

Polycomb complexes act redundantly to repress genomic repeats and genes

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Polycomb complexes establish chromatin modifications for maintaining gene repression and are essential for embryonic development in mice. Here we use pluripotent embryonic stem (ES) cells to demonstrate an unexpected redundancy between Polycomb-repressive complex 1 (PRC1) and PRC2 during the formation of differentiated cells. ES cells lacking the function of either PRC1 or PRC2 can differentiate into cells of the three germ layers, whereas simultaneous loss of PRC1 and PRC2 abrogates differentiation. On the molecular level, the differentiation defect is caused by the derepression of a set of genes that is redundantly repressed by PRC1 and PRC2 in ES cells. Furthermore, we find that genomic repeats are Polycomb targets and show that, in the absence of Polycomb complexes, endogenous murine leukemia virus elements can mobilize. This indicates a contribution of the Polycomb group system to the defense against parasitic DNA, and a potential role of genomic repeats in Polycomb-mediated gene regulation.

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Polycomb complexes establish epigenetic patterns for maintaining gene repression. On the cellular level, Polycomb complex-mediated epigenetic patterns are thought to be important for establishing and maintaining cellular identity. A crucial role for cell identity has been deduced from pioneering studies in flies, where Polycomb group (PcG) genes were identified as regulators of homeotic gene expression (for review, see Ringrose and Paro 2007; Schwartz and Pirrotta 2008). In the fly, Polycomb response elements that act as memory modules for maintaining specified gene expression patterns have been identified. In mammals, PcG proteins function in *Hox* gene regulation, development, and tumorigenesis, and contribute to genomic imprinting and dosage compensation (for reviews, see Schuettengruber et al. 2007; Pietersen and van Lohuizen 2008; Schwartz and Pirrotta 2008). Histone-modifying activities that establish chromatin modifications for the regulation of gene repression have been associated with two distinct PcG complexes. The Polycomb-repressive complex 2 (PRC2) contains the PcG proteins Ezh2, Eed, and Suz12 and mediates di- and trimethylation of histone H3 Lys 27 (H3K27me3) (Czermin

et al. 2002; Kuzmichev et al. 2002; Muller et al. 2002). H3K27me3 can act as a signal for recruitment of PRC1 (Cao et al. 2002; Fischle et al. 2003; Min et al. 2003). PRC1 composition is heterogeneous, depending on the cellular context, and contains several PcG proteins, including the RING finger protein Ring1B, which catalyzes ubiquitination of histone H2A (de Napoles et al. 2004; Fang et al. 2004), thereby inhibiting transcription (Stock et al. 2007). PRC1 has also been shown to mediate chromatin compaction (Francis et al. 2004). Currently, it is thought that PcG-mediated gene repression requires the sequential activity of PRC2 and PRC1, whereby H3K27me3 serves as a stable and self-perpetuating epigenetic mark (Hansen et al. 2008) for the recruitment of PRC1 (Fischle et al. 2003; Min et al. 2003; Wang et al. 2004). This view might be too simplistic, as considerable biochemical heterogeneity has been demonstrated for complexes containing PcG proteins. Ezh1 has been shown to form PRC2-like complexes with Eed and Suz12 that predominantly repress select genes by chromatin compaction, and, to a lesser extent, due to histone modifications (Margueron et al. 2008). In addition, Ezh2 complexes have been shown to carry histone H1 methylation activity (Kuzmichev et al. 2004). Also, the PRC1 protein Ring1B is present in different complexes, which also include the H3K36me2 demethylase Fbx110/dKDM (Sanchez et al. 2007; Lagarou et al. 2008). Ring1B has further been observed in the E2F6.com-1 complex, which also contains non-PcG proteins (Ogawa

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et al. 2002). These findings suggest considerable interactions between PcG complexes and chromatin regulators outside the strict definition of the Polycomb group.

In mice, mutations in *Ezh2* or *Eed* that abolish the function of PRC2 result in embryonic lethality soon after implantation (O'Carroll et al. 2001; Wang et al. 2002). Disruption of *Ring1B* causes a similar phenotype with arrest at gastrulation, demonstrating that both PcG complexes are essential for development (Voncken et al. 2003). The role of PcG complexes in differentiation has been investigated in pluripotent embryonic stem (ES) cells. ES cells can be maintained in culture, and retain the ability to differentiate into all embryonic cell types (for review, see Silva and Smith 2008). It is currently thought that the pluripotent state of ES cells is established by a network of transcription factors, including Oct4, Nanog, Sox2, and Klf4 (Takahashi and Yamanaka 2006; Silva et al. 2009). The contribution of epigenetic regulation to maintain ES cell identity, however, is less clear. Polycomb complexes are viewed to be critical for epigenetic regulation in ES cells, and the genome-wide distribution of PRC1 and PRC2 has been determined (Boyer et al. 2006b; Mikkelsen et al. 2007; Ku et al. 2008). In ES cells, ~2500 Polycomb target genes have been identified, including a significant number of transcription factors with lineage-specific expression profiles (Boyer et al. 2006a; Mikkelsen et al. 2007; Ku et al. 2008).

The dynamic repression of target genes by PcG complexes has been implicated in mediating lineage decisions (Bracken et al. 2006; Mikkelsen et al. 2007). During neuronal differentiation, Polycomb targets change between ES cells, lineage-restricted progenitors, and mature neurons (Mohn et al. 2008). This indicates that the promoter regulation by PcG complexes is highly plastic in a tissue- and differentiation state-dependent manner. In the epidermis, the PcG protein Ezh2 has been shown to be required for temporal and spatial control of gene expression during differentiation in a lineage-specific manner (Ezhkova et al. 2009). Furthermore, the PcG system is central in modulating expression from the *Ink4a/Arf* tumor suppressor locus, linking PcG-mediated repression to cell cycle control (Voncken et al. 2003; Chen et al. 2009; Dhawan et al. 2009). The PRC1 protein Bmi1 has also been shown to regulate mitochondrial function, DNA damage response, and antioxidant defense in mice (Chatoo et al. 2009; Liu et al. 2009). This indicates that PcG proteins are important for regulating several cellular processes that contribute to development.

We and others observed previously that lineage-specific genes are derepressed in ES cells lacking either the *Ring1B* or the *Eed* (Voncken et al. 2003; Boyer et al. 2006b; Leeb and Wutz 2007; Endoh et al. 2008; van der Stoop et al. 2008). These transcriptional changes destabilize ES cells, but are compatible with ES cell self-renewal. Furthermore, ES cells lacking either PRC1 or PRC2 function are capable of differentiating in culture, and *Eed*-deficient ES cells have been shown to form teratomas consisting of cells from the three germ layers and to contribute to chimeric embryos (Chamberlain et al. 2008). This is surprising, given the importance of the Polycomb system for epigenetic

regulation during embryogenesis. Currently, it is not clear whether Polycomb complexes are the main determinants of repression of target loci or whether they have a modulatory role for gene expression. Importantly, the extent to which they are required for the formation of differentiated cell types remains to be determined. In order to gain insight into the function of PcG-mediated transcriptional regulation, we sought to eliminate both chromatin modifications associated with the Polycomb system by simultaneous disruption of PRC1 and PRC2 function in mouse ES cells.

Results

Ring1B and Eed double-deficient (dKO) ES cells self-renew in culture

ES cells lacking PRC1 activity have been generated previously by disruption of the *Ring1B* gene (Leeb and Wutz 2007). Similarly, PRC2 function has been abolished in ES cells carrying a disruption of *Eed* (Schoeftner et al. 2006). To generate ES cells lacking both PRC1 and PRC2 activity, we followed a conditional gene targeting strategy to delete *Ring1B* in *Eed*-deficient ES cells. We disrupted the first allele of *Ring1B* by deleting the first three coding exons, which contain the catalytic RING domain, and inserting a splice acceptor-linked polyadenylation site to truncate the transcript. Subsequently, the selection cassette was removed using Cre recombinase-mediated excision. The second allele was targeted using a vector for flanking the first three coding exons with loxP sites. Deletion of *Ring1B* was then induced by transient expression of Cre recombinase. *Ring1B*^{-/-} *Eed*^{-/-} dKO ES cells were generated, but initially we were not able to establish lines, as dKO ES cells were extremely sensitive to culture conditions and showed a strong inclination to differentiate. However, after adaptations of the culture conditions, and using a Tamoxifen-inducible estrogen receptor fusion CreERT2 transgene to delete the second allele of the *Ring1B* gene, we were able to establish stable dKO ES cell lines with high efficiency. dKO ES cells self-renewed in culture and maintained a typical ES cell morphology for at least 20 passages (Fig. 1A; Supplemental Fig. 1A). We noted a pronounced dependence of dKO ES cells on fibroblast feeder cells, which was not overcome by a high dose of leukemia inhibitory factor (LIF). Apparently, lack of *Ring1B* and *Eed* caused an unstable ES cell phenotype, but did not block ES cell self-renewal.

dKO ES cells showed a clear nuclear immunofluorescence signal for Oct4 and Nanog, and expressed transcription factors known to be important for pluripotency of ES cells (Fig. 1B,C; Supplemental Fig. 1B). The PRC1 proteins Ring1B, Mel18, and Mph2 were abundant in control wild-type ES cells but were undetectable by Western analysis in dKO ES cells (Fig. 1C). Similarly, *Eed* was absent and the PRC2 proteins Suz12 and Ezh2 were reduced in dKO ES cells (Fig. 1C). Finally, dKO ES cells showed loss of genomic H3K27me3 and loss of ubiquitination of histone H2A (ubH2A) (Fig. 1D). In ES cell lines lacking *Eed*, we also detected a loss of dimethylation and a reduction of monomethylation of H3K27

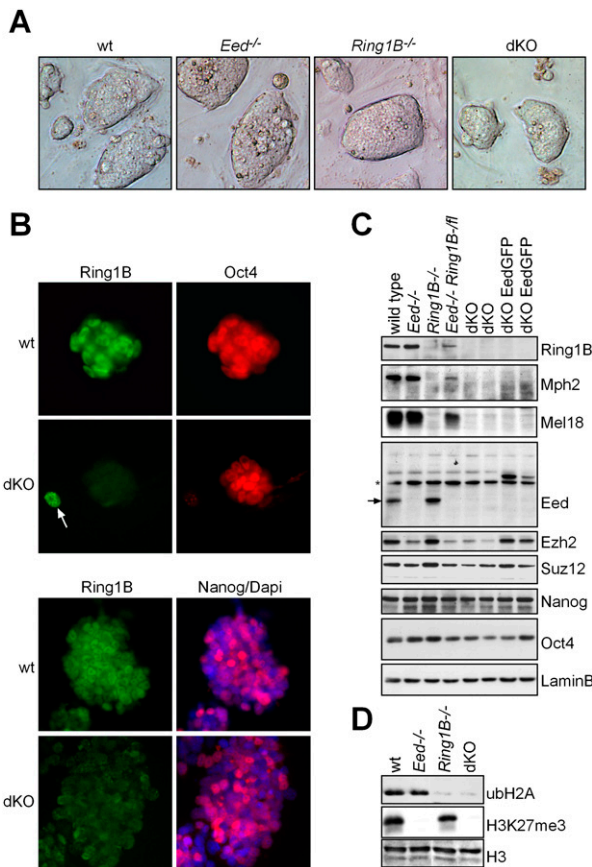


Figure 1. Establishment of dKO ES cells lacking PRC1 and PRC2 activity. (A) Morphology of wild-type (wt), *Ring1B*^{-/-}, *Eed*^{-/-}, and dKO ES cell colonies. (B) Immunofluorescence staining showing Oct4 and Nanog expression in dKO and wild-type ES cell colonies. (C) Western analysis of PcG proteins and transcription factors in ES cells of indicated genotypes shows that Mph2 and Mel18 are virtually absent in *Ring1B*^{-/-} and dKO ES cells. Ezh2 and Suz12 are reduced in *Eed*^{-/-} and dKO ES cells. An arrow indicates Eed (asterisk; nonspecific band). The pluripotency markers Nanog and Oct4 are largely unaffected. Lamin B was used as loading control. (D) Western analysis of ubH2A and H3K27me3 showing absence of PRC1 and PRC2 activity in dKO ES cells.

(Supplemental Fig. 2A). Immunofluorescence analysis showed that Lys 27 monomethylation formed foci over pericentric heterochromatin, consistent with previous observations (Supplemental Fig. 2B; Schoeftner et al. 2006). Expression of an *EedGFP* transgene rescued histone H3 lysine methylation patterns in dKO ES cells (Supplemental Fig. 2A,B). We conclude that ES cells lacking both *Ring1B* and *Eed* can be established, and Polycomb-mediated chromatin modifications are not essential for ES cell self-renewal in culture.

Simultaneous loss of *Ring1B* and *Eed* function impairs ES cell differentiation

To investigate the function of the PcG system in differentiation, we analyzed the ability of wild-type, *Eed*^{-/-},

Ring1B^{-/-}, and dKO ES cells to form teratomas (Fig. 2A). For this, we injected 5×10^5 ES cells into the flanks of immune-deficient recipient mice using Matrigel as a carrier. Injection of wild-type, *Eed*-deficient, and *Ring1B*-deficient ES cells resulted in teratomas in all injection sites, showing that tumor formation was efficient. Tumors were excised after 3 wk and were analyzed. Histological analysis showed that wild-type, *Ring1B*-deficient, and *Eed*-deficient teratomas were composed of differentiated structures. Immunohistochemistry further showed that a range of cell types and tissues from all three germ layers was present in all teratomas (Fig. 2B). However, teratomas from *Eed*- and *Ring1B*-deficient ES cells were significantly smaller (Supplemental Fig. 3A), and we noted a larger proportion of cells with an endodermal or ectodermal origin, respectively, when compared with wild-type teratomas. The ability of wild-type, *Eed*-deficient, and *Ring1B*-deficient ES cells to form tumors consisting of cells from the three germ layers demonstrates that

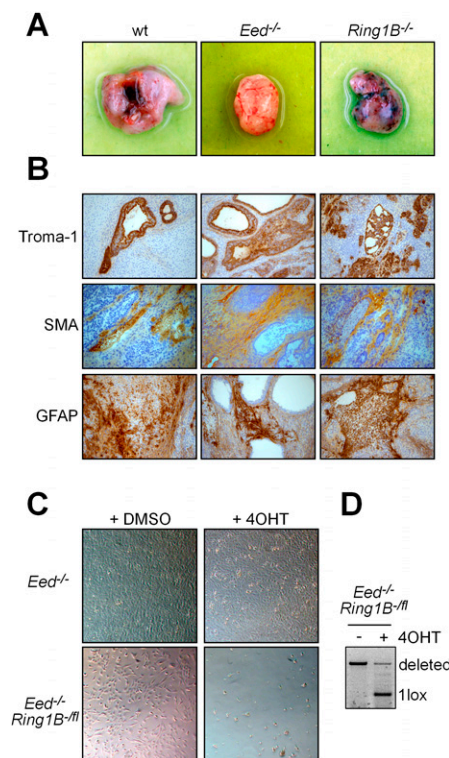


Figure 2. *Eed* and *Ring1B* are redundantly required for differentiation. (A) Teratomas formed by wild-type (wt), *Eed*^{-/-}, and *Ring1B*^{-/-} ES cells 3 wk after injection. dKO ES cells did not give rise to teratomas. (B) Immunohistochemical analysis of wild-type, *Ring1B*^{-/-}, and *Eed*^{-/-} teratomas using markers for endoderm (Troma1), mesoderm (smooth muscle actin [SMA]), and ectoderm (GFAP). (C) Double deficiency for PRC1 and PRC2 is not compatible with NS cell viability. Deletion of *Ring1B* in *Eed*-deficient NS cells by induction of CreERT2 with 4OHT caused cell death, whereas 4OHT had no effect on control *Eed*-deficient NS cells. (D) PCR analysis showing that induction of CreERT2 results in deletion of the conditional *Ring1B*^{fl} allele in *Eed*^{-/-} *Ring1B*^{fl} NS cells.

neither PRC1 nor PRC2 activity is essential for differentiation (Fig. 2B). In stark contrast, injection of dKO ES cells did not result in the formation of teratomas. When dKO grafts were excised after 6 mo, only Matrigel with a negligible amount of cells remained. This indicated that dKO cells were not maintained in the injection site. To ascertain that this defect is due to loss of both PcG complexes, we rescued PRC2 activity by expression of an *EedGFP* transgene in dKO ES cells. The resulting dKO^{*EedGFP*} ES cells efficiently formed teratomas in all injection sites (Supplemental Fig. 3BC). Although these teratomas developed slower than wild-type, *Eed*-deficient, or *Ring1B*-deficient teratomas, they contained cells expressing endodermal and ectodermal markers consistent with the development of distinct lineages (Supplemental Fig. 3D). We therefore conclude that loss of *Eed* and *Ring1B* causes a synthetic phenotype, and that at least one PcG complex is required for tumor formation.

To further test the differentiation potential of dKO ES cells, we used a protocol for directed differentiation into the neural lineage (Pollard et al. 2006). Nestin-positive neural stem (NS) cells could readily be established from *Eed*-deficient ES cells that were either wild type for *Ring1B* or contained a conditional *Ring1B* allele over a null allele (*Eed*^{-/-} *Ring1B*^{*fl*}), which resulted in strongly hypomorphic *Ring1B* expression (Fig. 2C; Supplemental

Fig. 4AB). However, we did not obtain NS cells from dKO ES cells. Therefore, we aimed to delete *Ring1B* in *Eed*^{-/-} *Ring1B*^{*fl*} NS cells expressing an inducible CreERT2 recombinase. Three days to 4 d after deletion of *Ring1B* by addition of Tamoxifen (4-hydroxy-tamoxifen [4OHT]), we observed massive cell death (Fig. 2C). Control *Eed*-deficient NS cells were not affected by the addition of 4OHT. This showed that, in contrast to ES cells, the combined loss of PRC1 and PRC2 activity is not compatible with NS cell survival.

To study the differentiation defect more closely, we induced cell differentiation with retinoic acid in monolayer culture and followed the expression of pluripotency and differentiation genes (Fig. 3A). We observed the down-regulation of *Oct4* on day 2 of differentiation in dKO, *Eed*-deficient, and wild-type ES cells. Differentiation markers *Gata4*, *Gata6*, *Afp*, and *Brachyury* were up-regulated in dKO cells on days 2 and 4, indicating that the cells had entered differentiation. The cells also showed a differentiated cell morphology consistent with entry into differentiation (Fig. 3B). However, the cell number of dKO cells was drastically reduced compared with wild-type or *Eed*-deficient ES cells (Fig. 3C–F). In the course of differentiation, dKO cell numbers declined sharply, and the cells were progressively lost (Fig. 3C–F). Expression of an *EedGFP* transgene rescued the differentiation defect of

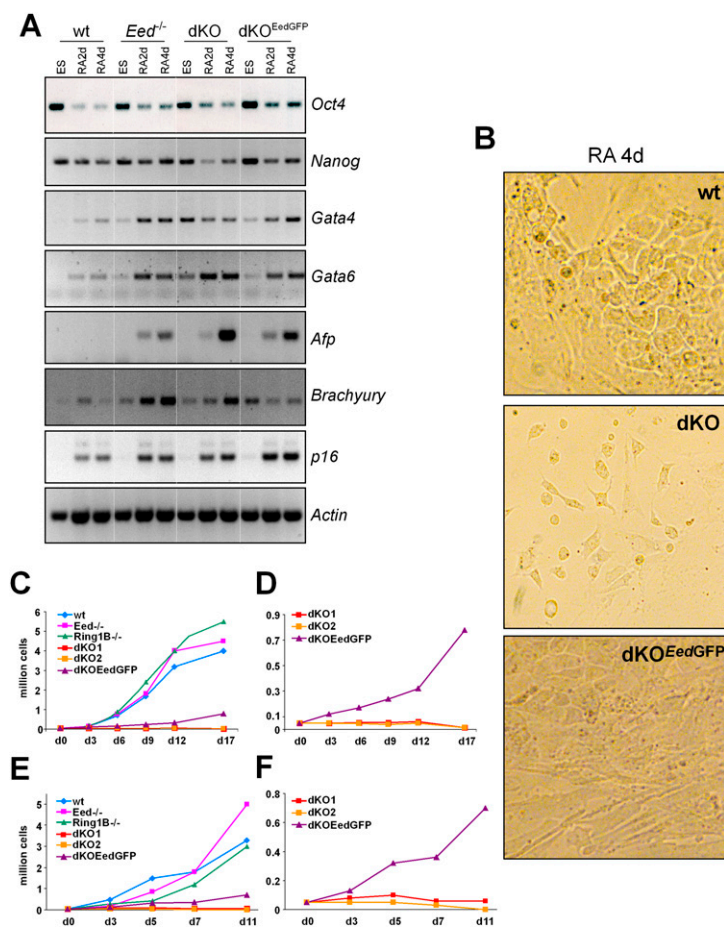


Figure 3. Differentiation of ES cells lacking PcG activity. (A) RT-PCR expression analysis of pluripotency markers (*Oct4* and *Nanog*) and differentiation markers (*Gata4*, *Gata6*, *Afp*, and *Brachyury*) and *p16* in wild-type, *Eed*-deficient, dKO, and dKO^{*EedGFP*} ES cells induced to differentiate with retinoic acid. Undifferentiated ES cells and cells after 2 and 4 d of differentiation are shown. *Actin* is used as loading control. (B) Microscopy image showing differentiated cell morphology on day 4 of retinoic acid-induced differentiation of wild-type, dKO, and dKO^{*EedGFP*} ES cells. (C–F) Cell numbers in differentiating cultures of wild-type, *Eed*-deficient, *Ring1B*-deficient, dKO, and dKO^{*EedGFP*} ES cells. (C,E) Two experiments are shown. (D,F) Rescaled representation of experiments in C and E showing that expression of an *EedGFP* transgene in dKO^{*EedGFP*} ES cells rescues the differentiation defect of dKO ES cells.

dKO ES cells to a large extent (Fig. 3A–F). Taken together, our data show that the simultaneous loss of *Ring1B* and *Eed* is not compatible with the formation of differentiated cells. Although differentiation of dKO ES cells was triggered by retinoic acid, differentiating cells were not viable and were lost.

To further assess the differentiation potential, we aggregated dKO ES cells into embryoid bodies. Compared with *Eed*-deficient and dKO^{*Eed*GFP} ES cells, dKO ES cells formed smaller embryoid bodies that became hollow spheres after 2 wk in suspension culture (Supplemental Fig. 5A). In these embryoid bodies, differentiation markers were up-regulated and expression of Oct4 was down-regulated (Supplemental Fig. 5B). This shows that dKO ES cells have a limited potential to form embryoid bodies and differentiate without a strong inducer like retinoic acid. Finally, we assessed the ability of dKO ES cells to contribute to embryos. We injected GFP-marked dKO ES cells into blastocysts, and observed efficient contribution to the inner cell mass similar to wild-type control ES cells (Supplemental Fig. 6). However, we could not detect a measurable contribution of dKO ES cells to chimeras at embryonic day 10.5, indicating that loss of *Ring1B* and *Eed* is not compatible with development. From our data, we conclude that, although PRC1 and PRC2 dKO ES cells can self-renew and express pluripotency markers, they are unable to differentiate properly. The aberrant differentiation potential of dKO ES cells is

most likely due to a defect in maintaining differentiated cells and not a failure to enter differentiation.

PRC1 and PRC2 act in parallel to repress target genes

The strong phenotype caused by the combined loss of *Eed* and *Ring1B* suggested a compensatory or redundant role for Polycomb complexes in differentiation. To understand the mechanistic basis behind this synthetic phenotype, we analyzed the effect of loss of PcG complex function on gene repression. For this, we established genome-wide gene expression profiles of wild-type, *Ring1B*-deficient, *Eed*-deficient, and dKO ES cells using Affymetrix microarrays (Fig. 4A). When compared with wild-type ES cells, 814 and 1151 genes were derepressed more than twofold ($P < 0.05$) in *Ring1B*-deficient and *Eed*-deficient ES cells, respectively. In dKO ES cells, the number of up-regulated genes increased to 2017 (Supplemental Fig. 7A). In order to assess the direct effect of loss of PcG function, we analyzed a gene set that was reported previously to be bound by PcG complexes (Ku et al. 2008). Two-hundred-seventy-nine (10% of annotated PcG targets) and 476 (18%) PcG target genes were derepressed more than twofold ($P < 0.05$) in *Ring1B*-deficient or *Eed*-deficient ES cells, respectively. In dKO ES cells, the number of derepressed PcG target genes nearly doubled (757; 29%) (Supplemental Fig. 7B). A set of 329 genes was derepressed only after the combined loss of PRC1 and PRC2, but

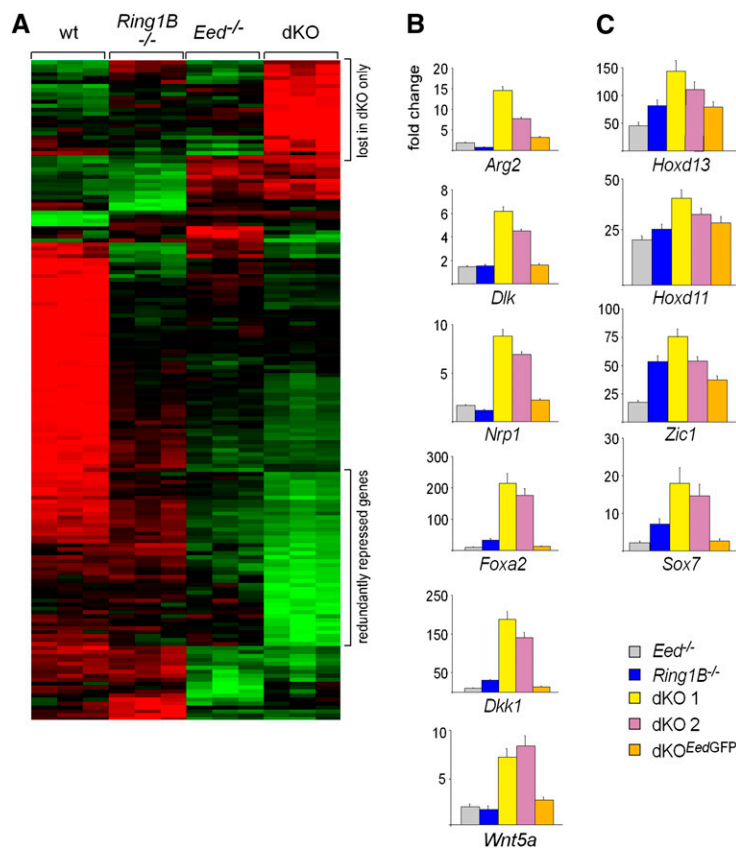


Figure 4. Analysis of transcriptional changes in ES cells lacking PcG activity. (A) A heat map showing high (green), intermediate (black), and low (red) expression of genes in wild-type, *Eed*^{-/-}, *Ring1B*^{-/-}, and dKO ES cells. The top regulated genes in all genotypes are shown. (B,C) Quantitative real-time PCR was used to confirm derepression of genes in PcG-deficient ES cells. Introduction of an *Eed*GFP transgene in dKO ES cells re-establishes repression largely to the levels of *Ring1B*^{-/-} ES cells. (B) A redundantly repressed set of PcG target genes is specifically derepressed in dKO ES cells. (C) PcG target genes derepressed in all PcG mutant ES cells.

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remained repressed in *Ring1B*-deficient and *Eed*-deficient ES cells (Supplemental Fig. 7B; Supplemental Table 1), suggesting that PRC1 and PRC2 are redundant for repression of these genes. We confirmed the specific derepression of the *Arg2*, *Dlk*, *Nrp1*, *Foxa2*, *Dkk1*, and *Wnt5a* genes in dKO ES cells by quantitative RT-PCR (Fig. 4B). Importantly, gene repression was restored after introducing an *EedGFP* transgene into dKO ES cells (Fig. 4B), indicating that derepression in dKO cells was reversible, and that genes remain marked for silencing in the absence of the PcG system in ES cells. These data show that a large set of genes is derepressed in dKO ES cells, but remains silenced in the absence of *Ring1B* or *Eed* alone. These redundantly silenced genes are repressed by both Polycomb complexes in parallel. This is in contrast to other PcG target genes such as *Hoxd13*, *Hoxd11*, *Zic1*, and *Sox7* that were already derepressed in the absence of a single PcG complex (Fig. 4C).

In dKO but not *Eed*-deficient and *Ring1B*-deficient ES cells, a set of 94 genes—including several transcription factors such as *Sall1*, *Rest*, *Pbx4*, *Nfat5*, *E2a*, *Aire*, *Hivep3*, and *Gli1*—was more than fivefold down-regulated (Supplemental Table 2). Loss of expression of these genes is most likely a secondary effect of deregulation of Polycomb target genes in dKO ES cells.

To further analyze the binding of Polycomb complexes on the promoters of redundantly silenced genes, we performed chromatin immunoprecipitation (ChIP). We detected *Ring1B*, *Suz12*, and H3K27me3 on the promoters of redundantly silenced genes as well as *Hox* genes in wild-type ES cells (Fig. 5). In *Ring1B*-deficient ES cells, the *Suz12* and H3K27me3 signal was maintained at wild-type level on redundantly silenced genes, whereas *Suz12* and H3K27me3 were reduced on *Hox* gene promoters (Fig. 5B,C). *Ring1B* was preferentially detected at a reduced level on the promoters of redundantly silenced genes in *Eed*-deficient ES cells, whereas it was lost from several *Hox* gene promoters (Fig. 5A). These observations show that both Polycomb complexes are recruited in parallel to redundantly silenced genes, and that recruitment of PRC1 and PRC2 is, at least in part, independent of each other. Although there is some preference for parallel recruitment of PRC1 and PRC2 to redundantly silenced genes, our data also show that the parallel recruitment is a general feature of PcG target genes, and suggest that some genes are more responsive to the loss of either of the two PcG complexes.

Stable repression of endogenous retroviruses requires the presence of PcG proteins

A redundant role for PRC1 and PRC2 in gene expression was unexpected, based on the existing models for PcG complex function. Hence, we wondered whether PcG complexes might contribute to a nuclear function apart from gene regulation, which could be lost in dKO ES cells. Previous studies have estimated that 15% of histone H3 is trimethylated on Lys 27 genome-wide (Peters et al. 2003; Schoeftner et al. 2006), and 10%–15% of histone H2A is ubiquitinated (de Napoles et al. 2004). Consider-

ing that genes constitute ~2% of the genome, the amount of PcG-catalyzed histone modifications cannot be attributed solely to gene promoters. Therefore, we analyzed dKO expression profiles for transcripts originating from genomic repeats. Affymetrix arrays contain several probes that map to repeat elements. We observed a strong increase in expression of long-terminal repeat (LTR) retrotransposons in dKO ES cells when compared with wild-type, *Eed*-deficient, and *Ring1B*-deficient ES cells (Supplemental Fig. 9A). Northern analysis confirmed the derepression of murine leukemia virus (MLV) retroelements in dKO ES cells (Fig. 6A). To investigate whether derepression of MLV sequences could result in mobilization of MLV elements, we measured the copy number of a specific MLV integration on chromosome 8 (Changolkar et al. 2008). We observed a 15-fold increase in the copy number of this provirus in *Eed*-deficient ES cells and a 30-fold increase in dKO ES cells (Fig. 6B). In order to define whether MLV elements are targets of PcG complexes, we performed directed ChIP. Using different primer sets spanning the proviral sequence, we found that MLV elements are enriched for H3K27me3 in wild-type ES cells to a level similar to the Polycomb target gene *Lef1* (Fig. 6C). The H3K27me3 enrichment on MLVs was lost in *Eed*-deficient ES cells, showing that the signal was specific. Enrichment of H3K27me3 over MLV elements was also maintained in *Ring1B*-deficient and differentiated ES cells (Supplemental Fig. 9C; data not shown). This demonstrates that Polycomb complexes contribute to the silencing of MLV retroviruses.

We also observed that intracisternal A particle (IAP) elements were derepressed in dKO ES cells (Fig. 6D). IAP repression could be restored by introduction of an *EedGFP* transgene in dKO ES cells (Supplemental Fig. 9B). Previously, it has been shown that DNA methylation is essential for maintaining repression of endogenous IAP retroelements during embryogenesis (Walsh et al. 1998). We investigated whether depletion of the PcG system would also affect DNA methylation of IAP elements. Using methylation-sensitive restriction enzyme analysis, we observed a reduction of DNA methylation of IAP elements in *Eed*-deficient and dKO ES cells (Fig. 6E). DNA methylation was largely maintained in *Ring1B*-deficient ES cells, which showed only slightly reduced methylation levels. This indicates that deficiency in PRC2 function leads to a loss of DNA methylation on IAP elements without affecting transcription. In conclusion, a subset of genomic repeats derived from LTR retrotransposons, including IAP and MLV elements, are repressed by PRC1 and PRC2 in ES cells. This indicates that, in ES cells, the Polycomb system contributes to the defense against parasitic DNA.

Discussion

Genomic repeats are targets of Polycomb complexes

We identify genomic repeat elements as a novel class of Polycomb targets. MLV and IAP retroelements are derepressed in dKO but not in PRC1- or PRC2-deficient ES

Redundant silencing by Polycomb complexes

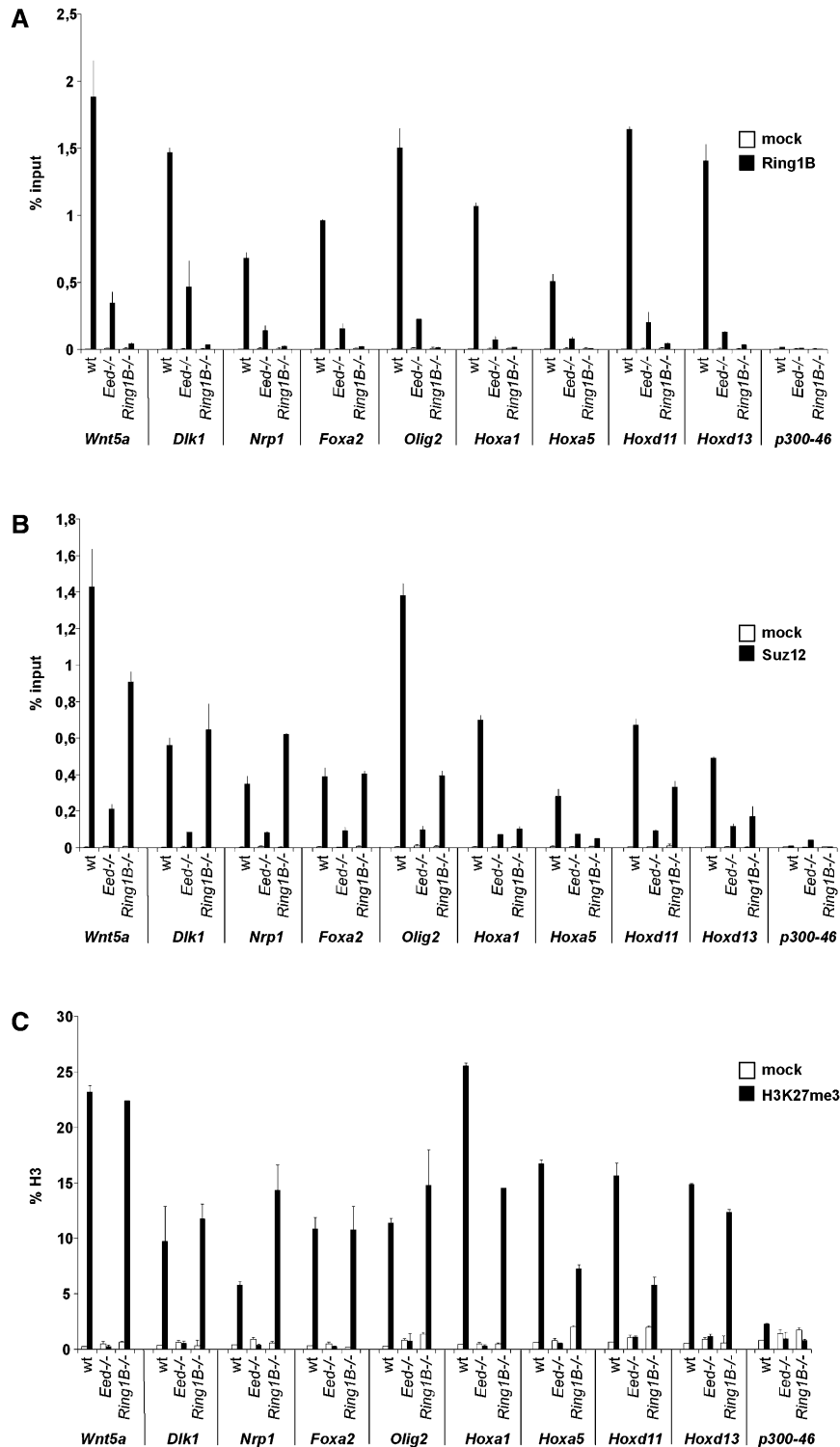


Figure 5. PRC1 and PRC2 bind to the promoters of redundantly silenced genes. ChIP analysis of Ring1B (A), Suz12 (B), and H3K27me3 (C) binding to gene promoters in wild-type, *Eed*-deficient, and *Ring1B*-deficient ES cells. H3K27me3 signal is normalized to histone H3, and Ring1B and Suz12 are represented as percent input. Error bars represent standard deviation.

cells, suggesting that both Polycomb complexes act in parallel in silencing of endogenous retroelements. The parallel recruitment of Polycomb complexes to genomic repeats is consistent with previous observations that genome-wide H3K27me3 is maintained in *Ring1B*-deficient ES cells (de Napoles et al. 2004; Schoeftner et al.

2006), and genomic ubH2A is maintained in *Eed*-deficient ES cells (Leeb and Wutz 2007). These data also suggest that by far the greatest proportion of Polycomb-mediated chromatin modifications is located in the nongenic regions of the genome. This might have important implications for understanding gene regulation by Polycomb

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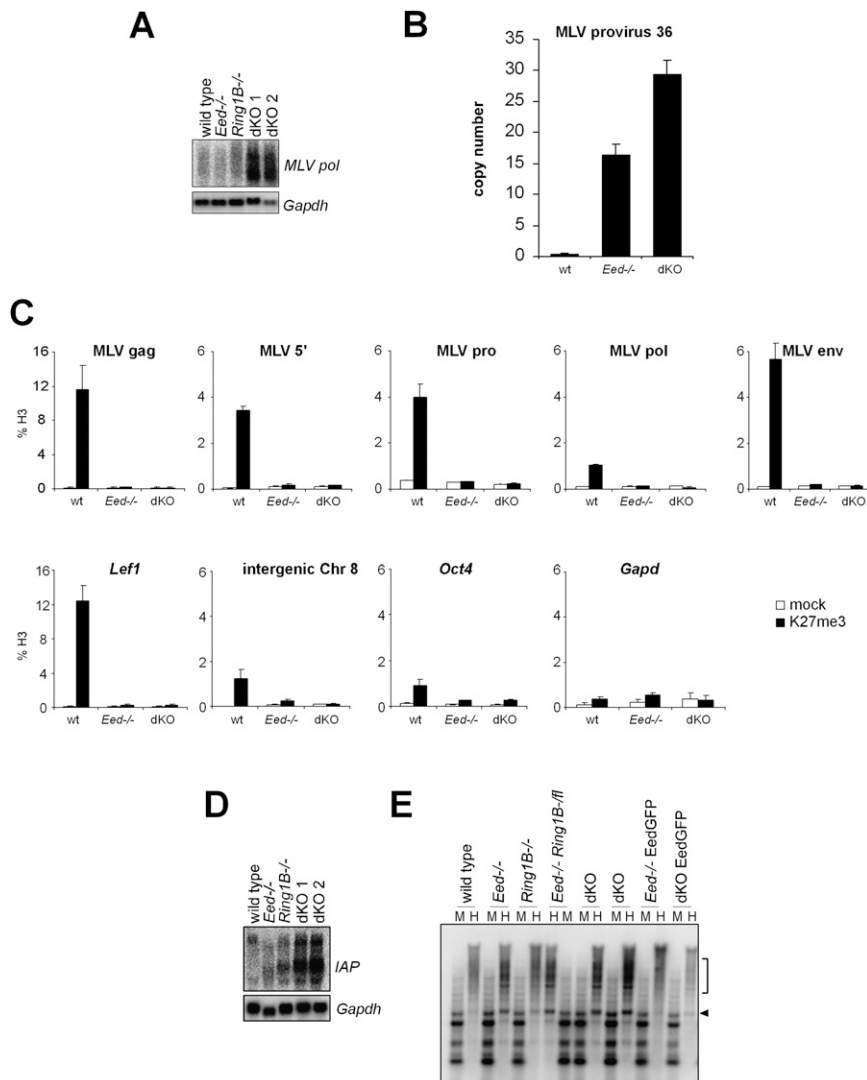


Figure 6. Redundant repression of endogenous retroviruses by PRC1 and PRC2. (A) Northern analysis showing derepression of endogenous MLV retroelements in dKO ES cells. *Gapdh* was used as a loading control. (B) Quantitative PCR analysis showing an increase in MLV provirus copy number in Polycomb-deficient ES cells. The copy number was quantitated relative to an intergenic genomic sequence using the standard curve method. (C) Directed ChIP showing enrichment of H3K27me3 over MLV elements in ES cells. (Top row) Different regions of the MLV provirus sequence were investigated by quantitative PCR. (Bottom row) *Lef1* was used as a positive control, and *Oct4*, *Gapdh*, and an intergenic sequence on chromosome 8 were used as negative controls. H3K27me3 signals are diminished in *Eed*-deficient ES cells demonstrating specificity. (D) Northern analysis showing the derepression of IAP retroviral sequences in dKO ES cells. *Gapdh* was used as loading control. (E) Restriction analysis with methylation-sensitive *Hpa*II (H) and methylation-resistant *Msp*I (M) and subsequent Southern analysis show loss of DNA methylation on IAP elements in *Eed*-deficient and dKO ES cells. Size of demethylated bands in *Hpa*II lanes are indicated by the arrowhead and square bracket.

complexes in mammals, as it indicates a potential role of repeat sequences in the PcG-mediated gene silencing mechanism. It is conceivable that genomic repeat sequences in mammals provide a binding platform for PcG proteins. Loss of both PRC1 and PRC2 leads to derepression of genomic repeats and, thus, could interfere with the function of such a repressive platform. We believe that this might underlie the severity of the phenotype of dKO ES cells, which show nearly twice as many genes derepressed when compared with ES cells lacking either PRC1 or PRC2 function. It will be exciting to investigate whether genomic repeats associate with Polycomb-regulated genes similar to the observed clustering of Polycomb-regulated genes and Polycomb response elements in *Drosophila* (Grimaud et al. 2006; Lanzaolo et al. 2007). In mammals, PRC1 and PRC2 are also recruited to the core of the inactive X chromosome, which contains genomic repeats (Chaumeil et al. 2006; Clemson et al. 2006). In X chromosome inactivation, PRC1 and PRC2 are recruited in parallel by the noncoding *Xist* RNA (Schoeftner et al. 2006; Leeb and Wutz 2007). Taken

together, these findings suggest that Polycomb complexes might have a role in compartmentalizing the nucleus by establishing chromatin domains for gene repression.

Redundancy of PRC1 and PRC2 in gene repression

We show that the combined disruption of *Ring1B* and *Eed* results in an aberration of cell differentiation. Given the molecular differences of the activities of PRC1 and PRC2, the severity of this synthetic phenotype is unexpected. PRC1-catalyzed ubH2A has been reported to inhibit transcription elongation (Stock et al. 2007), suggesting a direct function in gene repression. Yet, the removal of ubH2A during mitosis is required for cell cycle progression, which precludes this modification as a heritable epigenetic mark (Joo et al. 2007). In contrast, H3K27me3 is transmitted through the cell cycle, but a direct function of H3K27me3 in gene repression has not been shown, and its only reported function is to recruit other Polycomb proteins such as Cbx7 or PRC2 (Fischle et al. 2003;

Bernstein et al. 2006; Hansen et al. 2008; Margueron et al. 2009). The very different cell-biological properties of ubH2A and H3K27me3 indicate that the regulation of Polycomb-mediated silencing is a complex process. It is likely that considerable cross-talk exists between different Polycomb complexes, such as the reported binding of the PcG complex protein Rybp to ubH2A and Ring1B (Arrigoni et al. 2006), and other chromatin-associated complexes. PRC1 has additional functions in replication, and it has been shown that PRC1 proteins remain bound to chromatin when replicated in an *in vitro* system (Francis et al. 2009).

We find that Ring1B recruitment to PcG target genes depends quantitatively on PRC2, as most of the Ring1B signal on gene promoters is lost in *Eed*-deficient ES cells. Nevertheless, in the absence of PRC2, Ring1B remains detectable clearly over background level and is functionally relevant. In *Ring1B*-deficient ES cells, Suz12 and H3K27me3 signals are reduced on most nonredundantly silenced genes, but remain at wild-type levels on redundant gene promoters. These data could indicate that, to some extent, redundant gene silencing by PcG complexes reflects the recruitment of PRC1 and PRC2. However, we believe that additional factors might also contribute to and determine gene repression of PcG target genes.

Our data show that the deletion of *Ring1B* and *Eed* in ES cells disrupts the catalytic function of PRC1 or PRC2, respectively. Although we did not detect H3K27me3, we note that Ezh2 protein is still present at a reduced amount and could be functional to an extent below our detection. Since the same level of Ezh2 is also present in dKO cells, the differentiation defect does not arise due to further loss of Ezh2. *Ring1B*^{-/-} ES cells are deficient for PRC1 function, as Ring1A, a functional homolog of Ring1B, is not expressed in ES cells and does not restore genomic ubH2A levels (Leeb and Wutz 2007). In addition, *Ring1B* deletion leads to a loss of several PRC1 proteins, including Rybp, Cbx4, Mel18, and Bmi1. At present, it is not resolved to what extent the catalytic activity toward ubH2A and the structural role of PRC1 proteins contribute to the phenotype. From our observations, we suggest that the function of PRC1 and PRC2 is largely eliminated by disruption of *Ring1B* and *Eed*, respectively.

Activity of PRC1 and PRC2 defines the differentiation potential of ES cells

Ring1B-deficient or *Eed*-deficient ES cells can differentiate into cell types of all three germ layers, suggesting that the dynamic modulation of gene expression required for differentiation can be performed by a single PcG complex. However, the complete loss of PcG function abolishes the tumor formation potential of dKO ES cells. The ability of an *Eed*GFP transgene to restore tumor formation indicates that dKO ES cells can regain pluripotency. We find that, upon differentiation of dKO ES cells, pluripotency markers are down-regulated and differentiation markers are up-regulated. Thus, the differentiation defect of PcG-deficient ES cells does not result from a block to enter differentiation, but is due to a failure to maintain differ-

entiated cells. This suggests that the Polycomb system is critical for fine-tuning gene expression, and that epigenetic patterns required to progress through differentiation cannot be set up in the absence of PcG regulation.

Polycomb complex function in ES cells

ES cells lacking *Ring1B* and *Eed* can self-renew and maintain pluripotency marker expression. We also show that they can contribute to the inner cell mass when injected into blastocysts. However, these ES cells are unstable and tend to spontaneously differentiate in culture. Furthermore, they fail to execute differentiation programs appropriately. Thus, Polycomb complexes stabilize ES cell identity. We measured only a slight decrease in the proliferation rate of dKO ES cells compared with controls, indicating that ES cell self-renewal is largely independent of epigenetic gene regulation. This is also consistent with the finding that ES cells lacking DNA cytosine methyltransferases are viable (Tsumura et al. 2006). Recently, it has been suggested that ES cells represent the ground state of pluripotency, which would not require epigenetic regulation (Silva and Smith 2008). Our data support this notion by showing that the network of transcription factors in ES cells is stable enough to maintain self renewal in the absence of Polycomb regulation. The ability to maintain ES cells in the absence of PRC1 and PRC2 catalytic activity now provides an opportunity for studying the function of the PcG system in gene repression, chromatin organization, and genome stability. In the future, this will facilitate characterizing the role of the Polycomb system for the nuclear architecture of mammals.

Materials and methods

Cell culture and generation of ES cell lines

ES cells were cultured as described (Leeb and Wutz 2007). Cells were counted with a Casy cell counter (Schaefer System GmbH). Targeting of *Ring1B* was performed in *Eed*^{-/-} ES cells following a conditional strategy as described previously (Leeb and Wutz 2007). For deletion of *Ring1B*, an inducible pCAG-CreERT2:EF1-BSD transgene was randomly inserted into *Eed*^{-/-} *Ring1B*^{fl} ES cells. The Blasticidin concentration used for selection was 5 µg/mL. For establishing dKO cells, *Ring1B* deletion was induced with 1 µM 4OHT for 48 h. dKO ES cells were established with a frequency of 85%–95% and subcloned before further analysis. dKO ES cells were maintained on high-density mitotically inactivated low-passage mouse embryonic fibroblast feeder cells in ES-DMEM supplemented with 1000 U/mL LIF and 15% of a special selected batch of fetal bovine serum (FBS) (PAA) tested for optimal growth of mouse ES cells. To reconstitute *Eed* expression, dKO ES cells were transfected with a pCAG-EGFP-*Eed*-IRES-HygA transgene (Schoefner et al. 2006). Selection was performed using 140 µg/mL Hygromycin. NS cells were derived from ES cells using a monolayer differentiation protocol (Pollard et al. 2006). Differentiation of ES cells was induced by 100 nM all-*trans* retinoic acid in medium containing 10% FBS and no LIF. Embryoid body cultures were established by aggregation of ES cells in a low-adherent tissue culture dish in the absence of LIF. ES cells for blastocyst injection experiments were GFP-labeled by transfection using Lipofectamine2000 (Invitrogen) with a linearized pCAC-EGFP-pA transgene. Stable ES

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cell lines were generated by FACS sorting 3 and 12 d after transfection.

Teratoma formation assay

We injected 5×10^5 ES cells into the flanks of nude mice in Matrigel Basement Membrane Matrix (BD Bioscience). We performed three injections each for wild-type, *Eed*^{-/-}, *Ring1B*^{-/-}, two independent dKO, and dKO^{*Eed*GFP} ES cell lines. After 3 wk, teratomas were excised, embedded in O.C.T. compound (Tissue Tek) for cryosections, and subsequently analyzed using immunohistochemistry. dKO^{*Eed*GFP} teratomas grew slowly and appeared after ~10 wk of growth. Further characterization included H3K27me3 immunofluorescence analysis and PCR genotyping. dKO ES cells did not give rise to teratomas within 6 mo after injection.

Immunofluorescence, immunohistochemistry, and image acquisition

For immunofluorescence, ES cells were grown on Roboz slides (CellPoint Scientific). Immunostaining was performed as described previously (Leeb and Wutz 2007). Briefly, cells were fixed for 10 min in 4% PFA in PBS, permeabilized for 5 min in 0.1% Na citrate/0.5% Triton-X100, and blocked for 30 min in PBS containing 5% BSA and 0.1% Tween-20. DAPI (4',6'-diamidino-2-phenylindole) was used to stain the DNA. Vectashield (Vector Laboratories) was used as imaging medium. Immunohistochemistry was performed on frozen sections using the Ventana Discovery System. Immunofluorescence images were obtained at 20 \times magnification at room temperature with a fluorescence microscope (Zeiss Axioplan 2) using a Coolsnap fx CCD camera (Photometrics) and the MetaMorph image analysis software (Universal Imaging). Images of ES cell colonies and retinoic acid-differentiated cells were taken with a Zeiss Axiovert 200 microscope. A Zeiss Axioplan 2 microscope was used to analyze immunohistochemistry staining at a 20 \times magnification.

RNA and protein analysis

Northern analysis was performed using 5–15 μ g of RNA purified with Trizol (Invitrogen) as described previously (Leeb and Wutz 2007). Primers used to generate Northern probes are listed in Supplemental Table 3. Images were obtained using a Storm 860 Scanner (Molecular Dynamics) and Image Quant TL software. Proteins were extracted in RIPA buffer. Histones were acid-extracted in 0.2N HCl. α -Lamin B1, α -H3, and Ponceau S stainings were used to control for loading. The following antibodies were used: α -Ring1B (Atsuta et al. 2001), α -Mph2 (Isono et al. 2005), α -Mel18 (Santa Cruz Biotechnologies), α -Suz12 (Upstate Biotechnologies), α -Ezh2, α -H3K27me3 (Kohlmaier et al. 2004), α -H2AK119ub1 (Upstate Biotechnologies), α -H3 (Abcam), α -Nestin (Developmental Studies Hybridoma Bank), α -Lamin B1 (Abcam), smooth muscle actin (Thermo Scientific), glial fibrillary acidic protein (GFAP; Dako), α -1-Fetoprotein (Dako), and Troma-1 (Developmental Studies Hybridoma Bank). Secondary antibodies used were Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 488 goat anti-mouse IgG (Molecular Probes), HRP-conjugated Affinipure goat α -rabbit IgG, and HRP-conjugated, Affinipure goat α -mouse IgG (Jackson ImmunoResearch Laboratories).

Quantitative gene expression analysis

cDNA was generated from 2–4 μ g of DNase-treated total RNA using the SuperScript II Reverse Transcription kit (Invitrogen) and random hexamere primers. Quantitative real-time PCR

analysis was performed in triplicate using the Bio-Rad iQ Taq SYBR Green master mix on a Bio-Rad iCycler machine. The standard curve method was used for quantification of gene expression. Expression levels were normalized to L32 ribosomal protein. Error bars represent the standard deviation. Sequences for all primers used in this study are listed in Supplemental Table 3.

Affymetrix transcription analysis

ES cell cultures were carefully analyzed for ES cell morphology before microarray analysis. Gene expression profiles were established from Trizol-extracted total RNA of biological triplicates by Atlas Genomics using Affymetrix GeneChip 430 2.0 arrays, and were deposited in Gene Expression Omnibus (GEO) with the accession code GSE19076 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19076>). Normalization was performed using the MAS 5 algorithm. Pairwise comparisons between all genotypes were performed. Genes regulated at least twofold with a *P*-value of <0.05 were analyzed further. PcG target gene sets, CpG promoter classes, and pluripotency transcription factor-bound genes were annotated from published data sets (Boyer et al. 2006b; Kim et al. 2008; Ku et al. 2008). PcG target genes were defined as genes with a bivalent promoter status (Ku et al. 2008). One-hundred-sixty-eight genes, which were most regulated between the different genotypes, were selected, and a hierarchical clustering of the log-transformed, median-centered, and normalized expression was performed and represented as a heat map.

ChIP

ChIP was performed as described previously with minor modifications (Sado et al. 2005). For each experiment, 2×10^6 ES cells were used. Rabbit IgG was used as mock control. Immunoprecipitation was performed using Dynabeads Protein G (Invitrogen). Primer sequences for MLV provirus sequences were reported previously (Changolkar et al. 2008). Quantitative PCR was performed in duplicates. Error bars represent standard deviation. ChIP results were confirmed in two independent experiments. The ChIP data presented in Figure 5 was generated as described previously (Pasini et al. 2008).

The following antibodies were used: rabbit H3K27me3 (Upstate Biotechnologies), rabbit monoclonal H3K27me3 (Cell Signaling), rabbit H3 (Abcam), rabbit IgG (Sigma), rabbit Suz12 (Cell Signaling), Ring1B (K Helin and D Pasini, in prep.).

MLV copy number analysis

The copy number was analyzed using primers specific for one MLV integration site on chromosome 8. The fold change between wild-type and *Eed*-deficient or dKO ES cells was calculated using the standard curve method. Normalization between samples was performed using an intergenic region on chromosome 8. The MLV copy number was then set to 1 in wild-type ES cells.

DNA methylation analysis

DNA samples of wild-type, *Eed*^{-/-}, *Ring1B*^{-/-}, *Eed*^{-/-} *Ring1B*^{*fl*}, dKO, *Eed*^{-/-} *Eed*^{GFP}, and dKO^{*Eed*GFP} ES cells were digested with MspI and the CpG methylation-sensitive isoschizomere HapII. Primers used to generate the IAP-specific Southern probe can be found in Supplemental Table 3.

Accession of data sets

Gene expression profiles were deposited at GEO with the accession code GSE19076 (<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19076>).

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