest that co-occupancy of H2AZ and PcG proteins at promoters is a hallmark of ES cells and that global H2AZ enrichment patterns change during development and differentiation.

H2AZ Is Not Required for ES Cell Self-Renewal and Pluripotency

In order to investigate the role of H2AZ, ES cell lines that stably expressed sequence specific hairpins were generated by retroviral transduction. The expression of two unique hairpins (Figure 4A) resulted in efficient and specific reduction in H2AZ mRNA levels in ES cells as compared to a control cell line expressing a nontargeting GFP-specific hairpin (Figure 4B; Figure S2). Notably, a reduction in mRNA levels resulted in suppression of H2AZ protein abundance but did not alter the levels of the major histone H2A or the essential pluripotency transcription factor Pou5f1/Oct4 as compared to the control ES cell line (Figure 4C). Moreover, H2AZ-depleted ES cell lines showed typical colony morphology and Oct4 expression patterns

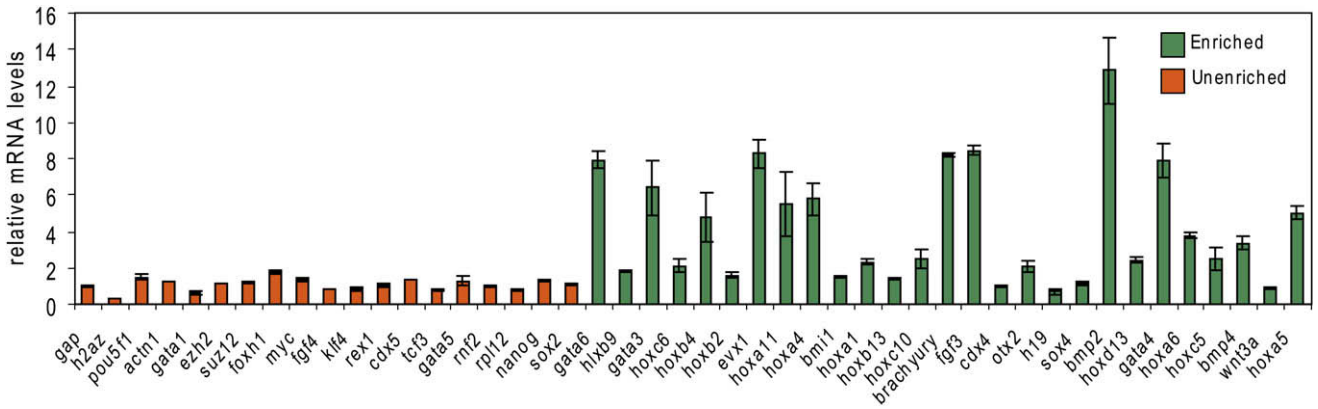

Figure 5. H2AZ Is Necessary for Control of Target Gene Expression

Target genes display a propensity to become derepressed upon RNAi-mediated suppression of H2AZ in ES cells. Real-time PCR analysis of mRNA levels in H2AZ-depleted ES cells for genes that are enriched (green) or unenriched (orange) is shown. Data were normalized to Gapdh and are shown relative to control ES cell lines. Reactions were performed in triplicate, and error bars represent two standard deviations of the mean.

(Figure 4D), displayed normal cell cycle kinetics, proliferated normally under ES cell growth conditions (Figures S3 and S4), and maintained a normal karyotype in prolonged culture (Figure S5). This suggests that H2AZ-depletion per se does not affect maintenance of the ES cell state.

H2AZ Levels Are Necessary for Regulation of Target Gene Expression

The observation that H2AZ occupied promoter regions in ES cells suggested a role in gene regulation. To examine whether H2AZ occupancy influences target gene expression, we used real-time polymerase chain reaction (RT-PCR) to compare the mRNA levels of H2AZ target genes in control and H2AZ-depleted ES cell lines. Notably, enriched genes displayed an overall tendency to become derepressed in H2AZ-deficient ES cells, whereas no significant difference was observed at a set of unenriched genes (Figure 5; Table S12). Importantly, these data are similar to that derived from the loss of PRC2 components such as Suz12 and Eed in ES cells (Boyer et al., 2006; Chamberlain et al., 2008; Pasini et al., 2007). The finding that H2AZ contributes to the regulation of target genes with known roles in development may explain, at least in part, the essential requirement for H2AZ during early mammalian development.

H2AZ and PcG Protein Occupancy at Target Promoters Is Interdependent in ES Cells

The co-occupancy of H2AZ and Suz12 at promoters in ES cells suggested that the localization of one could be dependent on that of the other. To investigate this, we performed ChIP and site-specific RT-PCR for a subset of genes in control, H2AZ-deficient, and *suz12* null ES cells (Figure 6; Table S13). Although significant Suz12 enrichment was detected at target genes in control ES cells, this enrichment was reduced in H2AZ-deficient ES cells (Figure 6A). The low level of Suz12 detected at these genes is most likely the result of the incomplete depletion of H2AZ due to RNAi. Surprisingly, H2AZ enrichment was also reduced to background levels (similar to the negative controls *Pou5f1/Oct4* and *Tcf3*) in *suz12* null ES cells (Figure 6B), suggesting that the localization of these regulators is mutually interdependent at target genes. Importantly, both H2AZ and Suz12 protein levels were unchanged in the reciprocal mutant ES cell line (Figure S6). Although components of PRC2 (Suz12, Eed) and PRC1 (Rnf2/Ring1b, Phc1) co-occupy a highly similar set of genes in ES cells (Boyer et al., 2006), PRC1 can be recruited independently to target regions (Pasini et al., 2007; Schoeftner et al., 2006). Therefore, enrichment for the essential component Rnf2/Ring1b was also analyzed in control and H2AZ-deficient ES cells. Similar to Suz12, Rnf2/Ring1b enrichment was significantly reduced at target genes (Figure 6C). We next tested whether Suz12 and H2AZ could directly interact by coimmunoprecipitation (Figure S7). Although an interaction was detected between the PRC2 core components Suz12 and Ezh2, an association with H2AZ was not observed in the ES cell extracts. These data suggest that H2AZ and PcG proteins may not directly interact and that their interdependence at promoters might be mediated at the level of chromatin or by an unknown factor.

H2AZ Is Necessary for ES Cell Differentiation

Because H2AZ is essential for early embryonic development and given that *suz12* null ES cells display derepression of target genes and an inability to initiate differentiation programs in vitro (Pasini et al., 2007), we asked whether H2AZ was also required for lineage specification. To test this, we cultured control and H2AZ-depleted ES cells under nonadherent conditions in the absence of leukemia inhibitory factor (LIF) to induce aggregation, a process that results in embryoid body (EB) formation. EBs are similar to, albeit more disorganized than, egg cylinder stage embryos and are capable of differentiation into the three germ layers. Of note is the finding that H2AZ-depleted ES cells failed to support normal development in vivo by tetraploid complementation and chimeric analysis (see the Supplemental Data and Figure S8), indicating that the knockdown cells represent a good model for assessment of the functional role of H2AZ during ES cell differentiation. In contrast to control ES cells, H2AZ-depleted EBs failed to give rise to typical structures representing

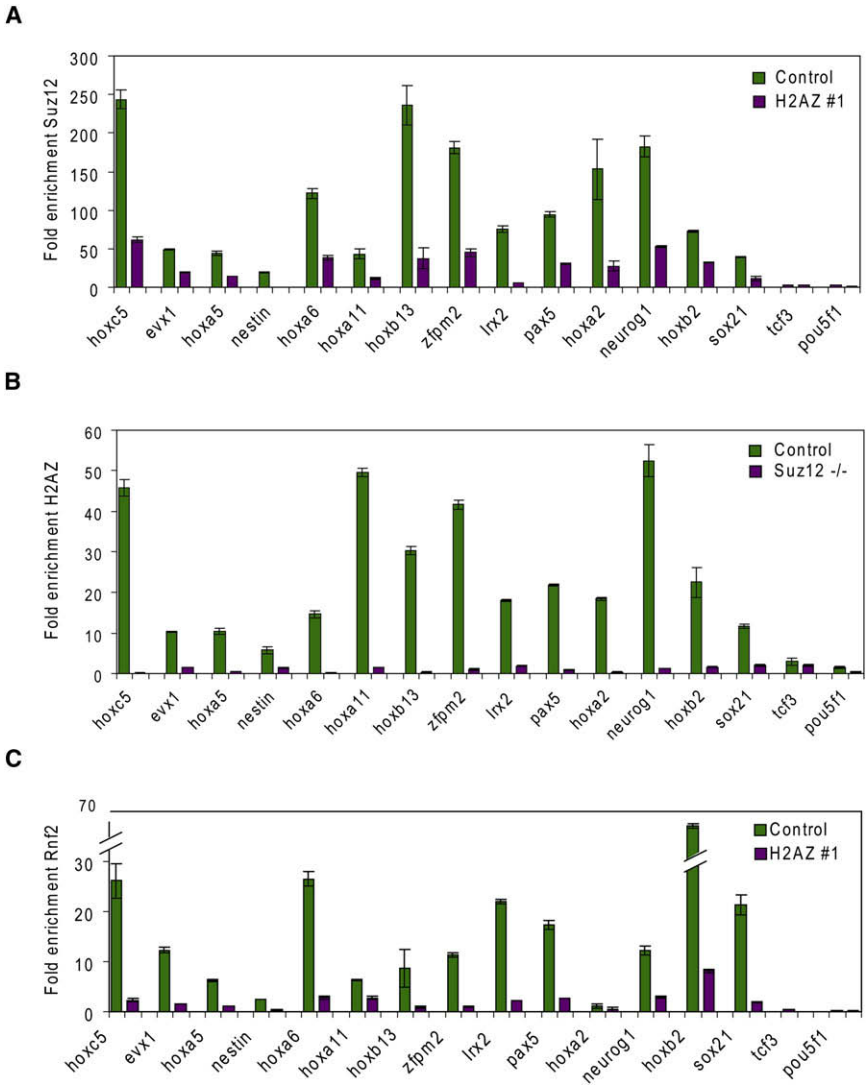

Figure 6. Interdependent Localization of H2AZ and Polycomb Complexes at Target Promoters in ES Cells

ChIP combined with real time PCR for Suz12 in control (green bars) or H2AZ-depleted ES cells (purple bars) (A), H2AZ in control (green bars) or *suz12* null ES cells (purple bars) (B), and Rnf2/Ring1b in control (green bars) or H2AZ-depleted ES cells (purple bars) (C) for the promoter regions of the indicated genes. All ChIP-qPCR reactions were performed in triplicate, and error bars represent two standard deviations of the mean. Primers were designed to amplify a region within 1 kb of the TSS and are listed in Table S13. Tcf3 and Oct4 (Pou5f1) represent negative controls. Similar results were obtained in both H2AZ-depleted ES cell lines.

differentiated cell types (Figure 7A). Moreover, induction of early differentiation markers such as *Brachyury*, *Pax3*, and *Wnt3a* required normal H2AZ levels (Figure 7B). Consistent with this differentiation failure, higher levels of the pluripotency genes *Oct4* and *Nanog* were observed in the H2AZ-depleted EBs. Thus, although target genes displayed a propensity to become derepressed in H2AZ-depleted ES cell lines, these genes fail to become activated in the proper spatiotemporal manner during differentiation.

Although the EBs did not differentiate under standard conditions, it was possible that H2AZ-depleted cells could be induced to differentiate into specific lineages when given particular developmental cues and culture support. To test this, we treated EBs derived from control and H2AZ-depleted cells with retinoic acid (RA) to induce neuronal differentiation. Under these conditions, the control cells underwent morphological changes consistent with neural differentiation (Glaser and Brustle, 2005; Li et al., 1998), whereas the H2AZ-depleted aggregates failed to do so (Figure 7C). Similarly, RA-treated control cells showed induction of early neuronal markers such as *Sox1* and *Nestin*, whereas the H2AZ-depleted cells did not display significant expression of these markers (Figure 7D). ES cells can also be robustly differentiated into hematopoietic lineages, as evidenced by the expression of classical hematopoietic markers (Kyba et al., 2002). Further experiments revealed that H2AZ-depleted cells were similarly defective when induced to differentiate into hematopoietic lineages (Figure S9). These studies argue that H2AZ plays a role with PcG proteins in arbitrating cell fate transitions and lineage commitment during ES cell differentiation.

DISCUSSION

How chromatin organization in ES cells contributes to the preservation of cell identity while maintaining the genome in a flexible state to allow for differentiation into multiple lineages remains an open question. In ES cells, the promoters of a subset of silent genes with roles in development are marked with "bivalent" histone modifications that correlate with both transcription initiation (H3K4me3) and gene silencing (H3K27me3) (Azuara et al., 2006; Bernstein et al., 2006; Mikkelsen et al., 2007). These chromatin marks are a result of the activity of Trithorax- (Trx) and Polycomb-group proteins, respectively. Core components of Polycomb Repressive Complex PRC2 (Suz12 and Eed, as well as the modification it catalyzes, H3K27me3) and PRC1 (Rnf2 and Phc1) have been found to significantly overlap in ES cells (Boyer et al., 2006; Lee et al., 2006). Our finding that H2AZ and Suz12 occupy the promoter regions of the same genes in ES cells suggests that this variant is an additional regulatory component at bivalent promoters and links H2AZ to the regulation of a large class of genes with known roles in development. Consistent with the existence of a role for H2AZ in gene regulation, target genes are derepressed and PcG proteins are lost from promoters upon

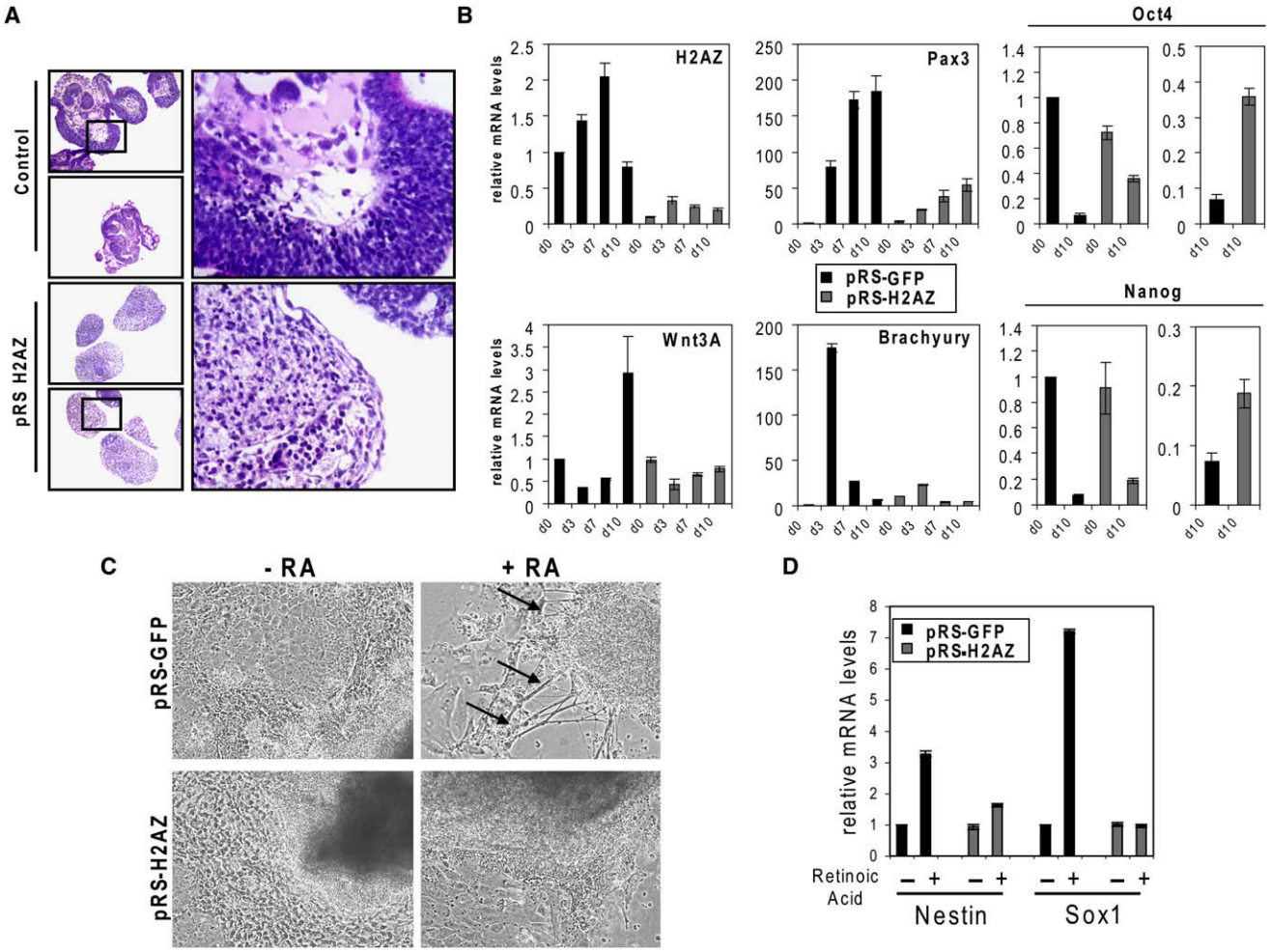

Figure 7. H2AZ Is Necessary for ES Cell Differentiation

(A) Hematoxylin and eosin staining of sectioned day 10 embryoid bodies (EBs) derived from control (upper panel) or H2AZ-depleted (lower panel) ES cells.

(B) Real-time PCR on mRNA isolated from control (black bars) or H2AZ-depleted (gray bars) EBs for the indicated genes. Relative mRNA levels are normalized to Gapdh. Days shown indicate the time EBs were maintained in culture prior to RNA isolation. The far-right panel shows end-point comparison of Oct4 and Nanog levels. Reactions were performed in triplicate, and error bars represent two standard deviations. Similar results were obtained with both H2AZ-depleted ES cells lines.

(C) Panels show EB images cultured for 7 days in the absence (left panels) or presence (right panels) of retinoic acid. Upper panels display control cells, whereas the lower panels represent EBs derived from H2AZ-depleted ES cells. Arrows indicate formation of neuron like structures typical for retinoic acid-induced differentiation.

(D) Real-time PCR of mRNA levels for neural differentiation markers Sox1 and Nestin in EBs as shown in (C). Reactions were performed in triplicate, and error bars represent two standard deviations. Similar results were obtained with both H2AZ-depleted ES cells lines.

RNAi-mediated H2AZ depletion in ES cells. It remains unclear, however, whether H2AZ incorporation is necessary for gene repression similarly to PcG proteins or whether it may be required for subsequent gene activation upon induction as has been observed in yeast (Li et al., 2005; Zhang et al., 2005).

Prior studies in metazoans have shown that H2AZ enrichment is directly proportional to gene activation (Barski et al., 2007; Mavrich et al., 2008; Schones et al., 2008), so it was surprising that H2AZ was enriched at a large subset of silent genes in ES cells. Recent studies have revealed that bivalent promoters experience transcription initiation but show no evidence of elongation, suggesting that they may be regulated at postinitiation steps (Guenther et al., 2007). An additional feature of these promoters is the presence of a paused RNA Polymerase II (Pol II) complex (Guenther et al., 2007; Stock et al., 2007). Interestingly, Polycomb-mediated ubiquitination of histone H2A, as catalyzed by the PRC1 component Rnf2/Ring1b (de Napoles et al., 2004), is necessary for this configuration to be maintained because its loss results in release of the paused polymerase and in gene derepression (Stock et al., 2007). The colocalization of PcG proteins and H2AZ suggests that H2AZ may be the target of this activity in ES cells. As such, it will also be of interest to investigate whether H2AZ loss or deubiquitination is required for release of a paused polymerase and transcriptional elongation.

It has been also proposed that the incorporation of H2AZ favors nucleosome eviction (Mavrich et al., 2008), which would promote histone turnover and chromatin accessibility. Interestingly, a recent study in *Arabidopsis* suggests that H2AZ may protect genes from DNA methylation, a permanent mark of silent chromatin (Zilberman et al., 2008). Thus, H2AZ incorporation may be a key mechanism allowing developmental genes to remain silent, yet poised for activation in ES cells.

Although the majority of H2AZ-bound regions encompass narrow intervals (<2 kb) at gene promoters, a small proportion of these regions extended from the promoter into the coding region and in some cases included contiguous genes such as those located in the *Hox* gene clusters (Lee et al., 2006). Biophysical analyses of H2AZ-containing nucleosomal arrays suggest that H2AZ inhibits the formation of highly compacted chromatin fibers (Fan et al., 2004). Thus, it is possible that, H2AZ incorporation into large regions with PcG proteins may also allow these genes to remain primed for activity by contending with Polycomb-mediated chromatin compaction (Francis et al., 2004). Together, these observations suggest that H2AZ and PcG proteins together may establish a specialized, structurally distinct chromatin conformation that has important consequences on gene regulation.

The promoter configuration at developmental regulators where both H2AZ and PcG proteins occupy a highly similar set of genes appears to be a hallmark of ES cells. We find that H2AZ and H3K27me3 are exclusively coenriched at genes in ES cells, whereas this pattern is not maintained upon differentiation into multipotent neural precursors. Rather, in NPs, H2AZ is found most highly enriched at active genes with a wide range of roles in metabolic processes. This observation is in accordance with previous studies in human T cells in that H2AZ localized to discrete regions at the promoters of active genes (Barski et al., 2007; Schones et al., 2008). Given that bivalent domains are also present in lineage-committed cells, it will be important to determine whether there is any enrichment of H2AZ at these regions. How can H2AZ mediate seemingly opposing functions? Interestingly, the acetylated form of H2AZ is enriched at the 5′ regions of active genes in yeast and vertebrates (Bruce et al., 2005; Millar et al., 2006), suggesting that posttranslational modification of H2AZ may underlie its differential distribution in ES cells compared to lineage-committed cells. Alternatively, H2AZ and the histone variant H3.3 can co-occupy the same nucleosome, and this has been shown to impact nucleosome stability (Jin and Felsenfeld, 2007). Thus, the function of H2AZ may depend on the composition of the nucleosome into which it is incorporated. As such, it will be of tremendous interest to determine how H3.3 is distributed in pluripotent and lineage-committed cells. Together, these studies suggest that H2AZ distribution patterns change during development and that its specific localization and function likely depend on additional modifiers.

We find that the association of H2AZ with PRC1 and PRC2 is mutually interdependent at promoters, because loss of one leads to depletion of the other. Although their mutual loss may be the result of gene derepression and ongoing transcription, the data suggest an obligate relationship between these regulators and their effect on chromatin structure, and ultimately on target gene regulation in ES cells. Consequently, their highly similar distribution patterns also suggest that these regulators are specifically recruited to genomic sites. Although considerable evidence exists that PcG proteins are targeted by DNA-binding factors in *Drosophila* (Ringrose and Paro, 2007), it is currently unknown how this class of repressors is localized to target genes in mammals. It is intriguing to speculate that H2AZ may play such a role in ES cells. However, we failed to detect an interaction between these proteins by coimmunoprecipitation assay, suggesting that recruitment may occur through a different mechanism. Interestingly, a recent report showed that long noncoding RNA facilitated PcG protein localization to *Hox* genes in human cells (Rinn et al., 2007), highlighting a potential global role for noncoding RNA in this process. Given that H2AZ was also redistributed during ES cell differentiation, how H2AZ is conscripted at target promoters is an equally important question. H2AZ deposition is catalyzed by the ATP-dependent activity of the SRCAP/SWR1 complex in mammals (Wong et al., 2007). Thus, it is possible that H2AZ incorporation is mediated by recruitment of this complex to promoters independently of PcG proteins. Given that both H2AZ and PcG proteins have been linked to cancer progression (Hua et al., 2008; Sparmann and van Lohuizen, 2006), it will be of extreme interest to elucidate the mechanisms by which these regulators are recruited to genomic sequences.

Our study demonstrates that H2AZ is necessary for execution of developmental programs but that it is dispensable for maintenance of the ES cell state, suggesting an important role for H2AZ in mediating cell fate transitions. This phenotype is similar to loss of Suz12, where null ES cells display derepression of target genes and failure to differentiate into multiple lineages (Pasini et al., 2007). Notably, although the PRC2 component Eed is essential for early development, null ES cells can contribute to tissues of all three germ layers in chimeric assays (Chamberlain et al., 2008), so it will be important to further dissect the roles of H2AZ and PRC2 in ES cell differentiation. Nonetheless, our findings demonstrate an important functional interaction between these two chromatin regulatory pathways in ES cells that is necessary for the control of developmental gene expression programs. In support of this, genetic interactions between H2AZ or its deposition complex and PcG proteins have been reported in *Drosophila* (Ruhf et al., 2001; Swaminathan et al., 2005), although this remains to be explored in mammals. Collectively, this work suggests that the association between H2AZ and PcG proteins at target genes provides an important functional switch controlling the initial stages of lineage commitment.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture

V6.5 (129SvJae and C57BL/6) ES cells were cultured as previously described (Boyer et al., 2006). H2AZ-depleted ES cell lines were similarly cultured with the addition of 1 μg/ml puromycin (Sigma) to maintain selection of hairpin expression. Neural precursors were derived from V6.5 ES cell lines as described in the Supplemental Data. The *suz12* null ES cell lines were cultured in standard ES cell media.

Antibodies, Chromatin Immunoprecipitation, and DNA Microarray Analysis

The antibodies used in this study are detailed in the Supplemental Data. ChIP was combined with DNA microarrays, and results were analyzed as described (Boyer et al., 2005; see also the Supplemental Data). In brief, a whole-chip error

model was used for the calculation of confidence values from the enrichment ratio and signal intensity of each probe (probe p value) and of each set of three neighboring probes (probe-set p value). Probe sets with significant p values ($p < 0.001$) and significant individual probe p values were assessed as enriched at high confidence. For promoter arrays, a bound region was assigned to an EntrezGene ID if this region mapped to within 4 kb of a transcription start site. All coordinates are based on NCBI Build 34 (mm6) of the mouse genome.

Gene Ontology

Gene ontology analysis was performed with DAVID (http://david.abcc.ncifcrf.gov/). EntrezGene IDs were used for the generation of enrichment statistics for the biological process category on the basis of a background list of all represented genes on the promoter microarray design.

K-Means Cluster Analysis of Binding Data

The spatial pattern of H2AZ and Suz12 enrichment was compared at the high-confidence set of 1655 H2AZ-enriched genes by K-means clustering (Table S8). For each start site, the raw enrichment ratio for the probe closest to that start site were selected in 250 bp increments over a 7 kb region (± 3.5 kb relative to the TSS). Promoters where more than 25% of probes were not represented on the array (typically because of repeat masking) were removed from the analysis. The ratios from the two experiments were used to create a single vector for each start site. The data were processed and the cluster diagram was generated with Cluster software (http://rana.lbl.gov/EisenSoftware.htm).

RNAi-Mediated Silencing of H2AZ in ES Cells

Nineteen-base pair hairpin oligonucleotides for H2AZ#1 (5′-TGGAGATGAA GAATTGGAT-3′) and H2AZ#2 (5′-GGTAAGGCTGGAAAGGACT-3′) and for GFP (5′-GCTGACCCTGAAGTTCATC-3′) were cloned into pRetro-Super as described previously (Brummelkamp et al., 2002). Ecotropic retroviral supernatants were generated by transfection of phoenix packaging cells with FuGENE 6 (Roche) according to the manufacturer's protocol. This viral supernatant was used for the infection of ES cells after the addition of 4 μg/ml polybrene (Sigma). Cells were infected overnight and allowed to recover for 48 hr in fresh ES cell medium. Single colonies from stable viral integrants were picked and clonally expanded in media containing 1 μg/ml puromycin.

Quantitative Real-Time PCR

RNA was isolated from 10^7 ES cells with the TRIzol Plus RNA Purification System (Invitrogen). First-strand cDNA synthesis was done with the SuperScript III First-Strand Synthesis SuperMix (Invitrogen). Relative transcript levels were measured in a triplicate set of reactions for each gene in control ES cells, as well as in both H2AZ-depleted ES cell lines, where relative Gapdh levels were used for normalization. qRT-PCR was performed with SYBR Green Master Mix (Invitrogen) on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The transcript levels in H2AZ-depleted ES cells were calculated as fold change relative to control ES cells expressing a nonspecific hairpin. Data analysis was performed with the manufacturer's software (Applied Biosystems 7900HT Sequence Detection Systems [SDS] version 2.3). Primers specific for H2AZ enriched (E) and unenriched (U) genes are listed in Table S12. Primers for the retinoic acid differentiation analysis are as follows: Sox1 forward, 5′-GGAAAACCCCAAGATGCACAAC-3′, reverse, 5′-CGCAGTC TCTTGGCCTCGTC-3′; and Nestin forward, 5′- GCCTATAGTTCAACGCCC CC-3′, reverse, 5′- AGACAGGCAGGGCTAGCAAG-3′.

ChIP and Quantitative Site-Specific Real-Time PCR Analysis

ChIP was performed in control (GFP-hairpin) and in both H2AZ-depleted ES cell lines with antibodies directed against H2AZ, Suz12, or Rnf2/Ring1b via our standard protocol (Boyer et al., 2006). For relative quantification by real-time PCR, dilutions of the whole-cell extract (WCE) DNA were used for construction of standard curves. One nanogram ChIP DNA or WCE control DNA was used per reaction and performed in triplicate with the SYBR Green PCR master mix (Invitrogen) on the ABI 7900HT Sequence Detection System. Primers were designed to amplify a region within 1 kb upstream of the transcription start site and are listed in Table S13.

ES Cell Differentiation

ES cells were diluted to 10,000 cells/ml in complete growth medium (see growth conditions for ES cells) lacking LIF, and 20 μl drops were arranged over the surface of a Petri dish. The plate was gently inverted and incubated at 37°C with 5% CO_2 for 3 days to allow aggregation and EB formation. EBs were transferred to low-attachment cell culture plates (Corning) with the addition of media for the remainder of the experiment. EBs were allowed to grow for up to 10–12 days, and samples were analyzed at various time points by hematoxylin and eosin staining after fixation in 10% formalin or by real-time PCR after mRNA isolation. For retinoic acid induction, EBs were formed as described above. At day 3, aggregates were transferred to complete growth medium lacking LIF and supplemented with 0.1 μM all-*trans* retinoic acid (Sigma) for the induction of neuronal differentiation (Glaser and Brustle, 2005; Li et al., 1998).

ACCESSION NUMBERS

Microarray data are available at ArrayExpress under the accession designation E-TABM-556.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, nine figures, and 13 tables and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08)01252-X.

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