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Histone H2A Mono-Ubiquitination Is a Crucial Step to Mediate PRC1-Dependent Repression of Developmental Genes to Maintain ES Cell Identity

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Abstract

Two distinct Polycomb complexes, PRC1 and PRC2, collaborate to maintain epigenetic repression of key developmental loci in embryonic stem cells (ESCs). PRC1 and PRC2 have histone modifying activities, catalyzing mono-ubiquitination of histone H2A (H2AK119u1) and trimethylation of H3 lysine 27 (H3K27me3), respectively. Compared to H3K27me3, localization and the role of H2AK119u1 are not fully understood in ESCs. Here we present genome-wide H2AK119u1 maps in ESCs and identify a group of genes at which H2AK119u1 is deposited in a Ring1-dependent manner. These genes are a distinctive subset of genes with H3K27me3 enrichment and are the central targets of Polycomb silencing that are required to maintain ESC identity. We further show that the H2A ubiquitination activity of PRC1 is dispensable for its target binding and its activity to compact chromatin at Hox loci, but is indispensable for efficient repression of target genes and thereby ESC maintenance. These data demonstrate that multiple effector mechanisms including H2A ubiquitination and chromatin compaction combine to mediate PRC1-dependent repression of genes that are crucial for the maintenance of ESC identity. Utilization of these diverse effector mechanisms might provide a means to maintain a repressive state that is robust yet highly responsive to developmental cues during ES cell self-renewal and differentiation.

Introduction

Embryonic stem cells (ESCs) can undergo unlimited self-renewal while maintaining their pluripotent and undifferentiated states, features that are consistent with their origin within the inner cell mass of the blastocyst. Increasing evidence suggests that in addition to the core gene regulatory circuitry composed of Oct3/4, Sox2, Nanog and other transcription factors, Polycomb group proteins critically contribute to maintain the undifferentiated state of ESCs by silencing genes that are involved in development and/or transcription [1,2,3,4,5,6]. Polycomb-mediated repression of these genes is also essential to preserve the ability of ES cells to differentiate in response to extracellular cues [7,8,9].

Polycomb group proteins are chromatin-modifiers that mediate transcriptional repression. They form at least two types of multimeric complexes, the Polycomb repressive complexes-1 (PRC1) and -2 (PRC2), the core components of which are conserved from Drosophila to human [10,11,12,13,14]. PRC2 contains Ezh2 or -1, which catalyze trimethylation of histone H3 lysine 27 (H3K27me3), a posttranslational modification that is thought to be recognized by the chromo-domain (CHD) protein components of PRC1 [12,13,14,15,16]. Within PRC1, Ring1B and –A act as major E3 ubiquitin ligases for histone H2A mono-ubiquitination at lysine 119 (H2AK119u1) [17,18]. Conditional depletion of Ring1B and –A in ESCs leads to global loss of H2AK119u1 and concurrent derepression of ‘bivalent’ genes enriched for both H3K27me3 and H3K4me3 [5,19]. H2AK119u1 deposition has been shown to localize to the inactive X chromosome (X), the XY body, and several silenced ‘bivalent’ loci in mouse ESCs [19,20,21]. Recent genome-wide H2AK119u1 analysis in MEFs (mouse embryonic...
Results

Ring1-mediated H2AK119u1 demarcates central targets of PRC1 in ESCs

Global H2AK119u1 distribution has been reported only for MEFs and the human teratocarcinoma NT2 cell line [22,27], but not for mouse ESCs. We, therefore, used ChIP-on-chip analysis to clarify H2AK119u1 deposition around transcription start sites (TSS) in mouse ESCs by using an Agilent mouse promoter array and an E6C5 monoclonal antibody (mAb) or a rabbit polyclonal antibody [29]. Ring1A/B-dKO ESCs, in which H2AK119u1 is apparently undetectable, were used as a negative control [5,9,28]. Ring1A/B-dKO ESCs were induced by treating Ring1A<sup>-/-</sup>;Ring1B<sup>-/-</sup>;Rosa26::CreERT2 ESCs with 4-hydroxy-tamoxifen (OHT), which rapidly activates CreERT2 and catalyzes loxP recombination at Ring1B locus [5]. Distribution of Ring1B, H3K27me3 and H2A were re-examined to obtain a reference data set.

E6C5-ChIP signals at the promoter regions of known target loci, Hoxa9, Pax9 and Tis8 show that H2AK119u1 deposition is readily detectable in Ring1A<sup>-/-</sup> ESCs but not in Ring1A/B-dKO (dKO) ESCs (Figure S1). These results were validated by ChIP-qPCR as shown in Figure S2A. We calculated the averages of E6C5-ChIP signals in Ring1B-bound and −unbound genes and found higher H2AK119u1 deposition in Ring1B-bound genes in Ring1A<sup>-/-</sup> than in Ring1A/B-dKO (dKO) whereas little difference was noted for unbound genes (Figure 1A; Figure S3A and S3B). These data therefore appear to reflect H2AK119u1 deposition that depends on Ring1B. For detailed investigation of the genes that exhibit H2AK119u1 enrichment, we examined the gene-wise distribution of E6C5-ChIP signals after subtraction of the background enrichment value and identified 538 target genes (Figure 1B; Figure S3C; the list of genes is shown in Table S1). The results with the E6C5 mAb were re-confirmed by using a rabbit antiserum that recognizes H2AK119u1 [28]. With this reagent, 524 genes were found to be bound by H2AK119u1, and these genes significantly overlapped with those identified by the E6C5 mAb (Figure S3D; Table S1). Taken together, using the above methods we determined a set of genes in ESCs that have H2AK119u1 deposition around their TSS.

We went on to examine the correlation of genes enriched for H2AK119u1 (H2AK119u1+) with those having Ring1B (Ring1B+) and H3K27me3 (H3K27me3+) depositions. We found that genes bound by Ring1B and H3K27me3 identified in this study were significantly overlapped with those reported in previous studies (Figure S3E, F). We identified 1721 and 3668 genes bound by Ring1B and H3K27me3, respectively, and found H2AK119u1+ genes as a subset of the Ring1B+ genes (Figure 1B; Figure S3C; Table S1). Since most Ring1B+ genes define a subset of H3K27me3+ genes, H3K27me3+ genes could be subdivided into three distinct layers, H2AK119u1+Ring1B+H3K27me3+ (Triple positive; TP), H2AK119u1+Ring1B- H3K27me3+ (Double positive; DP) and H2AK119u1+Ring1B- H3K27me3+ (Single positive; SP). We finally confirmed the quantitative differentiation of H2AK119u1 level at TP genes against DP or SP genes by ChIP-qPCR analysis at selected genes (Figure S2A). Although we cannot exclude a possibility that we failed to detect a low level of H2AK119u1 at some DP genes, our data demonstrate that H3K27me3+ gene promoters are not uniformly occupied by Ring1B and H2AK119u1.

We investigated functional properties of H2AK119u1+ genes among Polycomb targets. Scattered plot analysis for gene-wise deposition of H3K27me3 and Ring1B revealed that H2AK119u1 targets were significantly enriched among genes that have high...
levels of both Ring1B and H3K27me3 occupancy (Figure S4). This suggests that TP genes represent the central targets for Polycomb repression. We compared the impact of PRC1 loss among these subsets by examining the gene expression profiles in Ring1A/B-dKO ESCs (Figure 1C; Figure S2B). We found significant de-repression (p < 0.001) of TP, DP and SP genes but no significant changes in H3K27me3-negative genes. It is worth noting that the degree of de-repression of the TP genes was significantly higher than that of the DP and SP genes (Figure 1C). Gene ontology (GO) based analyses confirmed that TP genes are most significantly enriched for functions in transcription and/or development (Figure S5). Of note, Cdx2 and Gata6, which are known to be repressed by Oct3/4 and Nanog [29,30], are occupied by H2AK119u1, suggesting that H2AK119u1 might be involved in maintaining ESC properties by suppressing differentiation of ESCs.

E3 activity of Ring1B and H2AK119u1 are dispensable for PRC1 target binding

Above data suggest a potential importance of H2AK119u1 for repression of key developmental regulators which is required to maintain the undifferentiated status of ESCs. This however does not necessarily prove the importance of the E3 ligase activity and H2A ubiquitination independent functions of PRC1 in chromatin compaction and gene silencing both in vitro [24], and in vivo [23] has been
reported in previous studies. To investigate this question, we expressed mutant Ring1B proteins that are defective in the interaction with the E2 component in Ring1A/B-dKO ESCs. In this experimental setup, we have first stably expressed exogenous WT or mutant Ring1B in Ring1A<sup>−/−</sup>;Ring1B<sup>fl/fl</sup>;R26::CreERT2 ESCs and then endogenous Ring1B was depleted by OHT treatment (Figure 2A). Similar experiments have been described previously [23] but have made use of Ring1B single KO ESCs, and therefore could not exclude the contribution of low levels of Ring1A (Figure S6A) and associated H2AK119u1 that occur in ESCs [5,17,20,25]. We thus tested the role of Ring1A to mediate H2AK119u1 and repression of Polycomb targets in ESCs. We first compared global H2AK119u1 levels in Ring1B-KO ESCs with Ring1A/B-dKO and found significant amount of H2AK119u1 remained in Ring1B-KO (Figure S6B). Consistently, the expression of exogenous Ring1A obviously restored global H2AK119u1 level, ESC identity, and repression of TP genes in Ring1A/B-dKO ESCs (Figure S6B–E). We then performed ChIP-chip analysis to compare Ring1A distribution with Ring1B and found that Ring1A and Ring1B significantly shared target genes (Figure S6F, G). ChIP-qPCR analysis further confirmed Ring1A binding at promoter regions of representative TP genes such as Hoxd11 and Zic1 in the absence of Ring1B (Figure S6H). Importantly, binding of other PRC1 components such as Mel18 was concomitantly restored by the expression of exogenous Ring1A in Ring1A/B-dKO ESCs (Figure S6H). Therefore, Ring1A was shown to substitute for Ring1B functions in mediating H2AK119u1 and target gene repression. These results sufficiently justify the use of

Figure 2. Generation of ESCs expressing catalytically inactive Ring1B. (A) Schematic representation of 3xFlag-tagged Ring1B, showing wild-type and point-mutant derivatives. Each of these construct was stably transfected into Ring1A<sup>−/−</sup>; Ring1B<sup>fl/fl</sup>;R26::CreERT2 ESCs. (B) Immunoblot analysis of Ring1A, Ring1B, Flag, H2AK119u1 and Lamin B protein levels in whole cell lysates of wild-type and Ring1A<sup>−/−</sup>; Ring1B<sup>fl/fl</sup>;R26::CreERT2 ESC lines expressing mock, WT, I53S, or I53A Ring1B with or without OHT treatment (OHT+ and −, respectively). (C) Immunoprecipitation (IP) analysis showing the association of exogenous Ring1B WT, I53S, or I53A with an endogenous PRC1 component Mel18. Extracts of OHT-untreated (−) and +treated (+); day 2) Ring1A<sup>−/−</sup>; Ring1B<sup>fl/fl</sup>;R26::CreERT2 ESC lines expressing each of the constructs were immunoprecipitated with anti-Flag antibody. Resulting precipitates (IP) and lysates (Input) were immunoblotted with antibodies against Flag, Ring1B and Mel18. (D) Association of Flag-tagged proteins in Ring1A<sup>−/−</sup>; Ring1B<sup>fl/fl</sup>;R26::CreERT2 ESC lines expressing mock, WT, I53S, or I53A Ring1B with or without OHT treatment (OHT+ and −, respectively). (E) 3D FISH with probe pairs at Hoxb locus (Hoxb1 and Hoxb13) in PFA-fixed nuclei of Ring1A<sup>−/−</sup>; Ring1B<sup>fl/fl</sup>;R26::CreERT2 ESC lines stably expressing mock, Flag-tagged Ring1B WT, I53S, or I53A with promoter regions of their representative target genes before (−) or after (+) OHT treatment (day 2) as determined by ChIP and site-specific real-time PCR. Error bars represent standard deviations determined from three independent experiments. (E) 3D FISH with probe pairs at Hoxb locus (Hoxb1 and Hoxb13) in PFA-fixed nuclei of Ring1A<sup>−/−</sup>; Ring1B<sup>fl/fl</sup>;R26::CreERT2 ESC lines stably expressing mock, WT, I53S, or I53A Ring1B before (−) or after (+) OHT treatment (day 2). Scale bars indicate 1 μm. The boxes show the median and interquartile range of interprobe distances (μm) in the indicated cells. Open circles indicate outliers. The statistical significance of differences between the indicated two data was examined by the Mann-Whitney U test. doi:10.1371/journal.pgen.1002774.g002
**Role of H2A Ubiquitination in PRC1 Repression**

**E3 activity of Ring1B is required to repress Polycomb targets and maintain ESCs**

We next tested the phenotypes of the transfectants after deletion of endogenous Ring1B. We observed that the expression of either Ring1B (I53S) or Ring1B (I53A) was not sufficient to maintain ESCs in an undifferentiated state (Figure 3A). We obtained similar results in the presence of three inhibitors (3i) that target FGF receptor, MEK, and GSK3 (data not shown) [32]. This implies that the E3 activity of Ring1B is required to maintain ESC identity in the absence of Ring1A. Consistently, Ring1B (I53S) failed to repress expression of differentiation markers (Kdr, Gata6, Hnf4a and Sox2) and expression of undifferentiation markers (Pou5f1, Sox2 and Nanog) in Ring1A/B-dKO ESCs while WT Ring1B or Ring1A obviously restored (Figure S7A). We went on to examine differentiation ability of the respective ESC lines by forming embryoid bodies. We found that the progressive changes in expression of the marker genes upon induction of differentiation were considerably affected in Ring1A/B-dKO ESCs compared to wild-type or Ring1A/−/− ESCs (Figure S7B). These changes were restored by WT Ring1B but not by Ring1B (I53S). Together, Ring1B catalytic activity is required for maintenance and differentiation of ESCs.

We then examined the expression of H2AK119u1+ genes in these transfectants by using expression microarrays. In the mock transfectant, we observed that H2AK119u1+ genes were significantly de-repressed by depletion of Ring1 proteins whereas expression of H2AK119u1− genes was virtually unchanged (Figure 3B). De-repression of H2AK119u1+ genes in the Ring1A/B-dKO was mostly restored by the expression of WT Ring1B but only partially by Ring1B (I53S) and Ring1B (I53A) (Figure 3B; Figure S8). Moreover, the levels of restoration by Ring1B mutants were variable among target genes. To confirm the microarray results, we examined the expression of H2AK119u1 targets, Hoxa9, Hoxb13, Hoxd11, Zic1, and Pax3, by quantitative RT-PCR. These genes were de-repressed in Ring1A/−/− dKO compared to OHT-untreated control cells (Figure 3C). WT Ring1B was shown to fully restore the repression of these genes. Ring1B (I53S) and Ring1B (I53A) could slightly restore the repression of Hoxa9, Hoxb13 and Hoxd11, but almost failed to repress Zic1 and Pax3 (Figure 3C). Therefore, the E3 activity of Ring1B is required for efficient repression of its target genes. Our results also suggest that some genes, e.g., Zic1 and Pax3 are more dependent on the E3 activity than others, notably the Hox cluster genes such as Hoxd11.

**Ring1B mediates repression through H2AK119u1**

The above experiments strongly suggest that repression of developmental regulators in ESCs is attributable to PRC1 mediated H2AK119u1. Previous studies report that Ring1B regulates local H3K4me3 deposition and loading of RNA polymerase II (RNAP) in ESCs [5,19], and that H2AK119u1 has a role in suppressing MLL-mediated methylation of H3 lysine 4 (H3K4) and transcriptional initiation from nucleosomal templates [28]. We, therefore, examined whether the catalytic activity of Ring1B is involved in suppressing H3K4 methylation and RNAP loading at target gene loci. Consistent with the previous reports, we found that local levels of trimethylated H3K4 (H3K4me3) and RNAP loading were considerably up-regulated at target gene promoters in Ring1A/B-dKO ESCs, which could be repressed by expression of WT Ring1B in these cells (Figure 3D and 3E). In contrast, Ring1B (I53S) and Ring1B (I53A) failed to suppress local increases of H3K4me3 and RNAP levels. Therefore, the catalytic activity of Ring1B is required to repress H3K4me3 and RNAP loading. Consistent with these observations, the profound reduction in local H3K27me3 levels in Ring1A/B-dKO ESCs could not be restored by Ring1B (I53S) or Ring1B (I53A) (Figure S9). This may also suggest the contribution of Ring1B catalytic activity to maintain repressive chromatin. Collectively, our results demonstrate that Ring1B-dependent H2AK119u1 facilitates transcriptional repression of PRC1 target genes and thereby enables the maintenance of ESC identity.

**Discussion**

In the present study, we present genome-wide H2AK119u1 maps in ESCs and identify a group of genes at which H2AK119u1 is deposited in a Ring1-dependent manner. These genes are a distinct subset of genes with H3K27me3 enrichment and we suggest that these are the central targets of Polycomb silencing to maintain ESC identity. By using mutant versions of Ring1B, which can not bind to E2 components, we demonstrate the role of H2AK119u1 to facilitate the repression of these target genes. We
propose that H2AK119u1 contributes to capacitate Polycomb-mediated repression in a reversible manner because recognition and de-ubiquitination of H2AK119u1 have been shown to be linked with transcriptional activation [27,28,33]. This conclusion is different to a recent study which suggested that the catalytic mutant Ring1B could restore repression in Ring1B mutant ES cells [23]. A key difference in that study and our analyses shown here is that we assessed the function of catalytically inactive Ring1B in a background that is null for both Ring1B and the closely related homologue Ring1A. Ring1A potentially complements loss of Ring1B in ESCs, despite the fact that the expression level of Ring1A is relatively low compared to Ring1B (Figure S6) [5,20].

Our results are concordant with those of Eskeland et al. 2010 which reports that the ability of Ring1B to mediate the condensation of the Hoxb cluster is not dependent on its histone ubiquitination activity. In addition, in our study we have observed that the E3 activity of Ring1B contributes to the repression of Hox genes to a lesser extent than to Zic1 and Pax3 genes (Figure 3C). Based on these evidences, we propose that H2AK119u1-dependent repression is likely complemented by other PRC1-mediated mechanisms such as chromatin compaction [23]. The fact that H2AK119u1 independent repression is more prevalent at Hox loci compared to other Polycomb target genes may suggest that it is more effective when target loci are closely juxtaposed in cis. We indeed found a slight but a significant restoration of repression of H2AK119u1+ genes that are closely juxtaposed each other (<50 kb) by expression of mutant Ring1B in Ring1A/B-dKO, but this is not the case for H2AK119u1+ genes that are separated by ≥50 kb genomic regions (Figure S10). However, this tendency is not statistically significant once we excluded Hox cluster genes. Further studies are needed to elucidate the molecular nature for H2AK119u1-independent mechanisms.

Overall, our findings show that PRC1 mediates gene repression by combining multiple and different effector mechanisms, of which H2A ubiquitination is a major contributor (Figure 4). Such diverse PRC1 effector mechanisms might be required to make PRC1-mediated gene repression both flexible and robust. How H2A ubiquitination contributes to repress target gene transcription also awaits future studies, although mechanisms that

Figure 3. H2A ubiquitination activity of Ring1B is essential for the maintenance of ESC identity and repression of target gene expression. (A) Morphology of OHT-untreated and –treated (day 8) Ring1A+/−; Ring1Bfl/fl; R26::CreERT2 ESC lines expressing the indicated transgene. The images were acquired under a phase-contrast microscope. Scale bars indicate 200 μm. (B) Histograms showing the expression changes of H2AK119u1+ and H2AK119u1− genes in Ring1A+/−; Ring1Bfl/fl; Rosa26::CreERT2 ESCs expressing mock (blue line), WT Ring1B (red line), or mutant Ring1B (green dotted line) following OHT treatment. (C) Expression levels of Hoxa9, Hoxb13, Hoxd11, Zic1, Pax3 and Pou5f1 in Ring1A+/−; Ring1Bfl/fl; Rosa26::CreERT2 ESCs expressing mock, WT, I53S, or I53A Ring1B before (−) or after (+) OHT treatment (day 2). Expression levels were normalized to a Gapdh control and are depicted as fold changes relative to mock (OHT-untreated) ESCs. Error bars represent standard deviation determined from at least three independent experiments. (D) Local levels of trimethylated H3K4 (H3K4me3) at promoter regions of representative target genes in Ring1A+/−; Ring1Bfl/fl; R26::CreERT2 ESCs stably expressing mock, WT, I53S, or I53A Ring1B before (−) or after (+) OHT treatment (day 2) determined by ChIP and site-specific real-time PCR. The relative amount of immunoprecipitated DNA is depicted as a percentage of input DNA. Error bars represent standard deviation determined from at least three independent experiments. (E) As in (D), but showing local levels of RNA polymerase II (RNAP) detected with the 8WG16 antibody. doi:10.1371/journal.pgen.1002774.g003
antagonize against H2A ubiquitination have already been proposed [27].

**Materials and Methods**

**Cells and cell culture**

Ring1Bfl/fl; Rosa26::CreERT2, Ring1A<sup>2/2</sup>; Ring1Bfl/fl; Rosa26::CreERT2, and Eed-KO ESCs were described previously [5,19,20,26,34]. The ESCs were cultured in DMEM with 20% fetal bovine serum, MEM nonessential amino acids (Invitrogen), sodium pyruvate (Invitrogen), L-glutamine (Invitrogen), 2-mercaptoethanol (Sigma), and ESGRO (Chemicon) either on irradiated MEF as feeder layers or directly on gelatin-coated surfaces.

**Plasmids**

3xFlag-tagged wild-type Ring1A, wild-type Ring1B, mutated Ring1B (I53A [17,23] and I53S [31]) cDNAs were subcloned into the expression vector pCAG-IRES-Puro (a kind gift from Dr. Hitoshi Niwa in RIKEN CDB in Japan).

**Antibodies**

The following antibodies were used: Ring1B (clone #3) [35], Ring1A [36], Phc1 [37], Mel18 (Santa Cruz; sc-10744), Cbx2 [36], H3K27me3 (Millipore; 07-449), H3K4me3 (Millipore; 07-473), H2AK119u1 (E6C5; Millipore; 05-678; for ChIP), H2AK119u1 (Rabbit polyclonal; for ChIP) [28], H2AK119u1 (Rabbit polyclonal; Cell Signaling Technology; #8240; for western blot), H2A (Abcam; ab18255), RNAP (8WG16; Millipore; 05-952), Lamin B (Santa Cruz; sc-6216), mouse IgM (Millipore; 12-488), and Flag-tag (M2; Sigma; F1804).

**Stable transfection**

Ring1A<sup>2/2</sup>; Ring1B<sup>fl/fl</sup>; Rosa26::CreERT2 ESCs were stably transfected with tagged wild-type Ring1A, wild-type Ring1B, or mutated Ring1B. To establish stable transfectants, ESCs were electroporated (0.8 kV, 3 μF) with the respective expression vector and then selected for resistance to puromycin (1 μg/ml).

**Immunoprecipitation (IP) analysis**

Cells expressing each of tagged constructs were suspended in IP buffer [10 mM Tris-HCl (pH8.0), 1 mM EDTA, 140 mM NaCl, 0.4% NP-40, and 0.5 mM PMSF] and sonicated for several seconds. After centrifugation, the supernatant was collected, precleared with protein G Sepharose for 30 min at 4°C, and then incubated with anti-Flag antibody (M2) for 120 min at 4°C. The immune complexes were captured by protein G Sepharose for 60 min at 4°C. The Sepharose-bound proteins were washed with IP buffer, eluted in SDS sample buffer under reducing condition, separated on SDS-PAGE gels, and subjected to western blot analysis.

**Real-time PCR**

Quantitative real-time PCR was carried out with SYBR Green method and amplifications were detected with Mx3005P (Stratagene, La Jolla, CA, USA). The sequences of primers used in this study are shown in Text S1.

**Chromatin immunoprecipitation (ChIP) analysis**

ESCs were treated with 1% formaldehyde/PBS for 10 min at room temperature. Cells were washed with PBS, collected and...
resuspended in swelling buffer [20 mM Heps (pH 7.8), 1.5 mM MgCl2, 10 mM KCl, 0.1% NP-40, and 1 mM DTT] by pipetting and then kept on ice for 10 min. After Dounce homogenizing 10–20 times, the cells were centrifuged and then the pellets were resuspended in RIPA buffer [20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 140 mM NaCl, 1% Triton X-100, 0.1% SDS, and 0.1% deoxycholic acid] containing protease inhibitors and sonicated into fragments with an average length of 0.3–0.5 kb. After centrifugation, the supernatants were subjected to IP with specific antibodies as previously described [19,38]. For H2AK119u1-ChIP, pre-cleared chromatin (400 μg) was incubated with 50 μl of E6C5 antibody (overnight, 4°C) and then the chromatin-1st antibody complexes were immunoprecipitated with 2nd antibody (rabbit anti-mouse IgM) - preconjugated protein A dynabeads (Invitrogen). Purified immunoprecipitated and input DNA was quantified by real-time PCR, and, if necessary, was subjected to the linear amplification for ChIP-chip analysis.

ChIP-on-chip experiment

ChIP-on-chip analysis was carried out using the Mouse Promoter ChIP-on-chip Microarray Set (G4490A, Agilent, Palo Alto, Calif., USA). ESCs were subjected to ChIP assay using specific antibodies as described in the previous section. Purified immunoprecipitated and input DNA was subjected to T7 RNA polymerase-based amplification as described previously [39]. Labeling, hybridization and washing were carried out according to the Agilent mammalian ChIP-on-chip protocol (ver.9.0). Scanned images were quantified with Agilent Feature Extraction software under standard conditions. All of experiments were performed by using at least two biological replicates. The obtained data were analyzed as described in Text S1.

Gene expression microarray

Total RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) and purified with Qiagen RNeasy separation columns (Qiagen, Hilden, Germany). First strand cDNA was synthesized and hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 arrays (Affymetrix, Santa Clara, CA, USA) to assess and compare the overall gene expression profiles. The obtained data were analyzed as described in Text S1.

Three dimensional (3D)-DNA-FISH

3D-DNA-FISH with spatial preservation of chromatin architecture was performed as described previously [40]. Experimental details are described in Text S1.

Accession numbers

ChIP-chip and microarray data discussed in this publication have been deposited in NCBI’s Gene Expression Omnibus and is accessible through GEO Series accession number GSE38650.

Supporting Information

Figure S1  Examples of the distribution of H3K27me3, Ring1B, H2AK119u1 (E6C5), H2AK119u1 (Rabbit) and H2A at the promoter regions of representative genes. Plots display log2 values of unprocessed ChIP-enrichment ratios for all probes within a genomic region (ChIP-enriched versus input DNA). Green plots show the results from control ESCs (OHT–; OHT-untreated). Red plots for Ring1B, H2AK119u1 and H2A show the results from Ring1A/B-dKO ESCs (OHT–; 2 days after OHT treatment). Yellow plots for H3K27me3 show the results from Eed-KO ESCs. The transcription start sites (TSSs) are denoted by arrows. (PDF)

Figure S2  Local levels of H2AK119u1 and Ring1B at promoter regions of representative genes in Ring1A+/–; Ring1B+/+, Ring1A+/–; R26:CreERT2 ESCs before (−) or after (+) OHT treatment (day 2) were determined by ChIP and quantitative PCR. Those of H3K27me3 in wild-type and Eed-KO ESCs were also analyzed. Relative amount of immunoprecipitated DNA is depicted as a percentage of input DNA. Error bars represent standard deviation determined from at least three independent experiments. (B) Expression levels of representative genes in wild-type, Ring1A+/–, and Ring1A/B-dKO (2, 4, and 6 days after the start of OHT treatment) were determined by the quantitative RT-PCR. Expression levels were normalized to a Gapdh control and are depicted as fold changes relative to wild-type ESCs. Error bars represent standard deviation determined from at least three independent experiments. (PDF)

Figure S3  (A, B) ChIP-on-chip analysis showing the average of H2AK119u1 (E6C5) (A) and H2A (B) distributions at the promoter regions (from −5 kb to +5 kb relative to TSS) of Ring1B-bound and –unbound genes in Ring1A+/– (OHT–; green line) and Ring1A/B-dKO (OHT+; red line) ESCs. Enrichment of H2AK119u1 and H2A is expressed relative to input DNA. (C) Detection of ChIP-chip positive genes using approximated Gaussian distributions. Geometric mean of Ring1B and H2AK119u1 enrichment for each gene was subtracted from that in Ring1A+/– ESCs (OHT–) to provide histograms showing the distribution of Ring1B depletion-sensitive enrichment of Ring1B (green) and H2AK119u1 (E6C5; orange, rabbit polyclonal; brown). Geometric mean of H3K27me3 enrichment in Eed-KO ESCs for each gene was subtracted from that in wild-type ESCs to provide a histogram showing the distribution of PRC2 deficiency-sensitive enrichment of H3K27me3 (blue). Each histogram was approximated using two Gaussian distributions (pink), and the mean ±3σ value of the lower distribution was used as a threshold to determine positive genes (dotted blue). (D) Venn diagram representing the overlap of H2AK119u1 target genes identified by using two different antibodies (E6C5 and rabbit polyclonal, respectively). Numbers in parentheses represent the total number of genes occupied by each one. The probability of the overlap between these target genes is calculated and shown (Po; (E) Venn diagram representing the overlap of H3K27me3 target genes identified in this and a previous ChIP-seq study [Mikkelsen TS et al., Nature, 2007]. The probability of the overlap between these target genes is calculated and shown (Pr; (F) As in (E), but showing the overlap of Ring1B-bound genes identified in this and a previous ChIP-seq study [Ku M, Koche RP, Rheinbay E, Mendenhall EM, Endoh M, et al. (2008) Genomewide Analysis of PRC1 and PRC2 Occupancy Identifies Two Classes of Bivalent Domains. PLoS Genet 4(10): e1000242. doi:10.1371/journal.pgen.1000242]. (PDF)

Figure S4  Scatter plots demonstrating the overall correlation of occupancy levels between H3K27me3 and Ring1B for each gene. The geometric mean of H3K27me3 and Ring1B enrichment for each gene is depicted and Pearson’s correlation efficient (r) was calculated. H2AK119u1-positive and –negative genes are depicted as orange and blue dots, respectively. (PDF)

Figure S5  Gene ontology (GO) term analysis showing that genes related to transcription and/or development are highly over-represented among H2AK119u1-positive genes. H3K27me3-positive genes were classified according to the presence (+) or absence (−) of Ring1B and H2AK119u1 and the enrichment of...
respective GO terms in each subset of genes was calculated. The percentages of respective subsets of genes in a particular GO group are graphed along the right half of the x-axis, and p-values for the significance of over- or under-representation against total genes are graphed along the left half of the x-axis. Significant under-representation is indicated by asterisks beside the respective p-value bars.

**Figure S6** (A) Expression levels of Ring1A in wild-type, Ring1B/−/−, and Ring1A/−/− ESCs were determined by quantitative RT-PCR. Expression levels were normalized to a Gapdh control and are depicted as fold over wild-type ESCs. Error bars represent standard deviation determined from at least three independent experiments. (B) Immunoblot analysis of Ring1B, Ring1A, Flag, H2A-K119u1 and Lamin B protein levels in whole cell lysates of wild-type, Ring1B/−/−, and Ring1A/−/−; Ring1B/−/−; R26::CreERT2 ESC lines expressing mock or Flag-tagged Ring1A construct with or without OHT treatment (OHT+ and −, respectively). The locations of bands of endogenous and exogenous Ring1A are indicated by arrow heads. No sample was loaded on the lane indicated by an arrow. (C) Graph showing proliferation of the indicated ESC lines after OHT treatment. OHT-treated Ring1A/−/−; Ring1B/−/−; R26::CreERT2 ESC lines stably expressing mock or Flag-tagged Ring1A construct (2 transfectants; #1 & #4) were indicated as dKO and dKO*Ring1A, respectively. (D) Morphology of the indicated ESC lines. The images were acquired under a phase-contrast microscope. Scale bars indicate 200 μm. (E) Expression levels of Hoxa9, Hoxd11, Zic1 and Pax3 in wild-type, Ring1A/−/− and Ring1A/B−/−dKO ESCs expressing mock or Flag-tagged Ring1A construct (2 days after the start of OHT treatment) were determined by the quantitative RT-PCR. Expression levels were normalized to a Gapdh control and are depicted as fold over Ring1A/−/− ESCs. Error bars represent standard deviation determined from at least three independent experiments. (F) Venn diagram representing the overlap among genes occupied by Ring1B and Ring1A. Ring1B- and Flag-Ring1A-bound genes were determined by ChIP-on-chip experiments using Ring1B and Flag antibodies. Numbers in parentheses represent the total number of genes occupied by each one. (G) ChIP-on-chip analysis showing the average of 3×Flag-Ring1A distributions at the promoter regions (from −6 kb to +6 kb relative to TSS) in Ring1A/−/−; Ring1B/−/−; R26::CreERT2 ESCs with (OHT+ day2: green dotted line) or without (OHT−: orange line) OHT treatment. Enrichment of Flag-tagged Ring1A is expressed relative to input DNA. (H) Local levels of H2A-K119u1, Mel18, Flag-tagged Ring1A and Ring1B at promoter regions of Hoxd11 and Zic1 in the indicated ESC lines were determined by ChIP and quantitative PCR. Error bars represent standard deviations determined from three independent experiments.

**Figure S7** (A) Expression levels of undifferentiation and differentiation markers in wild-type, Ring1A/−/−, and Ring1A/B−/−dKO ESCs (2, 4, or 6 days after the start of OHT treatment) expressing mock, WT Ring1B, I53S Ring1B, or I53S Ring1B construct with OHT for 2 days to generate Ring1A/B−/−dKO ESCs expressing either of the constructs. Then, wild-type, Ring1A/−/−, and these OHT-treated ESCs were subjected to embryoid body formation and cultured for 3, 6, or 9 days in the absence of LIF and feeder cells. Expression levels were normalized to a Gapdh control and are depicted as fold changes relative to the undifferentiated wild-type ESCs. Error bars represent standard deviation determined from at least three independent experiments.

**Figure S8** A heat map with hierarchical clustering showing de-repressed (green), unchanged (black), or repressed (red) H2A-K119u1+ genes upon OHT treatment (day 2) in Ring1A/−/−; Ring1B/−/−; R26::CreERT2 ESCs stably expressing mock, WT, I53S, or I53A Ring1B construct was generated from the microarray data.

**Figure S9** Local levels of H3K27me3 at promoter regions of the representative target genes in wild-type and Ring1A−/−; Ring1B−/−; R26::CreERT2 ESCs stably expressing mock, WT, I53S, or I53A Ring1B construct before (−) or after (+) OHT treatment (day 2) were determined by ChIP and site-specific real-time PCR. The relative amount of immunoprecipitated DNA is depicted as a percentage of input DNA. Error bars represent standard deviation determined from at least three independent experiments.

**Figure S10** We arbitrarily divided H2AK119u1-positive genes into three groups based on the intergenic distances to the closest H2AK119u1-positive genes, and compared the level of de-repression upon OHT treatment in Ring1A/−/−; Ring1B/−/−; R26::CreERT2 ESCs expressing mock, wild-type, or mutant Ring1B construct using the microarray data. The distance between each H2AK119u1-positive gene was determined using the annotation of the reference mouse genome (NCBI version 36, mm8). The boxes show the median and interquartile range of the expression changes upon OHT treatment. Open circles indicate outliers. The differences of the expression changes between the indicated two groups were statistically evaluated using Mann-Whitney’s U-test, because the numbers of applied genes were too small to expect normal distribution.

**Table S1** Mean ChIP-chip enrichment score for each gene. Geometric mean of Ring1B and H2AK119u1 enrichment for each gene in Ring1A/B−/−dKO ESCs (OHT+) was subtracted from that in Ring1A−/− ESCs (OHT−) to provide Ring1B depletion-sensitive enrichment of Ring1B and H2AK119u1. Geometric mean of H3K27me3 enrichment in End-KO ESCs for each gene was subtracted from that in wild-type ESCs to provide PRC2 deficiency-sensitive enrichment of H3K27me3. The threshold between signal and noise in each ChIP-chip experiment was determined as described in Figure S3C, and the enrichment scores that are more than the threshold value are depicted as red.

**Text S1** Supporting Methods.

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Author Contributions
Conceived and designed the experiments: ME, HK. Performed the experiments: ME, TE, K-K. Analyzed the data: TA, EO, TT. Contributed reagents/materials/analysis tools: TI, RE, WAB, MV. Wrote the paper: ME, JS, WAB, BEB, HK.

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