

EZH1 Mediates Methylation on Histone H3 Lysine 27 and Complements EZH2 in Maintaining Stem Cell Identity and Executing Pluripotency

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SUMMARY

Trimethylation on H3K27 (H3K27me₃) mediated by Polycomb repressive complex 2 (PRC2) has been linked to embryonic stem cell (ESC) identity and pluripotency. EZH2, the catalytic subunit of PRC2, has been reported as the sole histone methyltransferase that methylates H3K27 and mediates transcriptional silencing. Analysis of *Ezh2*^{-/-} ESCs suggests existence of an additional enzyme(s) catalyzing H3K27 methylation. We have identified EZH1, a homolog of EZH2 that is physically present in a noncanonical PRC2 complex, as an H3K27 methyltransferase in vivo and in vitro. EZH1 colocalizes with the H3K27me₃ mark on chromatin and preferentially preserves this mark on development-related genes in *Ezh2*^{-/-} ESCs. Depletion of *Ezh1* in cells lacking *Ezh2* abolishes residual methylation on H3K27 and derepresses H3K27me₃ target genes, demonstrating a role of EZH1 in safeguarding ESC identity. *Ezh1* partially complements *Ezh2* in executing pluripotency during ESC differentiation, suggesting that cell-fate transitions require epigenetic specificity.

INTRODUCTION

Polycomb group (PcG) proteins serve in transcriptional control to preserve cellular identity and maintain epigenetic memory during metazoan development. Three PcG proteins, enhancer of zeste 2 (EZH2), embryonic ectoderm development (EED), and suppressor of zeste 12 homolog (SUZ12), comprise the core of Polycomb repressive complex 2 (PRC2), which mediates methylation on histone H3 lysine 27 (H3K27) (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002). EZH2, a SET domain-containing histone methyltransferase (HMTase), functions as the catalytic subunit of PRC2. EED contains five repeats of WD40 domains and functions as a scaffold protein

by physically linking EZH2 and histone H3 substrates (Tie et al., 2007). SUZ12 is required for nucleosome recognition and stability of EZH2 (Nekrasov et al., 2005; Pasini et al., 2004). In addition to the essential role of EED and SUZ12 in PRC2, histone-binding protein RBBP4 (or RbAp48/46) and the zinc finger protein AEBP2 bind to PRC2 and optimize its enzymatic activity in vitro (Cao and Zhang, 2004).

Mouse embryos lacking *Eed*, *Ezh2*, or *Suz12* show defects in gastrulation around 7–9 days postcoitus (Faust et al., 1995; O'Carroll et al., 2001; Pasini et al., 2004). In addition to essential roles in early development, PRC2 is required for diverse physiological functions including B lymphoid development, myogenic differentiation, imprinted X chromosome inactivation, and reprogramming of migrating primordial germ cells (Caretti et al., 2004; Kalantry et al., 2006; Seki et al., 2007; Su et al., 2003). Moreover, PRC2 members are associated with cancer (Valk-Lingbeek et al., 2004).

Three states of methylation, mono-, di-, and tri-, are observed on H3K27. H3K27me₃ is characteristic of PcG target genes and is functional in repression (Schwartz and Pirrotta, 2007). Genome-wide location analysis in embryonic stem (ES) cells has revealed that many PcG target genes encode transcription factors important in development (Boyer et al., 2006). Derepression of PcG target genes in *Eed*^{-/-} ESCs suggests a requirement of PRC2 in maintaining ESC pluripotency and plasticity. *Suz12* is implicated in regulating ESC differentiation (Pasini et al., 2007). However, *Ezh2*^{-/-} ESCs have not been isolated and *Ezh2*-deficient blastocysts have been reported to be impaired in outgrowth (O'Carroll et al., 2001). It has been speculated that EZH2 may have independent functions that do not involve H3K27 methylation (Martin and Zhang, 2005; Pasini et al., 2007; Rajasekhar and Begemann, 2007; Schwartz and Pirrotta, 2007).

Higher eukaryotes often have multiple HMTases with similar specificity, for example, six HMTases acting on H3K4 and seven H3K9 HMTases (Glaser et al., 2006; Martin and Zhang, 2005). There are two sequence homologs of EZH, including EZH2 and EZH1. Human *Ezh1* has been reported to interact with *Eed* by yeast two-hybrid and coimmunoprecipitation (coIP) assays (Jones et al., 1998; van Lohuizen et al., 1998). Expression of

murine *Ezh1* restores gene repression in *Saccharomyces cerevisiae* mutants impaired in telomeric silencing (Laible et al., 1997). However, whether EZH1 is part of PRC2 or contains enzymatic activity is unknown.

As EZH2 has been reported as the sole HMTase acting on H3K27, we have investigated the role of *Ezh2* in mouse ESCs in an effort to determine whether *Ezh2* is directly required for establishment and maintenance of ESC identity and pluripotency. We generated a conditional knockout allele of *Ezh2* and established *Ezh2*^{-/-} ESCs. Despite global loss of di- and trimethylation on H3K27, *Ezh2*^{-/-} ESCs preserve H3K27me3 at a subset of developmental genes and show robust H3K27me1, indicating that the EZH2-containing PRC2 complex is not the sole HMTase responsible for methylation on H3K27. Importantly, we have demonstrated that EZH1 is part of a noncanonical PRC2 complex that catalyzes addition of methyl groups on H3K27 and prevents the derepression of PRC2 target genes.

RESULTS

Residual H3K27me3 and Robust H3K27me1 in *Ezh2*^{-/-} ESCs

Previously, O'Carroll et al. failed to establish *Ezh2*^{-/-} ESCs through direct outgrowth of the inner cell mass, and hypothesized an essential role in ESC derivation (O'Carroll et al., 2001). To investigate the role of *Ezh2* in ESC pluripotency, we generated a conditional knockout allele of *Ezh2* by inserting loxP (*fl*) sites flanking exons 14 and 15, which encode part of the catalytic SET domain (Figure 1A). Deletion of exon 14-15 introduces a frameshift and subsequent termination mutation, which effectively deletes the SET domain.

By either sequentially targeting both alleles of the *Ezh2* locus or in vitro *Cre* excision of the *fl* allele of *Ezh2*^{fl/fl} (see the Supplemental Data available online), we were able to establish *Ezh2*^{-/-} ESCs, showing a nonessential role of *Ezh2* in ESC maintenance. Western blot analysis confirmed the absence of EZH2 (or a shorter form) in *Ezh2*^{-/-} cells (Figure 1B), suggesting that the deletion of the SET domain in vivo may adversely affect protein folding and result in degradation of truncated EZH2. Levels of EED and SUZ12 are normal in *Ezh2*^{-/-} cells. SUZ12 coimmunoprecipitates with EZH2 in *Eed*^{-/-} but not in *Ezh2*^{-/-} cells (Figure S1A).

Ezh2^{-/-} mouse embryos are deformed and underdeveloped as compared to wild-type littermates and die at ~E8.5 (Figure S1B). To reassess whether *Ezh2* is required for de novo establishment of ESCs, we sought to isolate null ESCs directly from knockout embryos. Out of 50 blastocysts obtained from mating of *Ezh2*^{+/-} mice, we established 28 ES lines, including 13 (46.4%) heterozygous lines, 8 (28.6%) wild-type, and 7 lines (25%) that are *Ezh2*^{-/-} (Figure S1C). Thus, contrary to expectations based on prior work, *Ezh2* is required neither for establishment nor maintenance of ESCs. *Ezh2*^{-/-} ESCs generated by the above methods are indistinguishable. The discrepancy between O'Carroll et al. and our work may reflect improved contemporary methods for derivation of ESC lines from blastocysts. It is also possible that in the O'Carroll et al. allele an in-frame fusion of LacZ with the first 200 amino acids of EZH2 (which contain an

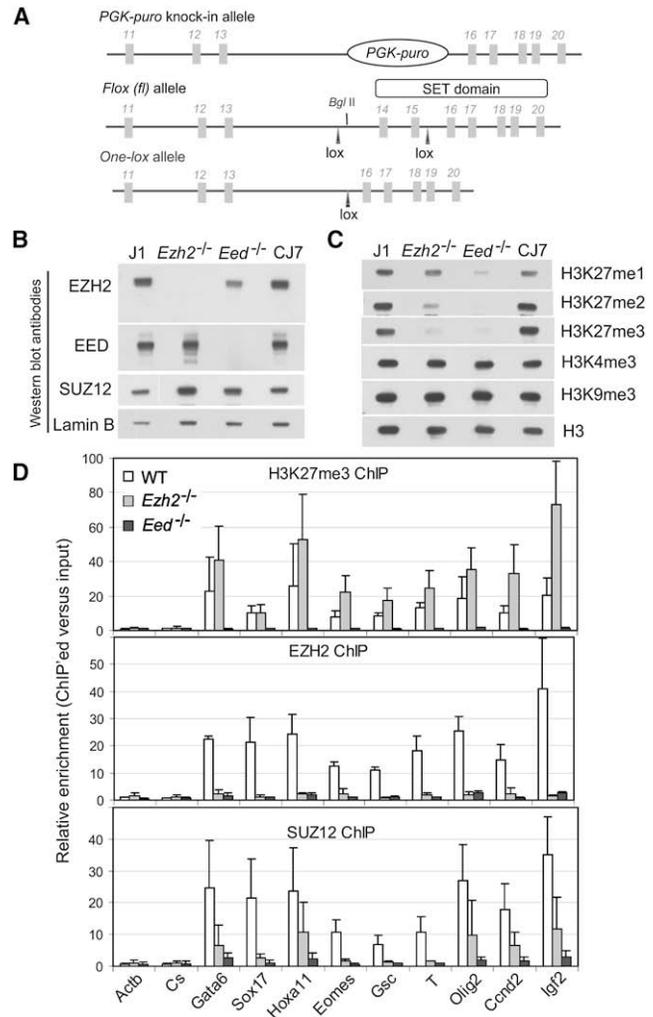


Figure 1. Altered Methylation on H3K27 in *Ezh2*^{-/-} Cells

(A) Targeting strategy to generate *Ezh2* conditional and knockout alleles. Exon number is indicated above each exon shown in boxes. Lines represent introns. (B and C) Western blot analysis of nuclear extracts (B) and histones (C) isolated from wild-type (J1 and Cj7) and mutant (*Ezh2*^{-/-} and *Eed*^{-/-}) ESCs. (D) ChIP-qPCR analysis of H3K27me3, EZH2, and SUZ12 binding at promoters of PcG target genes in wild-type (WT), *Ezh2*^{-/-}, and *Eed*^{-/-} ESCs. The y axis shows relative enrichment of ChIPed DNA versus the inputs. The *Actb* promoter and an intergenic region (*Cs*) serve as negative controls. Error bars represent standard deviations of relative enrichments.

EED-interacting domain) generated a dominant-negative protein or perturbed functions beyond PRC2.

In contrast to *Eed*^{-/-} cells that lack di- and trimethylation on H3K27 and show dramatic reduction of H3K27me1, *Ezh2*^{-/-} cells show decreased H3K27me2 and H3K27me3, yet retain robust H3K27me1 (Figure 1C), indicating an additional HMTase(s) catalyzing H3K27me1 in an EED-dependent manner. To confirm the loss of the H3K27me3 mark on individual PcG targets (Boyer et al., 2006), we performed chromatin immunoprecipitation (ChIP) and quantitative PCR (qPCR) (Figure 1D). Surprisingly, the H3K27me3 mark is still significantly enriched at promoters of PcG target genes in *Ezh2*^{-/-} cells, but is entirely lost in

Eed^{-/-} cells. The loss of EZH2 binding at these loci rules out possible residual activity of EZH2 in *Ezh2*^{-/-} cells, indicating that an additional HMTase(s) catalyzes H3K27me3. In addition, SUZ12 binding at PcG sites is reduced in *Ezh2*^{-/-} cells but abolished in *Eed*^{-/-} cells. It was reported that G9a, the HMTase catalyzing methylation on H3K9, might mediate H3K27 methylation (Tachibana et al., 2001). However, we observed no binding of G9a at PcG target loci in either wild-type or *Ezh2*^{-/-} ESCs (data not shown). Therefore, robust H3K27me1 and residual H3K27me3 imply the presence of an additional HMTase(s) beyond EZH2 acting on H3K27.

EZH1 Interacts with Core Components of PRC2

To identify another HMTase(s) associated with EED, we generated ESC lines stably expressing subendogenous levels of EED containing a biotin tag and a FLAG epitope at its amino terminus (herein referred to as bFEED; Figures S2A–S2C). Five components of PRC2 are pulled down by FLAG-antibody and streptavidin-mediated tandem colP and identified by mass spectrometry (MS) sequencing (Figure 2A). Interestingly, in addition to EZH2 (represented by a total of 224 peptides), bFEED also pulls down EZH1 (72 peptides), a sequence homolog of EZH2 (Table S1). More peptides corresponding to EZH2 than EZH1 are recovered. Although the numbers of peptides identified by MS analysis provide only a rough estimate of protein abundance, consistent differences in peptide numbers in multiple pull-down experiments suggest a greater abundance of EZH2 than EZH1 polypeptides in association with EED.

By using a similar strategy, we constructed ESC lines stably expressing subendogenous levels of bFEZH2 or bFEZH1 (Figures S2B and S2C). Core components of PRC2 including SUZ12, EED, RBBP4, and AEBP2 are pulled down with both EZH2 and EZH1 (Figure 2A; Table S1). However, neither bFEZH2 nor bFEZH1 tandem pull-down identifies any peptides representing each other, suggesting the presence of EZH2 and EZH1 in alternative protein complexes. In addition, five core components of the EZH1-containing complex have similar stoichiometry as found in the canonical EZH2-containing PRC2 complex (Figure 2A).

To confirm these data, we performed one-step streptavidin-mediated colP of bFEZH1. EZH2 is detected in the colP fraction as a weak protein band with about half of the intensity as the input. However, both EED and SUZ12 show ~5-fold higher intensity in the colP fraction than in the inputs (Figure 2B). In addition, bFEZH1 is pulled down with endogenous EZH2 (Figure 2C). Therefore, the interaction of EZH2 and EZH1 is evident in a less stringent one-step colP but not in tandem purification, suggesting that EZH1 and EZH2 may indirectly interact through their binding to EED and SUZ12. Thus, we have identified an alternative PRC2 complex containing a different subunit, EZH1, which might provide catalytic activity.

To examine the integrity of PRC2 complexes in the absence of EZH2, we generated *Ezh2*^{-/-} ESCs stably expressing subendogenous bFEZH1 (Figure S2B). Although bFEZH1 efficiently pulls down SUZ12 in the absence of EZH2, the amount of EED pulled down by bFEZH1 in *Ezh2*^{-/-} cells is about 10-fold less than that in wild-type cells (Figures 2B and 2D). Thus, the interaction between EED and bFEZH1 appears dramatically reduced,

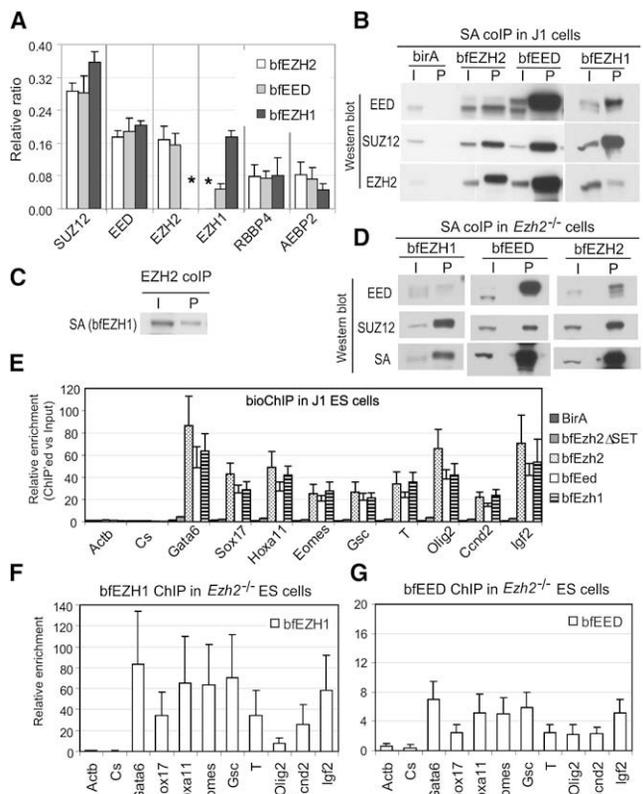


Figure 2. EZH1 Interacts and Colocalizes with EZH2 and EED at PcG Target Loci

(A) Proteins interacting with bFEZH2, bFEED, or bFEZH1 were pulled down by FLAG and biotin-streptavidin (SA)-mediated tandem colP and subsequently identified by MS sequencing. The y axis shows the relative ratio of peptides representing an identified protein (x axis) in the total number of peptides identified by MS. Error bars represent standard deviations of ratios of peptides identified by MS in independent pull-down experiments. Neither bFEZH2 nor bFEZH1 pulls down the other (indicated by asterisks).

(B) One-step SA colP and western blotting of J1 ESCs expressing birA alone or with any of bFEZH2, bFEZH1, or bFEED. I, inputs; P, colP fraction.

(C) bFEZH1 is colP with endogenous EZH2.

(D) SA colP and western blot analysis of *Ezh2*^{-/-} ESCs expressing bFEZH1, bFEED, or bFEZH2.

(E) Colocalization of bFEZH1 with bFEZH2 and bFEED at promoters of PcG targets shown by bioChIP-qPCR analysis in J1 ESCs.

(F and G) The binding of bFEZH1 (F) and bFEED (G) to PcG target loci in *Ezh2*^{-/-} ESCs.

Error bars in (E)–(G) represent standard deviations of relative enrichments.

although detectable in *Ezh2*^{-/-} cells. This result suggests a possible role of EZH2 in regulating EZH1-containing PRC2 by promoting EZH1-EED protein interactions.

EZH1 Colocalizes with H3K27me3 in Wild-Type and *Ezh2*^{-/-} ESCs

To investigate the role of EZH1 within the alternative PRC2 complex, we performed biotin-streptavidin-mediated ChIP (bio-ChIP) and qPCR to analyze EZH1 association with chromatin. bFEZH2 and bFEED, but not birA control and SET domain-deleted EZH2 (bFEZH2ΔSET), show enrichment on PcG target genes

(Figure 2E), demonstrating the specificity of the bioChIP procedure. Interestingly, bfEZH1 shows significant enrichment at PcG target loci not only in wild-type but also in *Ezh2*^{-/-} cells (Figures 2E and 2F). H3K27me3 modification on PcG target loci is unaltered irrespective of *bfEzh1* expression (Figures S2D and S2E), suggesting that exogenous bfEZH1 does not disturb the balance of methylation. In addition, *Ezh2*^{-/-} cells do not upregulate *Ezh1* transcripts to compensate the loss of *Ezh2* (Figure S2B). Moreover, bfEED enrichment at PcG loci in *Ezh2*^{-/-} cells is dramatically decreased compared to that in wild-type cells (Figure 2G), a finding consistent with an attenuated interaction of EED and bfEZH1 in the absence of EZH2.

We then determined whether EZH1 colocalizes with H3K27me3 in a genome-wide manner and where the residual H3K27me3 mark is located in *Ezh2*^{-/-} ESCs. Whole-genome ChIP-chip analysis of H3K27me3 detects 7045 peaks ($p < 1e^{-7}$) which are associated with 3012 genes in wild-type ESCs, but 1997 peaks associated with 1077 genes in *Ezh2*^{-/-} cells, suggesting a 65% or 72% loss of H3K27me3 at peak or gene level, respectively (Figure 3A; Tables S2–S6). BioChIP-chip analysis of bfEZH1 reveals 7478 peaks ($p < 1e^{-7}$) associated with 3851 genes, overlapping with 92% of H3K27me3 targets in wild-type cells. The average probe signals of H3K27me3 and bfEZH1 are dramatically reduced in *Ezh2*^{-/-} cells (Figure S3B). *Ezh2*^{-/-} cells display a 70% or 68% loss of bfEZH1 binding at peak or gene level, respectively. Importantly, target genes of H3K27me3 and bfEZH1 in *Ezh2*^{-/-} cells are largely overlapping (79%–84%).

We observed that H3K27me3 and bfEZH1 binding on DNA was often broad in wild-type ESCs covering distal, proximal promoters and gene bodies, but became focused at proximal promoters near transcription start sites in *Ezh2*^{-/-} ESCs (Figure 3B; Figure S3A). Indeed, the widths of corresponding H3K27me3 or bfEZH1 peaks in *Ezh2*^{-/-} cells are narrower than those in wild-type cells despite similar positioning on chromatin (Figures 3C and 3D). Therefore, loss of H3K27me3 or bfEZH2 binding is underestimated if the narrowed binding pattern of these marks in mutant cells is not taken into account.

Consistent with ChIP-chip, enrichment patterns of bfEZH1 and H3K27me3 in a few target regions shown by ChIP-qPCR are very similar (Figures 3B–3F). For example, in *Ezh2*^{-/-} cells, genes such as *Cdkn2a* and *Wnt9a* lose the association of both H3K27me3 and bfEZH1 marks, whereas enhancer or 3'UTR regions of *Eomes*, *Gsc*, and *T* show reduced binding. H3K27me3 and bfEZH1 signals appear higher at promoters of *Ccnd2*, *Gata6*, *Hoxa11*, and *Igf2* in *Ezh2*^{-/-} cells than in wild-type cells. However, only 91 out of 1077 genes that retain the H3K27me3 mark in *Ezh2*^{-/-} cells show higher ChIP-chip signals (Table S7), suggesting that greater enrichment of H3K27me3 in selected regions is not general to all targets.

Thus, colocalization of EZH1 and H3K27me3 on chromatin in both wild-type and *Ezh2*^{-/-} ESCs implies a direct role of EZH1 in formation of the H3K27me3 mark. Loss of *Ezh2* negatively affects EZH1 association with chromatin, indicating that DNA binding ability of the EZH1-interacting complex is at least partially dependent on EZH2. Furthermore, we found that over 94% target genes revealed by bfEZH2 bioChIP-chip analysis were co-occupied by bfEZH1 and H3K27me3 (data not shown),

suggesting that EZH2 and EZH1 bind overlapping sets of target genes, perhaps in a coordinated manner.

To gain insights into the functions of genes that preserve or lose the H3K27me3 mark in *Ezh2*^{-/-} ESCs, we performed gene ontology (GO) analysis. The overlapping target genes of H3K27me3 and bfEZH1 in wild-type (WT) ESCs are referred to as WT^{H3K27me3} genes, representing overall target genes marked by H3K27me3 (Table S8). Consistent with previous reports (Boyer et al., 2006; Lee et al., 2006), WT^{H3K27me3} genes are enriched in development-related GO terms including cell-fate commitment, embryonic development, and transcription regulator activity, and so forth (Figure 3G). Interestingly, of WT^{H3K27me3} genes, the subset of genes (referred to as *Ezh2*^{-/-}, H3K27me3⁺) that retain both H3K27me3 and bfEZH1 marks in *Ezh2*^{-/-} cells shows even higher enrichment scores on developmental terms. On the contrary, the subset (referred to as *Ezh2*^{-/-}, H3K27me3⁻) of genes that lose both marks in *Ezh2*^{-/-} cells shows no enrichment on development-related GO terms, but is enriched in cyclic nucleotide metabolic process, potassium ion binding, and metal ion transport. In comparison, none of the three sets shows significant enrichment in metabolic processes. Therefore, *Ezh2*^{-/-} ESCs preferentially preserve EZH1 binding and the H3K27me3 mark on genes related to development, suggesting a possible role of EZH1 in ESC function.

***Ezh2* Is Dispensable for the Maintenance of ESC Identity**

To determine functional consequences of residual H3K27me3 possibly mediated by EZH1 in maintaining ESC identity, we compared microarray expression profiles of wild-type and mutant ESCs and performed gene set enrichment analysis (GSEA) on three predefined subsets of H3K27me3 target genes that are activated at day 6 differentiation of wild-type ESCs including WT^{H3K27me3}, day 6 up genes; *Ezh2*^{-/-}, H3K27me3⁻, day 6 up genes; and *Ezh2*^{-/-}, H3K27me3⁺, day 6 up genes (Table S9). All three gene sets remain suppressed in *Ezh2*^{-/-} ESCs despite substantial reduction in the H3K27me3 mark, but are upregulated in *Eed*^{-/-} ESCs which lose all H3K27me3 (Figures 4A and 4B; Figure S4).

Reverse-transcription and qPCR analysis confirm the loss of *Ezh2* transcripts in *Ezh2*^{-/-} cells and dramatic upregulation of differentiation-induced, H3K27me3 genes in *Eed*^{-/-} cells but not in *Ezh2*^{-/-} ESCs, despite normal expression of pluripotency markers in both mutant cells (Figures 4C and 4D). To rule out the possibility of derepression as a consequence of partial differentiation due to the unstable nature of *Eed*^{-/-} ESCs, we analyzed a number of single colonies grown on feeders that showed undifferentiated ESC morphology. Of 52 H3K27me3 and PRC2 target genes analyzed, 28 genes are upregulated in *Eed*^{-/-} cells as compared to wild-type ESCs (Figure S5), suggesting that derepression is intrinsic to the loss of *Eed*. Thus, complete loss of the H3K27me3 mark in the absence of *Eed* leads to global derepression of polycomb genes, whereas *Ezh2*^{-/-} ESCs appear to be normal in this respect. These data suggest that the repression of developmental genes by residual H3K27me3 mediated by EZH1 may contribute to the maintenance of ESC identity in the absence of *Ezh2*.

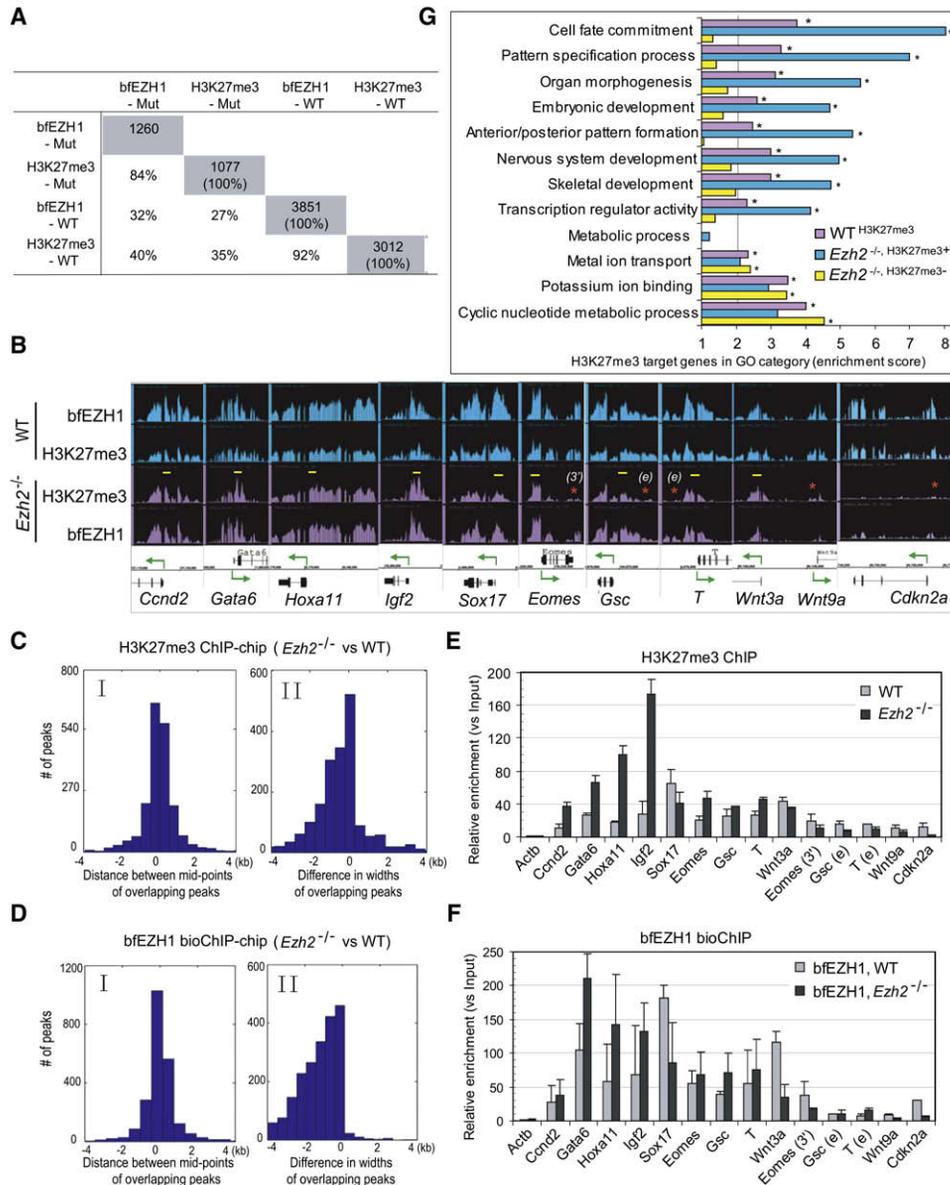


Figure 3. bfEZH1 Colocalizes with H3K27me3 on Chromatin

(A) The overlapping percentages of target genes identified by H3K27me3 and bfEZH1 ChIP-chip in wild-type (WT) and *Ezh2*^{-/-} mutant (Mut) ESCs. The total numbers of target genes ($p < 1e^{-7}$) are highlighted in gray.

(B) Representative view of bfEZH1 and H3K27me3 occupancy at various loci in WT (shown in blue) and *Ezh2*^{-/-} ESCs (in purple) by the Affymetrix Integrated Genome Browser. Arrows indicate transcription starts and directions. Yellow bars indicate regions retaining strong binding in *Ezh2*^{-/-} cells. Asterisks in red indicate regions losing ChIP peaks in *Ezh2*^{-/-} cells. qPCR primers used in (E) and (F) were designed in the above marked regions.

(C and D) Comparisons of peak positions and widths of H3K27me3 (C) or bfEZH1 (D) ChIP-chip in *Ezh2*^{-/-} and wild-type ESCs. Panel I shows deviations of midpoints of corresponding ChIP peaks in *Ezh2*^{-/-} and wild-type cells. The distribution is centered at 0 and the deviations, in large part, are within 1 kb on both sides. Panel II shows the distribution of width differences of corresponding ChIP peaks in *Ezh2*^{-/-} and wild-type cells. The distribution is heavily skewed to the left, indicating less broad peaks in *Ezh2*^{-/-} cells than in wild-type cells in general.

(E and F) qPCR verification of H3K27me3 ChIP (E) in WT and *Ezh2*^{-/-} ESCs without exogenous *bfEzh1* and bfEZH1 bioChIP (F) in cells expressing *bfEzh1*. Error bars represent standard deviations of relative enrichments.

(G) Gene ontology (GO) analysis of H3K27me3 target genes. GO terms with an enrichment score larger than 2 (i.e., 1-fold above genome background) and a p value less than $1e^{-6}$ are considered significantly enriched and are indicated by asterisks. The WT^{H3K27me3} gene set comprises all of the H3K27me3 target genes. *Ezh2*^{-/-}, H3K27me3⁺ or *Ezh2*^{-/-}, H3K27me3⁻ genes represent a subset of H3K27me3 target genes which retain or lose this mark in *Ezh2*^{-/-} cells, respectively.

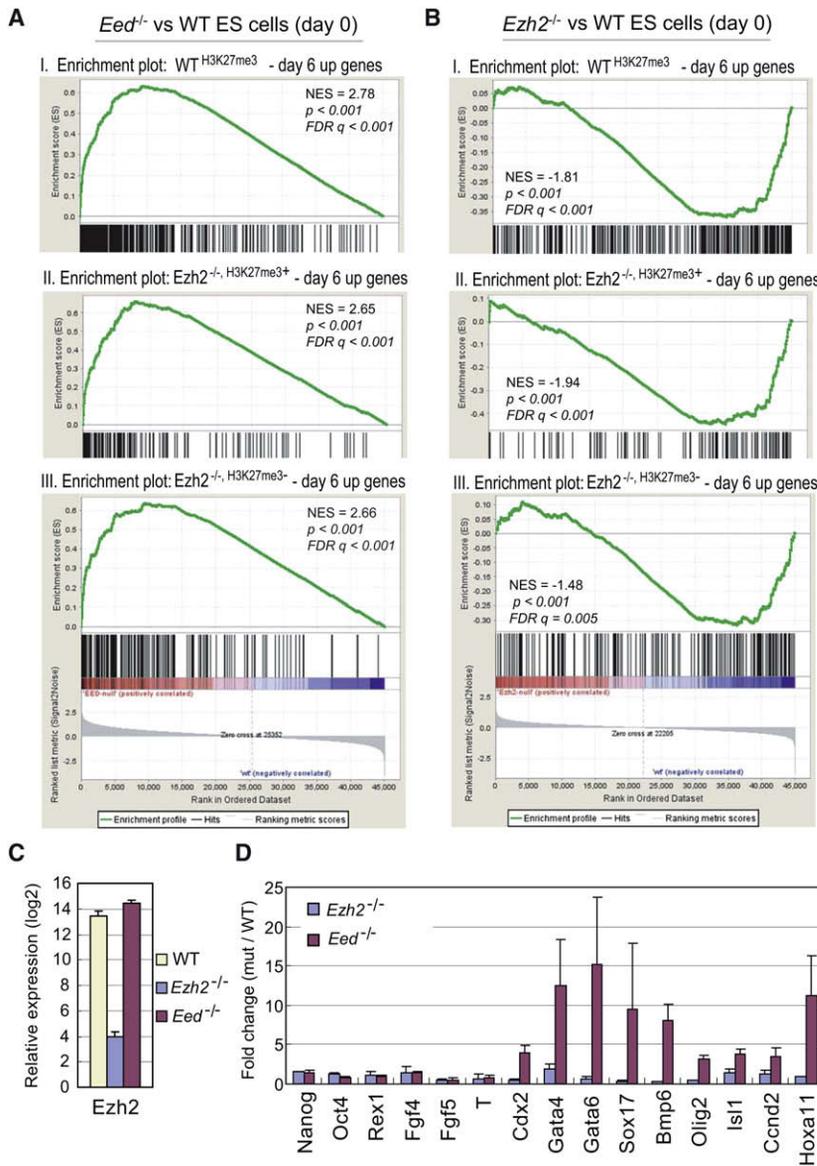


Figure 4. *Ezh2* Is Dispensable for the Maintenance of ESC Identity

(A and B) GSEA profiles of three subsets of H3K27me₃ targets by comparing undifferentiated *Eed*^{-/-} (A) or *Ezh2*^{-/-} ESCs (B) to wild-type (WT) ESCs. Panel I: WT^{H3K27me₃}, day 6 up genes; panel II: *Ezh2*^{-/-}, H3K27me₃⁺, day 6 up genes; panel III: *Ezh2*^{-/-}, H3K27me₃⁻, day 6 up genes. All three subsets show positive normalized enrichment scores (NES) in *Eed*^{-/-} ESCs but negative NES in *Ezh2*^{-/-} ESCs, suggesting derepression in *Eed*^{-/-} ESCs but repression in *Ezh2*^{-/-} ESCs. (C) The *Ezh2* transcript is reduced ~1000-fold in *Ezh2*^{-/-} cells compared to that in WT and *Eed*^{-/-} cells. (D) Fold changes of gene expression in mutants versus wild-type cells. Error bars represent standard deviations of relative expression (C) or fold changes (D).

(Figure 5B), demonstrating a role of EZH1 in catalyzing mono-, di-, and trimethylation on H3K27 in the absence of *Ezh2*. Moreover, in *Ezh2*^{-/-} cells depleted of *Ezh1*, the H3K27me₃ mark is dramatically reduced at PcG target loci (Figure 5C; Figure S6D), and PcG targets are upregulated, despite normal expression of pluripotency markers (Figure 5A; Figure S6B). Therefore, cells lacking *Ezh2* and depleted of *Ezh1* resemble *Eed*^{-/-} ESCs. These findings demonstrate an *Eed*-dependent, complementary role of *Ezh1* in suppressing differentiation and maintaining the identity of *Ezh2*^{-/-} ESCs.

EZH1 Is a Histone Methyltransferase

To directly evaluate the enzymatic activity of EZH1, we coexpressed FLAG-tagged EZH1 with EED, SUZ12, RBBP4, and AEBP2 in Sf21 cells. These four proteins are colPeped with FLAG-tagged EZH1, indicating their existence in a core complex (Figures S7A and S7B). The EZH1 complex is capable of transferring [³H]methyl to core histones and shows enzymatic activity at a level roughly comparable to the EZH2 complex (Figure 5D).

We next determined how the other four components of PRC2 affect the catalytic activity of EZH1. Both EZH1 and EZH2 require the presence of EED and SUZ12 for enzymatic activity (Figure 5E). The presence of RBBP4 and AEBP2 significantly enhances the activity of both enzymes. Similar to EZH2 protein, EZH1 is unstable and does not interact with RBBP4 and AEBP2 outside of PRC2 (Figure S6C). Coexpression of EED or SUZ12 greatly enhances the stability of EZH1 and EZH2. SUZ12, but not EED, is required for the interaction of either EZH protein with RBBP4 and AEBP2. Therefore, EZH1 and EZH2 show

EZH1 Complements EZH2 in Mediating H3K27 Methylation and Gene Repression

To assess whether EZH1 is responsible for methylation on H3K27 and required for repression of developmentally regulated PcG target genes in *Ezh2*^{-/-} cells, we used RNA interference (RNAi) to knock down *Ezh1* expression. Compared to cells treated with mock RNAi, *Ezh1* RNA transcripts decrease 50%–70% in *Ezh1* RNAi (Figure 5A; Figures S6A and S6B). In wild-type cells depleted of *Ezh1*, no change in global methylation on H3K27 is observed despite modest reduction of the H3K27me₃ mark at PcG target loci (Figures S6C and S6E). In addition, *Ezh1* RNAi did not affect the expression of PcG target genes (Figure S6A), supporting EZH2 as the major histone methyltransferase acting on H3K27.

Interestingly, all three forms of methylation on H3K27 are barely detected in *Ezh2*^{-/-} cells treated with *Ezh1* RNAi

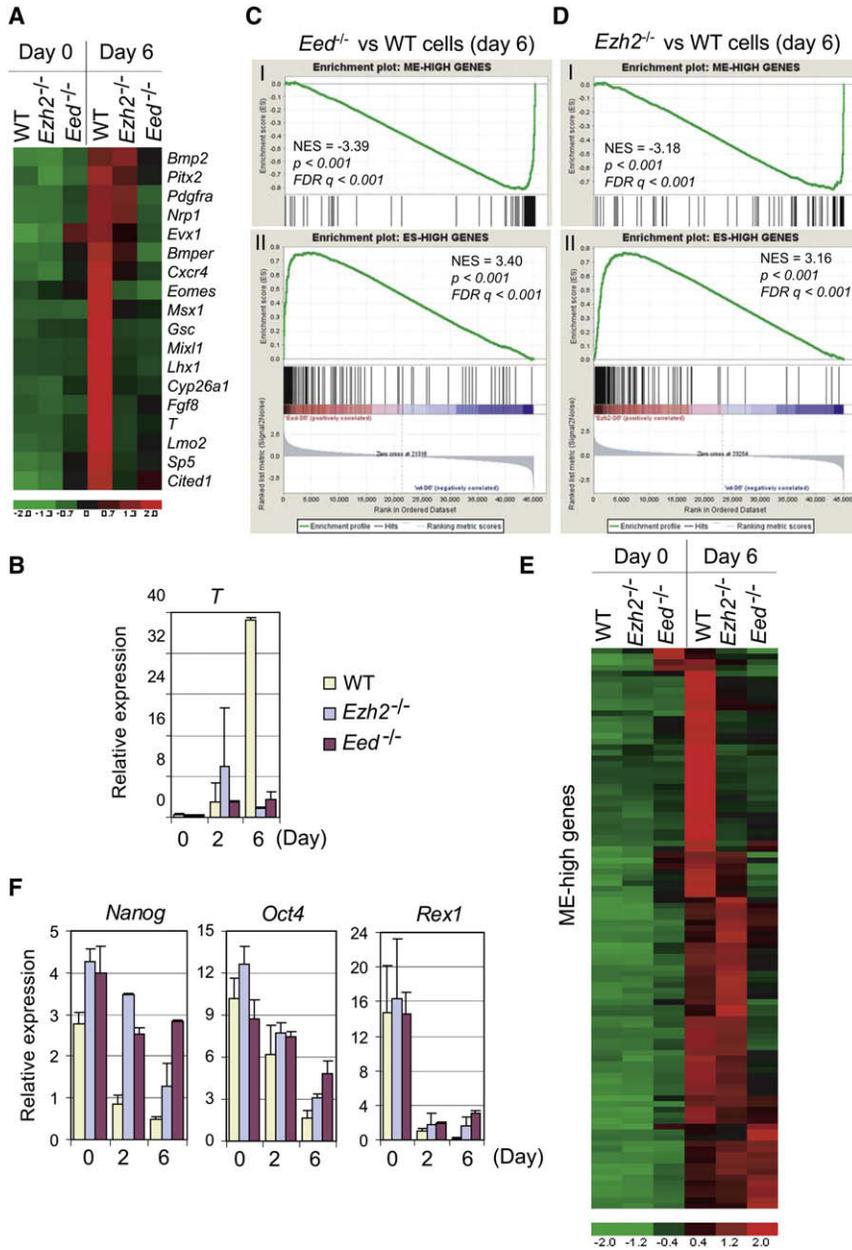


Figure 6. *Ezh2* and *Eed* Are Required for Mesoendodermal Differentiation

(A) Heat map showing impaired activation of known mesodermal markers in *Ezh2*^{-/-} and *Eed*^{-/-} cells as compared to wild-type (WT) cells. (B) RT-qPCR analysis of *T* expression along ESC differentiation by LIF withdrawal. (C and D) GSEA profiles of ME-high (panel I) and ES-high (panel II) genes by comparing day 6 differentiated *Eed*^{-/-} (C) or *Ezh2*^{-/-} (D) cells to wild-type cells. (E) Heat map of ME-high genes in wild-type and mutant ESCs. (F) RT-qPCR analysis of pluripotency markers along ESC differentiation. Error bars represent standard deviations of relative expression.

High correlation between EZH1 and H3K27me3 target genes suggests possible roles of EZH1 in both initiating and spreading the H3K27me3 mark. We cannot formally exclude, however, the possibility that exogenously introduced bfEZH1 may overrepresent EZH1 protein. Against this possibility, however, we note that exogenously introduced bfEZH1 does not rescue the methylation defect shown in the absence of endogenous EZH2, suggesting that we have not appreciably perturbed protein equilibrium in vivo. Extensive loss of H3K27me2 and me3 in *Ezh2*^{-/-} cells and no global methylation change on H3K27 in wild-type cells depleted of *Ezh1* point to EZH2 as the major H3K27 HMTase in ESCs (Figure 7).

Consistent with H3K27me1 as the primary product of the EZH2-PRC2 reaction in vitro (Sarma et al., 2008), EZH2 is also involved in monomethylation in vivo. However, EZH1 can fully compensate the loss of EZH2 in forming H3K27me1 (Figure 7B). A recent genome-wide study

conducted in human T cells revealed an inverse correlation between H3K27me1 and me3 (Barski et al., 2007). The H3K27me1 mark is associated with actively transcribed regions, whereas H3K27me3 is enriched at silenced promoters. As both EZH1 and EZH2 colocalize with H3K27me3 on chromatin, we suspect the EZH proteins may be regulated to leave the sites after the addition of one methyl group to certain nucleosomes (Figure 7). Monomethylation may occur on free H3 nucleosomes prior to their assembly into nucleosomes during DNA replication. It is unclear whether H3K27me1 is simply an intermediate state or is functionally important.

Previous studies have shown that at limiting concentrations, EED may be preferentially assembled into a monomethylase

DISCUSSION

Complementary Roles of *Ezh1* and *Ezh2* in H3K27 Methylation

Mammalian *Ezh1* has been an “orphan” since its discovery a decade ago. EZH1 has not previously been identified within a Polycomb repressive complex by biochemical purifications or coimmunoprecipitation in HeLa or 293T cells (Cao et al., 2002; Kuzmichev et al., 2002). A trivial explanation is possibly low-level expression of *Ezh1* in these cells. Here we have demonstrated in vivo association of EZH1 with PRC2. The noncanonical EZH1-mediated PRC2 coexists with the classic EZH2-mediated PRC2 in catalyzing H3K27me3 at overlapping genes (Figure 7).

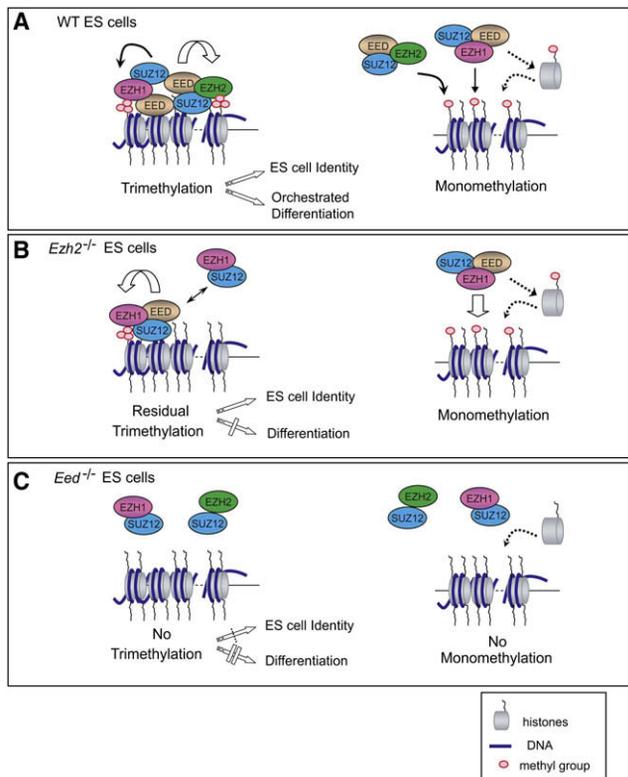


Figure 7. Complementary but Nonredundant Roles of EZH1 and EZH2 in Mediating Methylation on H3K27 and ESC Function

(A) In wild-type (WT) ESCs. Both EZH2 and EZH1 bind to EED and SUZ12, forming alternative but interacting PRC2 complexes containing HMTase activity. EZH2-mediated PRC2 is more abundant in ESCs and plays a major role in forming H3K27me3, which is required for ESC identity and proper differentiation. Both EZH1- and EZH2-containing PRC2 complexes may mediate monomethylation on H3K27 in a nontargeted manner by either acting transiently on nucleosomes across the genome or on free H3 histones prior to nucleosomal assembly during DNA replication.

(B) In *Ezh2*^{-/-} ESCs, EZH1-PRC2 mediates monomethylation and residual trimethylation on H3K27. EED may regulate the EZH1-PRC2 complex in an EZH2-dependent manner. EZH1 mediates H3K27me3 on a set of developmental genes and prevents derepression of PRC2 target genes from global loss of the H3K27me3 mark in *Ezh2*^{-/-} ESCs. Hereby, EZH1 complements EZH2 in the maintenance of ESC identity. However, EZH1-PRC2 cannot fully rescue defects in mesoendodermal development during *Ezh2*^{-/-} cell differentiation, suggesting nonredundant functions of these two PRC2 complexes in lineage commitment.

(C) *Eed*^{-/-} ESCs lack all levels of methylation on H3K27, indicating the essential role of EED in EZH1- or EZH2-mediated PRC2 complexes. Complete loss of H3K27me3 in the absence of *Eed* confounds ESC identity by derepressing a set of differentiation-regulated PRC2 target genes, and blocks the proper execution of pluripotent programs.

complex and the removal of H3K27me1 may occur passively (Chamberlain et al., 2008; Montgomery et al., 2005). We show detectable, but significantly reduced, association between bEZH1 and EED in *Ezh2*^{-/-} cells. Consistent with the essential role of EED in all methylation on H3K27, our data support EED as the rate-limiting factor regulating both EZH1- and EZH2-containing PRC2 complexes. It is also possible that posttranslational

modifications or conformation switches might affect the ability of EED to interact with the EZH proteins.

Robust H3K27me1 in *Suz12*^{-/-} cells suggests a dispensable role of SUZ12 in mediating H3K27me1 (Pasini et al., 2004). Intriguingly, SUZ12 is required for the HMTase activity of both EZH1 and EZH2 in vitro. It is possible that another gene(s) or *Suz12* homolog(s) in the mammalian genome are yet to be identified and might compensate for the loss of *Suz12* in vivo. Of potential relevance, in *Caenorhabditis elegans*, EZH2 and EED homologs are present in a complex that retains H3K27 methyltransferase activity yet lacks a SUZ12 homolog (Bender et al., 2004).

H3K27me3 and PRC2 Complexes in Stem Cell Maintenance

Our success in isolating *Ezh2*^{-/-} ESCs directly from knockout blastocysts, and by inactivating *Ezh2* in established ESC lines, demonstrates that *Ezh2* is dispensable for both establishment and maintenance of mouse ESCs. Our results are consistent with postimplantation lethality and the ability of pathenogenetic embryos lacking maternal and embryonic *Ezh2* to develop to the blastocyst stage in vitro (Erhardt et al., 2003; O'Carroll et al., 2001).

Interestingly, deletion of *Ezh2* does not result in transcriptional activation of approximately two thirds of H3K27me3 target genes that lose this mark. EZH1 in *Ezh2*^{-/-} ESCs preferentially binds to and transfers the repressive H3K27me3 mark onto developmental genes whose expression promotes differentiation. Inactivation of EZH1 in *Ezh2*^{-/-} ESCs resembles the loss of *Eed*, leading to the derepression of a set of PcG target genes. As complete loss of H3K27me3 leads to epigenetic instability where some genes are expressed and some are not, EZH1 thus provides an additional layer of protection to safeguard the pluripotent state by preventing aberrant gene expression.

We, and others, have observed that *Eed*^{-/-} cells are prone to differentiate, even when maintained on feeders (Boyer et al., 2006; Chamberlain et al., 2008). In addition, we have observed that *Eed*^{-/-} ESCs grow more slowly than *Ezh2*^{-/-} cells. Therefore, the maintenance of H3K27me3 on a subset of development-related PcG genes is crucial to prevent the expression of conflicting epigenetic programs which may be detrimental to self-renewal and survival (Figure 7). Of note, the extent of derepression is insufficient to drive full differentiation. *Eed*^{-/-} ESCs thus retain limited capacity to self-renew and differentiate upon stimulation. We speculate that *Eed*^{-/-} ESCs may reflect an epigenetic state intermediate between undifferentiated and partially differentiated ESCs.

EZH2 is essential for the proliferation of fibroblasts and tumor cells (Valk-Lingbeek et al., 2004), but is dispensable for ESC survival and proliferation, suggesting the lack of epigenetic restrictions in these cells. ESCs have a globally relaxed, hyperdynamic chromatin structure (Niwa, 2007). This permissive chromatin environment may be counterbalanced by repressive mechanisms, such as dynamic methylation at H3K9 and H3K27, repressive activities of NuRD, PRC2, and PRC1 complexes, and proteasomal degradation of cryptic transcripts in ESCs (Szutorisz et al., 2006; Wang et al., 2006). Together with transcriptional repressor activities of pluripotency regulators (Boyer et al., 2005), multiple

repressive regulatory mechanisms in ESCs cooperate to keep developmental genes silenced but poised for eventual upregulation as needed for cell lineage commitment.

EZH2-PRC2 in Stem Cell Differentiation

Suz12^{-/-} ESCs fail to upregulate selected markers during embryoid body formation (Pasini et al., 2007). However, the roles of PRC2 in ESC differentiation have not been investigated systematically. We show that *Ezh2*^{-/-} and *Eed*^{-/-} ESCs fail to fully activate the mesoendodermal transcription program, whereas neuronal gene activation is partially impaired in *Eed*^{-/-} ESCs. These results indicate profound disturbances of differentiation in the absence of *Ezh2* and *Eed*.

Although *Eed*^{-/-} ESCs are able to contribute to chimeric embryos, high-contribution chimeras display some of the same defects as homozygous *Eed*^{-/-} embryos showing allantois overgrowth, poor neurectoderm, and scarce embryonic mesoderm (Chamberlain et al., 2008; Faust et al., 1995). Non-cell-autonomous functions for *Eed* in morphogenesis in the chimeric setting might compensate for some developmental blocks, thereby confounding a strict definition of pluripotency. Nevertheless, defects in lineage specification shown by in vitro differentiation of *Ezh2*^{-/-} and *Eed*^{-/-} ESCs are consistent with in vivo gastrulation defects observed both in high-contribution chimeras of *Eed*^{-/-} ESCs and in homozygous knockout embryos lacking *Eed*, *Ezh2*, or *Suz12*. Therefore, our study demonstrates that *Ezh2* and *Eed* are required to orchestrate the sequence of events necessary to execute a pluripotency program.

The block in transcriptional activation observed in PRC2 mutants is initially paradoxical, as the loss of the H3K27me3 repressive mark should promote gene activation. Faulty gene activation observed during differentiation of *Ezh2*^{-/-} and *Eed*^{-/-} cells may disrupt normal developmental programs. It is also possible that the failure to extinguish pluripotency genes lies at the basis of differentiation failure.

Alternatively, ME induction may be initiated but cannot be sustained due to cell-cycle arrest and apoptosis in mutant cells. It has been reported that decreased *Ezh2* levels in senescent cells and aged stem cells lead to the activation of the *Ink4f-Arf* locus encoding *Cdkn2a*, a negative regulator of the cell cycle, and subsequent decreases in proliferation and stem cell self-renewal (Bracken et al., 2007). ESCs lacking Rb-E2F signaling are less sensitive to *Cdkn2a* activation (White et al., 2005). As somatic cells acquire Rb-E2F and G1 checkpoint regulations, high-level expression of *Cdkn2a* may be detrimental to cell proliferation. Indeed, *Suz12*^{-/-} embryos show decreased proliferation and increased apoptosis (Pasini et al., 2004). We suspect *Ezh2*^{-/-} embryos may die of the combined effects of differentiation failure and cell-cycle-related proliferation alterations.

Lastly, PRC2 binding to DNA regulatory elements could act positively to recruit specific transcription activators to initiate lineage commitment but repress transcription during terminal differentiation (Pasini et al., 2007). This hypothesis is intriguing, as it suggests an instructive role of the H3K27me3 mark mediated by PRC2 complexes in cell-fate switches beyond their well-known repressive functions in maintaining cellular memory during cell division.

Implications of EZH1-Mediated Noncanonical PRC2 in Development and Disease

Our work challenges the traditional view of EZH2 as the sole H3K27 HMTase in mammals. Coexisting canonical EZH2- and noncanonical EZH1-mediated PRC2 complexes are analogous to the SWI/SNF chromatin remodeling complex mediated by BRG1 or BRM, two mutually exclusive ATPase subunits (Roberts and Orkin, 2004). In addition, variants of the SWI/SNF complex exist where tissue-specific subunits provide distinct functions. For example, a switch in BAF45 and BAF53 subunit composition of the SWI/SNF complex is required for proliferating neural stem cells transiting into postmitotic neurons (Lessard et al., 2007). Similarly, *Ezh1* is upregulated upon the transition of the presomitic mesoderm into the somites in E9.5 mouse embryos, suggesting a role of *Ezh1* in somite patterning (Buttitta et al., 2003).

EZH1 cannot fully complement EZH2 during ESC differentiation, suggesting their nonredundant roles during epigenetic establishment of cell fates. De novo recruitment of alternative PRC2 complexes to distinct chromatin loci may be required for lineage commitment. Similar redundancy of the methyltransferases MLL and MLL2 acting on H3K4 has been reported. Despite regulation of different *Hox* complexes by MLL and MLL2 during differentiation, neither is essential for ESC self-renewal (Glaser et al., 2006; Lubitz et al., 2007). Nonredundant functions of EZH1 and EZH2 in lineage commitment further support the notion that development entails networks of epigenetic specificities. Future studies of genome-wide location analysis of EZH1 and EZH2 in purified lineage-committed cells and the identification of interacting proteins which direct PRC2 complexes to DNA should provide insights on whether and how the two PRC2 complexes are coordinated to regulate development.

It is also possible that low-level expression of *Ezh1* in embryonic cells, or temporal and spatial regulation of the EZH proteins (Laible et al., 1997; van Lohuizen et al., 1998), accounts for the failure of *Ezh1* to fully complement *Ezh2* in orchestrating proper differentiation and development in vivo and in vitro. For example, differential expression of *Ezh1* and *Ezh2* in B lymphoid lineages may explain the specific block at the Pro-B cell stage upon deletion of *Ezh2* in bone marrow cells (Su et al., 2003). We find that human *Ezh1* is widely expressed by analysis of expressed sequence tag (EST) counts (Figure S11). Some tissues including brain, kidney, prostate, and spleen express more *Ezh1* than *Ezh2*, and tissues such as adipose, parathyroid, and pituitary gland express *Ezh1* exclusively. Therefore, *Ezh1* may be physiologically more important in postembryonic development. In addition, human cancers including kidney tumor, prostate cancer, and skin tumor show similar *Ezh1* EST counts as *Ezh2*, which serves as a diagnostic marker for various human cancers. Our study reveals EZH1 residency in an alternative PRC2 complex and suggests potential mechanisms by which inappropriate levels of EZH1 may cause epigenetic dysregulation and contribute to cancer. A more detailed knowledge of the recruitment and target genes of EZH1 and EZH2 in maintaining cellular identity and during cell-fate switches will enable a better understanding of the role of PRC2 complexes during development and in disease.

EXPERIMENTAL PROCEDURES

ESC Lines and Culture

Generation of *Ezh2*^{-/-} ESCs, cells expressing biotin-FLAG-tagged proteins, and ESC culture and differentiation methods are described in [Supplemental Data](#).

ChIP and RNA Analysis

ChIP and bioChIP was performed as described previously (Kim et al., 2008). DNA levels at various loci were normalized to an internal control region in the first intron of *Actb*. Relative enrichments were calculated by dividing normalized ChIPed DNA to input DNA. Error bars in ChIP-qPCR analysis represent standard deviations of relative enrichments from three biological replicates of ChIP. Total RNA was reversely transcribed by SuperScript III (Invitrogen). Gene expression was normalized to *GADPH*. Error bars in RNA analysis represent standard deviations of mean expression or fold changes based on at least three cell and RNA isolations. Primer sequences are listed in [Table S11](#).

Microarray Hybridization and Data Processing

ChIPed DNA was amplified by ligation-mediated PCR (Kim et al., 2008) and hybridized to Affymetrix mouse tiling 2.0R array sets. Model-based analysis of tiling array (MAT) was used to predict target loci ($p < 1e^{-7}$) (Johnson et al., 2006). RNA profilings were performed on Affymetrix mouse genome 430 2.0 arrays. GSEA was performed as described (Subramanian et al., 2005). Detailed procedures are described in [Supplemental Data](#).

RNAi

RNAi was performed as described previously (Wang et al., 2006). Details are described in [Supplemental Data](#).

Baculoviral Expression, Protein Purification, and HMTase Assay

The Bac-to-Bac expression system (Invitrogen) was used to generate baculoviruses expressing FLAG-tagged EZH1 (GenBank accession number BC007135), FLAG-EZH2, FLAG-EZH2ΔSET, HA-tagged EED (isoform III), SUZ12, Myc-tagged RBBP4, and V5-tagged AEBP2 (GenBank accession number BC031376). Sf21 cells were harvested 70 hr postinfection. Proteins purified by anti-FLAG M2 agarose (Sigma) were assayed for HMTase activity on core histones (Millipore).

Coimmunoprecipitation and Antibodies

Tandem purification, colP, and protein identification by LC-MS/MS were described previously (Wang et al., 2006). About 5% of nuclear extracts used for colP were loaded as the input in western analysis. Antibodies are described in [Supplemental Data](#).

ACCESSION NUMBERS

Raw data for Affymetrix microarray expression profiling were deposited in the Gene Expression Omnibus under accession number GSE12982.

SUPPLEMENTAL DATA

The Supplemental Data include 11 figures, 11 tables, and Supplemental Experimental Procedures and can be found with this article online at [http://www.molecule.org/supplemental/S1097-2765\(08\)00732-6](http://www.molecule.org/supplemental/S1097-2765(08)00732-6).

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