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# The Polycomb Group Protein Suz12 Is Required for Embryonic Stem Cell Differentiation▽†

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**Polycomb group (PcG) proteins form multiprotein complexes, called Polycomb repressive complexes (PRCs). PRC2 contains the PcG proteins EZH2, SUZ12, and EED and represses transcription through methylation of lysine (K) 27 of histone H3 (H3). Suz12 is essential for PRC2 activity and its inactivation results in early lethality of mouse embryos. Here, we demonstrate that *Suz12*$^{-/-}$ mouse embryonic stem (ES) cells can be established and expanded in tissue culture. The *Suz12*$^{-/-}$ ES cells are characterized by global loss of H3K27 trimethylation (H3K27me3) and higher expression levels of differentiation-specific genes. Moreover, *Suz12*$^{-/-}$ ES cells are impaired in proper differentiation, resulting in a lack of repression of ES cell markers as well as activation of differentiation-specific genes. Finally, we demonstrate that the PcGs are actively recruited to several genes during ES cell differentiation, which despite an increase in H3K27me3 levels is not always sufficient to prevent transcriptional activation. In summary, we demonstrate that Suz12 is required for the establishment of specific expression programs required for ES cell differentiation. Furthermore, we provide evidence that PcGs have different mechanisms to regulate transcription during cellular differentiation.**

The evolutionarily conserved Polycomb group (PcG) proteins silence gene expression through the formation of multiprotein complexes. The Polycomb repressive complex 2 (PRC2) contains the PcG proteins EZH2, EED, and SUZ12 and catalyzes the di- and trimethylation of lysine 27 of histone H3 (H3K27me2 and H3K27me3, respectively) (8, 12, 19, 28). It has been suggested that this modification serves as a "docking-site" for the PRC1 complex and is required for maintaining transcriptional repression (8, 19). The three PcG subunits of PRC2 are all essential for embryonic development, and *Eed*$^{-/-}$, *Ezh2*$^{-/-}$, and *Suz12*$^{-/-}$ embryos all display severe defects during gastrulation (15, 30, 32). SUZ12 is required for PRC2 enzymatic activity, and *Suz12*$^{-/-}$ embryos die around 7 days postcoitus (32). Loss of Suz12 leads to global loss of H3K27me3 and to destabilization of Ezh2 (9, 32). These results demonstrate the critical role of Suz12 and PRC2 in regulating normal development and suggest that PRC2 controls the expression of genes essential for early embryogenesis.

Embryonic stem (ES) cells isolated from the inner cell mass (ICM) of preimplantation embryos (embryonic day 3.5 [E3.5]) are immortal and pluripotent. Their self-renewal requires leukemia inhibitory factor (LIF)-dependent Stat3 activation as well as the expression of ES cell-specific transcription factors like Nanog and Oct4 (2). ES cells can give rise to all the somatic cells of an organism and can be differentiated in vitro to all cell types. They are therefore attractive as a tool to study the molecular mechanisms that control cell fate decisions during development.

Cell fate decisions start taking place before embryo implantation, and ICM pluripotency is already lost during embryo gastrulation (~E7.5). Cell type specifications occur through the establishment of specific gene expression programs that require epigenetic-dependent transcriptional regulation (2). Epigenetic control of transcription involves modifications of both DNA and histones, and the factors that can "write" and "read" these modifications play a critical role during development (22).

Consistent with this model of development, it has not been possible to establish *Ezh2*$^{-/-}$ ES cell lines in tissue culture (30). In contrast, *Eed*$^{-/-}$ ES cells can be expanded in tissue culture even though the cells lack global levels of H3K27 methylation (26). *Eed*$^{-/-}$ ES cells have an increased expression of differentiation-specific genes, and the cells "tend" to lose pluripotency (4). The discrepancy between the phenotypes of *Ezh2*$^{-/-}$ and *Eed*$^{-/-}$ ES cells suggests that Ezh2 could have independent functions that do not involve H3K27 methylation. In addition, recent reports have shown that both PRC2 and the PRC1 are required to maintain the repression of differentiation-specific genes in mouse and humans ES cells as well as in human embryonic lung fibroblasts (TIG3) (4, 5, 21).

Here we describe the role of Suz12 in ES cell proliferation and differentiation. We show that Suz12 is essential for proper differentiation, but not proliferation, of ES cells. Importantly, we show that different mechanisms of PcG transcriptional regulation exist during development. This involves active recruitment of PcGs to repress gene expression; however, we also demonstrate that increased levels of H3K27me3 and PcG binding can also correlate with the activation of gene expression.

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## MATERIALS AND METHODS

**Derivation of ES cells, culture, genotype, and sex determination.** Blastocysts were isolated from the uterus of superovulated pregnant *Suz12*$^{+/-}$ female mice at 3.5 days postcoitus in M16 medium (Sigma). Single blastocysts were cultured on 0.5% gelatin-coated plates in Glasgow medium (Sigma) supplemented with glutamine (Gibco), nonessential amino acids (Gibco), sodium pyruvate (Gibco), 50 μM β-mercaptoethanol–phosphate-buffered saline (PBS; Gibco), and 20% ES-cell-tested fetal bovine serum (HyClone) in the presence of 2,000 U/ml of LIF (ESGRO). ICM outgrowths were expanded and kept in culture in the same basal medium with 10% fetal bovine serum and 1,000 U/ml of LIF. Genotyping of single clones was performed as described previously (32). Sex was determined by PCR using the following primers: Xist forward, 5′-GCTTTGTTTCACTTTC TCTGGTGC-3′; Xist reverse, 5′-ATTCTGGACCTATTGGGAAG-GGGC-3′; Sry forward, 5′-GCATTTATGGTGTGGTCCCGTG-3′; and Sry reverse, 5′-CC AGTCTTGCCTGTATGTGATGG-3′.

**ES cell karyotype and immunostaining.** Growing ES cells were treated with Colticin (10 μl/ml; Gibco) for 1 h. Cells were harvested, incubated for 18 min in hypotonic solution (75 mM KCl), and subsequently fixed in fixative solution (1 volume of acetic acid in 3 volumes of methanol). Metaphases were allowed to dry on slides overnight at 37°C and were stained with DAPI (4′,6′-diamidino-2-phenylindole; Sigma). For immunostaining, ES cells were cultured in normal ES cell medium on mitotically inactivated mouse embryonic fibroblasts (MEFs). Cells were fixed for 10 min in 4% buffered formaldehyde and stained with the antibodies indicated in the figure legends in the presence of 10% serum for 1 h in a humid chamber.

**Antibodies.** Immunoblotting and immunostaining were performed with the following antibodies: rabbit anti-Suz12, anti-H3K27me3, anti-H3K27me1 (where me1 indicates monomethylation), anti-H3K9me3, anti-H3K9me2, and anti-H3K4me2 from Upstate; rabbit anti β-tubulin from Santa Cruz; rabbit anti-H3K27me2 (33); mouse anti-EZH2 BD43 (32); mouse anti-H3K27me2/me3 (31); and rabbit anti-Nanog and rabbit anti-Oct4 from Abcam. Chromatin immunoprecipitation (ChIP) analysis was performed with the following antibodies: rabbit anti-Suz12 and anti-H3K27me3 from Upstate, rabbit anti-polymerase II and rabbit antihemagglutinin (Y11) from Santa Cruz, mouse monoclonal antibody to EZH2 (AC22) (32), mouse monoclonal antibody to BMI1 (AF27) (5), and two rabbit affinity-purified polyclonal antibodies to CBX8 (hPc3), LAST and GALD (5).

**Gene expression analysis.** Total RNA was extracted independently from three *Suz12*$^{+/-}$ (clones SBE 4, SBE5, and SBE6) and three *Suz12*$^{-/-}$ (clones SBE1, SBE7, and SBE8) ES cell clones. RNA was quantified, and equal amounts from the three *Suz12*$^{+/-}$ and the three *Suz12*$^{-/-}$ samples were pooled into one sample to reduce the experimental variation. Targets for microarray hybridization were synthesized according to the supplier's instructions (Affymetrix). Hybridization, washing, staining, scanning, and data analysis were performed at the Affymetrix microarray unit at the Institute of Molecular Oncology of the Italian Foundation for Cancer Research-European Institute of Oncology campus, Milan, Italy, according to the manufacturer's instructions. Expression levels were analyzed using Microarray Analysis Suite, version 5.0, statistical algorithm software (Affymetrix), using the default parameters and scaling (TGT value) signal intensities for all the GeneChip arrays to a value of 500. The *Suz12*$^{+/-}$ samples were used as a baseline condition for comparison with the *Suz12*$^{-/-}$ samples.

**Quantitative PCR and primers.** cDNA preparation and real-time quantitative PCR (qPCR) were performed following the manufacturer's instructions (Applied Biosystems). The analysis of the results was performed as described previously (32). For primer sequences see Table S2 in the supplemental material.

**EB formation and neuronal differentiation.** Embryoid bodies (EBs) were allowed to form in the absence of LIF in hanging drops containing 1,000 ES cells/20-μl drop on petri dish lids for 48 h. EBs were collected from the drops after 2 days and left in culture in noncoated petri dishes for the times indicated in the figure legends in ES medium in the absence of LIF. Medium was changed every 2 days. Neuronal differentiation followed the above EB formation protocol with the following modifications: EBs were treated from day 2 to day 5 with 0.5 μM all-*trans*-retinoic acid (ATRA) and plated on gelatin-coated dishes at day 7 to allow neuronal differentiation. For ChIP analysis EBs were formed as mass cultures by plating ES cells in suspension on noncoated petri dishes at the concentration of $5 \times 10^5$ cells/ml.

**ChIP.** ChIP assays were performed as described previously (6).

**Animal studies.** Animal care and experiments on live animals were performed at the University of Copenhagen in accordance with the Danish institutional and national guidelines (law number 726, 9 September 1993), and the studies were approved by the Dyreforsøgstilsynet committee (project number 2004/561-860).

## RESULTS

**Establishment of *Suz12*$^{-/-}$ ES cells.** To study the role of Suz12 in ES cell proliferation, we attempted to derive ES cells from the ICM of *Suz12*$^{-/-}$ blastocysts. ICM outgrowths isolated from blastocysts at E3.5 were expanded in tissue culture in the presence of LIF. Genotypes of the individual clones showed that both female and male *Suz12*$^{-/-}$ ES cells could be derived and maintained in culture (Fig. 1A). *Suz12*$^{-/-}$ ES cells do not display any morphological differences compared to wild-type and *Suz12*$^{+/-}$ cells (Fig. 1A and B, top panels). Moreover, the proliferation rate of *Suz12*$^{-/-}$ ES cells did not differ significantly from wild-type and *Suz12*$^{+/-}$ cells (data not shown). Importantly, *Suz12*$^{-/-}$ ES cells express normal levels of the ES cell markers *Oct4* and *Nanog* (Fig. 1C) and have a normal karyotype (Fig. 1D). Consistent with the requirement for Suz12 to stabilize Ezh2, *Suz12*$^{-/-}$ ES cells have reduced levels of Ezh2 (Fig. 1B). In order to further characterize the *Suz12*$^{-/-}$ ES cells, we stained for the expression of Oct4 and Nanog. Consistent with the expression data presented in Fig. 1C, *Suz12*$^{+/-}$ and *Suz12*$^{-/-}$ ES cells but not feeder cells expressed high levels of both Oct4 and Nanog (Fig. 2A and B), demonstrating that *Suz12*$^{-/-}$ ES cells present normal ES cell features.

**Suz12 is required for di- and trimethylation of H3K27 in ES cells.** To determine if Suz12 is required for the methylation of H3K27 in ES cells, we prepared cell lysates from proliferating *Suz12*$^{+/-}$ and *Suz12*$^{-/-}$ ES cells. Remarkably, *Suz12*$^{-/-}$ ES cells have no detectable H3K27me3 and strongly reduced levels of H3K27me2, whereas the methylation of other lysines on the histone tails remains unchanged (Fig. 1E). These results suggest that PRC2 is the major (if not the only) histone methyl transferase responsible for H3K27me3 in ES cells. In addition, they show that neither Suz12 nor of H3K27me is essential for the self-renewal of ES cells.

***Suz12*$^{-/-}$ ES cells express higher levels of differentiation-specific genes.** To further analyze the features of *Suz12*$^{-/-}$ ES cells, we compared the global gene expression profiles of *Suz12*$^{-/-}$ and *Suz12*$^{+/-}$ ES cells using Affymetrix oligonucleotide microarrays containing probes for more than 39,000 different mouse transcripts. Significantly, *Suz12*$^{-/-}$ ES cells contain increased levels of differentiation-specific genes, suggesting that *Suz12*$^{-/-}$ ES cells have differentiated features, despite displaying a normal stem cell phenotype (Fig. 3A). To validate the gene expression data, we determined the expression levels for 25 upregulated and 5 downregulated genes in *Suz12*$^{-/-}$ and *Suz12*$^{+/-}$ ES cells by real-time qPCR. As shown in Fig. S1A in the supplemental material, the microarray data were confirmed for all the tested genes, demonstrating the high accuracy of the expression profile data. Because these genes could be potential direct targets of the PRC2 complex, we tested Ezh2 and Suz12 binding and the presence of H3K27me3 modification within a 3-kb promoter region upstream of the transcription start site of the genes. ChIP analysis revealed that 10% (3/30) of the analyzed genes were direct targets of PRC2 (see Fig. S1 in the supplemental material) and that Ezh2 binding and accumulation of H3K27me3 at the *Tbx3*, *Satb2*, and *Otx2* promoters are dependent on Suz12 (see Fig. S1 in the supplemental material). These results are consistent with the requirement of Suz12 for Ezh2 enzymatic activity.

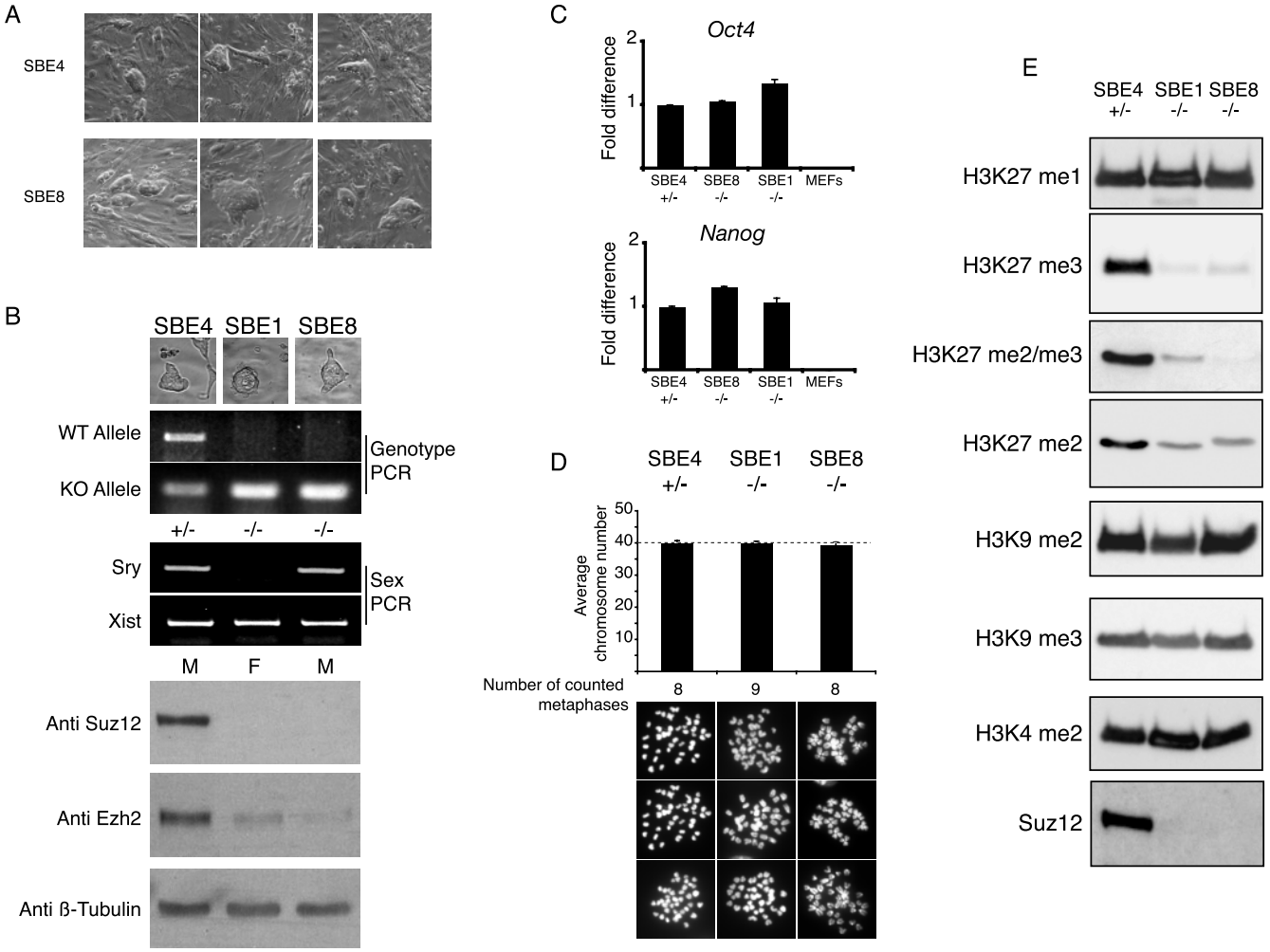


FIG. 1. Analysis of *Suz12*$^{-/-}$ ES cells. (A) Phase-contrast pictures of growing *Suz12*$^{+/-}$ (clone SBE4) and *Suz12*$^{-/-}$ (SBE8 8) ES cell clones growing on feeders cells. (B) Phase-contrast pictures (top) of growing *Suz12*$^{+/-}$ (clone SBE4) and *Suz12*$^{-/-}$ (SBE1 and SBE8) ES cell clones on gelatin-coated plates. Genotype and sex determination PCRs (middle) show that both male (M) and female (F) *Suz12*$^{-/-}$ ES cells can be derived. Immunoblots (bottom) using antibodies to Suz12, Ezh2, and β-tubulin are shown. β-Tubulin served as a loading control. KO, knockout; WT, wild type. (C) RNA expression levels of the ES cell markers *Oct4* and *Nanog* in Suz12$^{+/-}$ and Suz12$^{-/-}$ ES clones compared to MEFs. (D) Metaphase spreads showing a normal karyotype for Suz12$^{+/-}$ and Suz12$^{-/-}$ ES clones. At the top the average numbers of counted chromosomes per cell are given. The bottom panels are representative pictures of metaphase spreads from Suz12$^{+/-}$ and Suz12$^{-/-}$ ES clones. (E) Immunoblots of different histone H3 lysine (K) modifications using specific antibodies to the indicated proteins and their modifications.

In agreement with our results, *Tbx3*, *Satb2*, and *Otx2* were identified as direct PRC2 targets in whole genomic screenings aimed at identifying PRC targets genes in ES cells (4, 21). Surprisingly, several of the other PRC2 target genes identified in previous studies were not identified as upregulated in *Suz12*$^{-/-}$ ES cells. One explanation for this could be that the expression of these genes is not detectable in *Suz12*$^{+/-}$ ES cells by microarray technology, and they were therefore discarded in the statistical analysis. To address this possibility, we compared the expression of three differentiation-specific transcription factors, *Gata1*, *Gata4*, and *Hnf4*, in *Suz12*$^{+/-}$ and *Suz12*$^{-/-}$ ES cells. As shown in Fig. 3B, *Gata1*, *Gata4*, and *Hnf4* expression was indeed higher in the *Suz12*$^{-/-}$ ES cells than in *Suz12*$^{+/-}$ cells. Consistent with the repression by PRC2, the H3K27me3 repressive marker was present at the *Gata1*, *Gata4*, and *Hnf4* promoters and lost in *Suz12*$^{-/-}$ ES cells (Fig. 3B). These results are consistent with the model by which PRC2 represses the expression of differentiation-specific genes in ES cells (4, 21) and demonstrate that Suz12 is required for this activity.

Interestingly, we also found that the expression of the paternally imprinted *H19* gene is significantly increased in *Suz12*$^{-/-}$ ES cells relative to expression in to *Suz12*$^{+/-}$ ES cells (Fig. 3C; see also Table S1 and Fig. S1A in the supplemental material). This result is consistent with the requirement of *Eed* for the imprinting of different autosomal loci (24), suggesting that imprinting could also be affected in *Suz12*$^{-/-}$ embryos. To understand if the PRC2 complex and H3K27me3 are directly involved in the maintenance of *H19* repression, we analyzed the binding of Ezh2 and Suz12 and the presence of histone H3K27me3 modification both at the *H19* promoter (−5 to +1 kb with respect to the transcription start site) and at the CG regulatory element placed at the 3′ end of the *H19* locus. ChIP

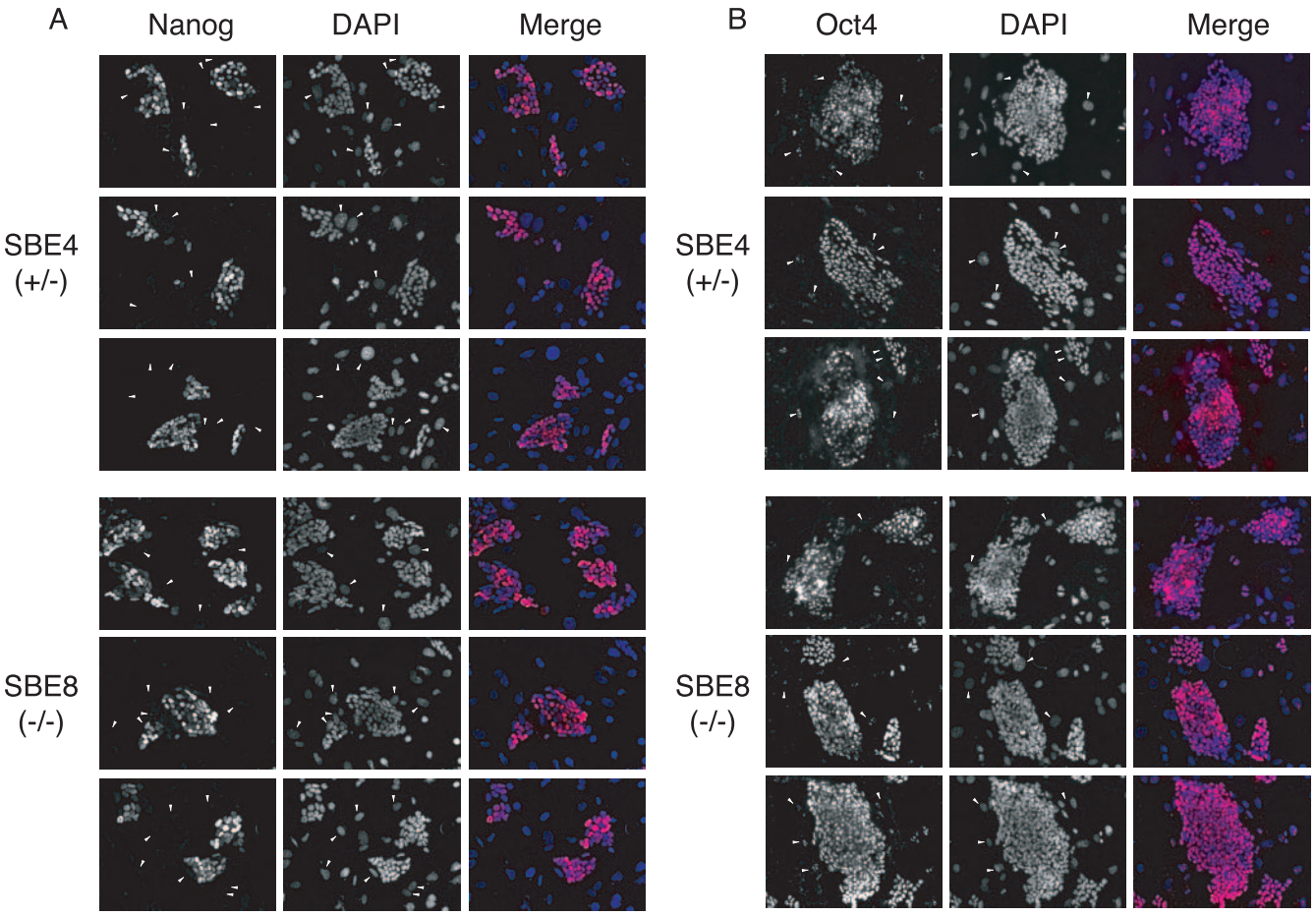


FIG. 2. Expression of Nanog and Oct4 in *Suz12*$^{-/-}$ ES cells. Immunostaining of Suz12$^{+/-}$ and Suz12$^{-/-}$ ES cells growing on feeders cells shown with anti-Nanog-specific (A) and anti-Oct4-specific (B) antibodies reveals expression of both ES cell markers in Suz12$^{+/-}$ and Suz12$^{-/-}$ ES cells but not in feeder cells (arrows).

analysis showed that neither PRC2 nor H3K27me3 was present in the analyzed genomic regions (see Fig. S1A in the supplemental material), suggesting that either the PRC2 regulation is indirect or that it involves the recruitment of PRC2 activity at different sites or in earlier developmental stages.

**Suz12 is required for differentiation of ES cells.** Our findings are consistent with the fact that *Suz12*$^{-/-}$, *Ezh2*$^{-/-}$, and *Eed*$^{-/-}$ embryos are all able to implant but fail to undergo further development, and they suggest that PRC2 and its enzymatic activity are required for the differentiation processes taking place during development. To investigate this, we took advantage of the fact that ES cells undergo neuronal differentiation in the presence of ATRA. Thus, we treated *Suz12*$^{+/-}$ and *Suz12*$^{-/-}$ ES cells with ATRA and found that while *Suz12*$^{+/-}$ ES cells efficiently form neurons, we were unable to detect any neurons in ATRA-treated *Suz12*$^{-/-}$ ES cells (Fig. 4A; see also Fig. S2A in the supplemental material). Consistent with this, the expression of two neuron-specific markers, *GluR6* and *Gad65*, was strongly activated in *Suz12*$^{+/-}$ differentiated cells, but not in *Suz12*$^{-/-}$ cells (Fig. 4B; see also Fig. S2B in the supplemental material).

The differentiation of ES cells in tissue culture first requires the formation of EBs, followed by terminal differentiation (18). When ES cells are cultured in suspension in the absence of LIF, they form EBs in which cells differentiate into the three germ layers. EBs are the in vitro developmental equivalent of the mouse embryo at the egg cylinder stage (E5.5 to E6). They contain an outer endoderm layer and differentiating cells in the center of the body, together with epithelial-like cavities. Because EBs represent the developmental stage when PRC2 mutant embryos display morphological abnormalities and die, we investigated whether EBs derived from *Suz12*$^{-/-}$ ES cells present any defects in differentiation. As shown in Fig. 4C and Fig. S2C in the supplemental material, EBs from *Suz12*$^{+/-}$ cells are morphologically normal, presenting an endodermal outer layer and organized epithelial structures (Fig. 4C, left frames). In contrast, *Suz12*$^{-/-}$ EBs display a disorganized structure and are often smaller. In addition, *Suz12*$^{-/-}$ EBs fail to form a proper endodermal layer and lack any form of internal organized structures (Fig. 4C, right frames; see also Fig. S2C in the supplemental material). Consistent with the ability of *Suz12*$^{-/-}$ ES cells to form EBs in vitro, *Suz12*$^{-/-}$ ES cells were able to form teratomas when subcutaneously injected into immunodeficient mice (data not shown).

To further support these results, we compared the expression of specific ES cell markers as well as the expression of

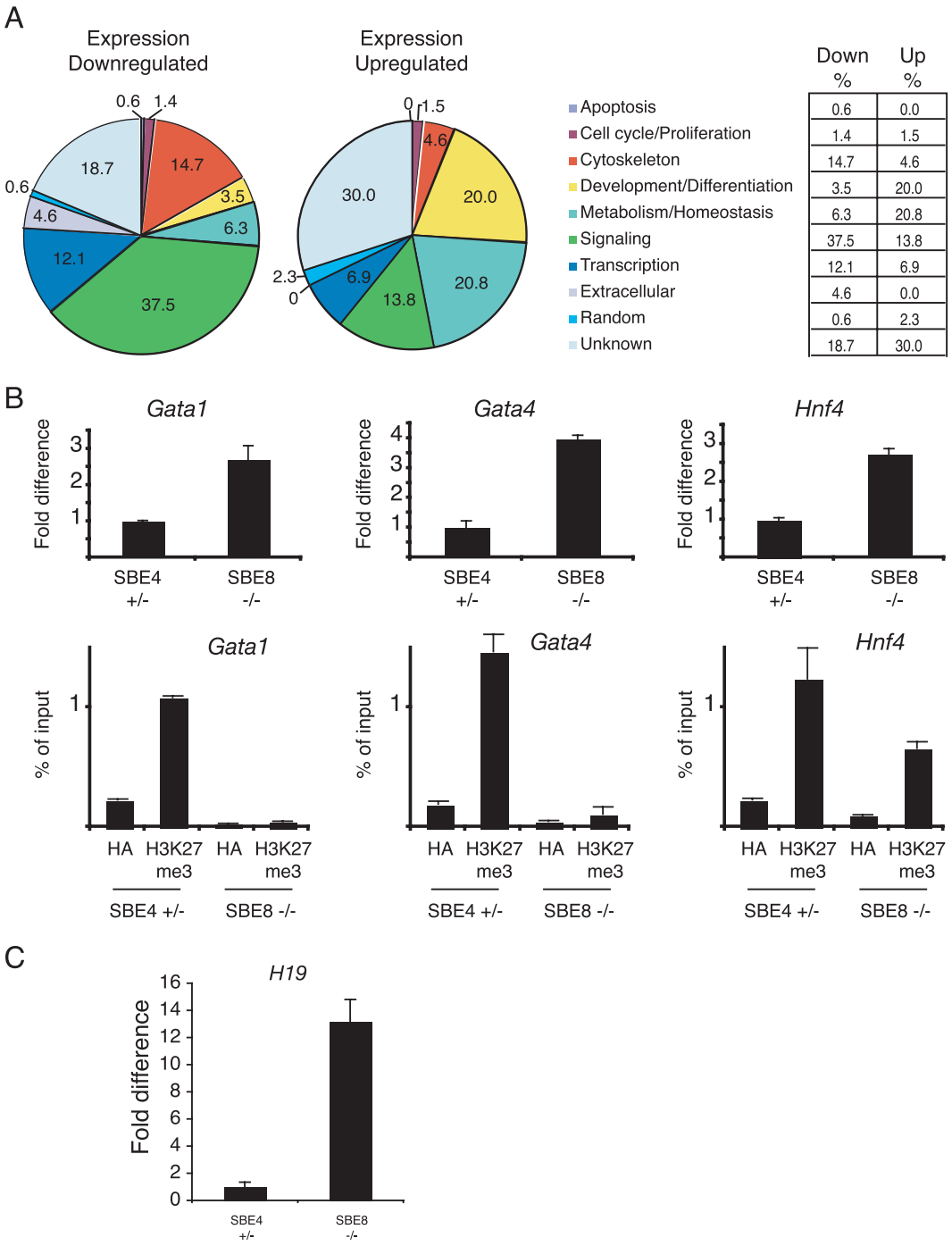


FIG. 3. Suz12 is required for the regulation of a large number of genes involved in development, differentiation, and homeostasis. (A) Functional clustering of gene expression changes between *Suz12*$^{+/-}$ and *Suz12*$^{-/-}$ ES cells. Expression downregulation refers to the functional clustering of genes whose expression was downregulated in Suz12$^{-/-}$ compared to Suz12$^{+/-}$ ES cells. Expression upregulation refers to the functional clustering of genes whose expression was upregulated in Suz12$^{-/-}$ compared to Suz12$^{+/-}$ ES cells. (B) Expression (top) and ChIP analysis using the indicated antibodies (bottom) were determined by real-time qPCR. HA, hemagglutinin. (C) Expression levels of H19 in *Suz12*$^{+/-}$ and *Suz12*$^{-/-}$ male ES clones.

genes specifically activated during gastrulation in *Suz12*$^{+/-}$ and *Suz12*$^{-/-}$ differentiating ES cells. Consistent with the lack of germ layer formation, the expression of gastrulation-specific genes (*Brachyury*, *Pax3*, *Pax7*, *Fgf3*, and *Wnt3a*) was activated only in *Suz12*$^{+/-}$ and not *Suz12*$^{-/-}$ EBs (Fig. 5B). Moreover, the expression of *Oct4* and *Nanog* was fully repressed during ES cell differentiation in *Suz12*$^{+/-}$ ES cells, whereas the expression of the two stem cell transcription factors was only partly repressed in *Suz12*$^{-/-}$ EBs (Fig. 5A; see Fig. S2D in the supplemental material), with expression levels approximately

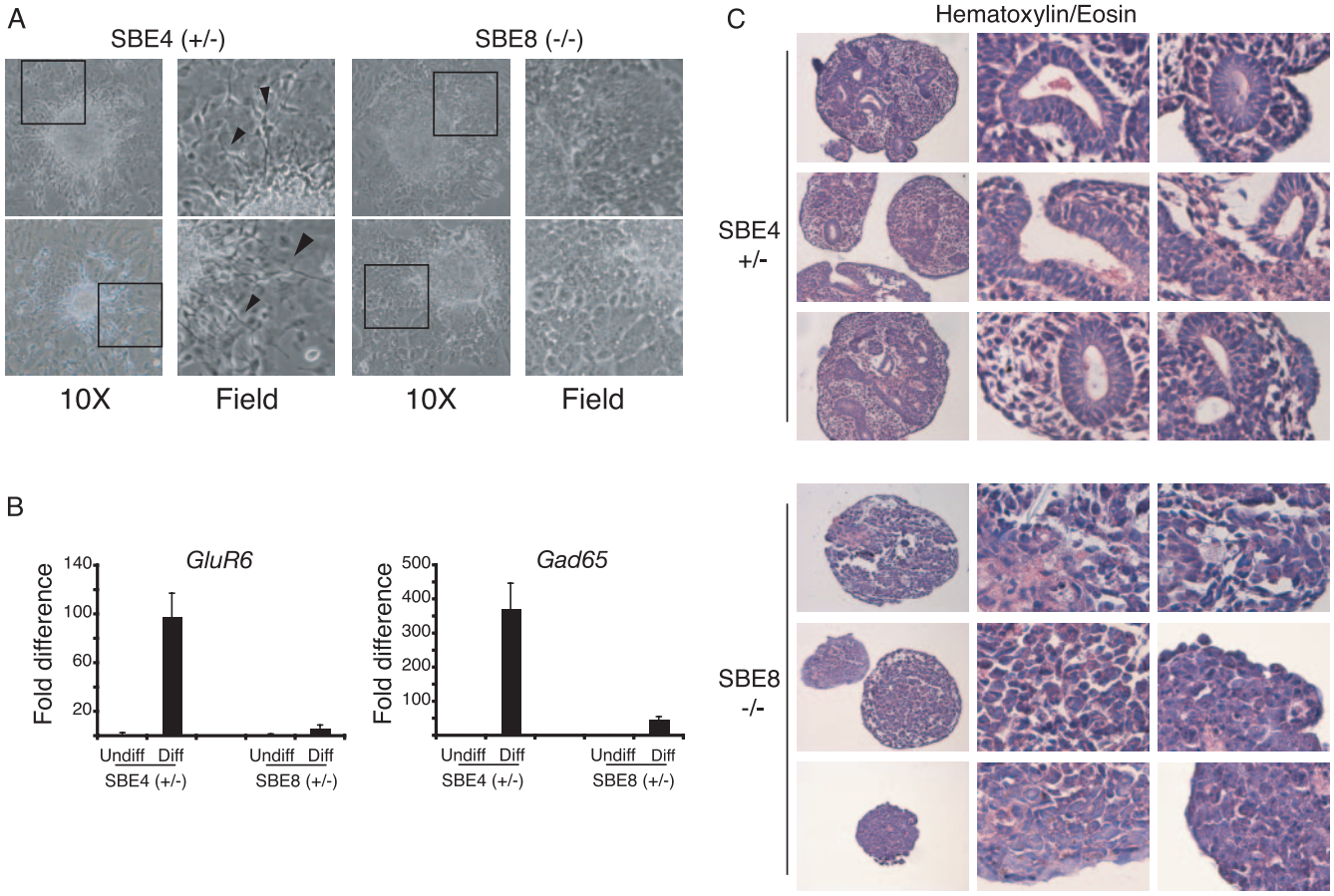


FIG. 4. *Suz12*$^{-/-}$ ES cells fail to undergo proper differentiation. (A) Phase-contrast pictures of neuronal differentiation of *Suz12*$^{+/-}$ and *Suz12*$^{-/-}$ ES cells. Arrows in the left panels show neuron formation in *Suz12*$^{+/-}$ cells, while the right panels show lack of neuron formation in *Suz12*$^{-/-}$ cells. (B) Expression levels of two specific neuronal markers *GluR6* and *Gad65* showing strong activation in differentiated *Suz12*$^{+/-}$ cells and no activation in *Suz12*$^{-/-}$ cells. Undiff, undifferentiated; diff, differentiated. (C) Hematoxylin and eosin staining of 7-day EBs formed by *Suz12*$^{+/-}$ and *Suz12*$^{-/-}$ ES cells. Top panels show normal morphology of *Suz12*$^{+/-}$ EBs. High-magnification fields highlight outer endodermal layers and epithelium-like cavities. Bottom panels show *Suz12*$^{-/-}$ EBs that lack forms of organized structures and that are often smaller.

50-fold higher in Suz12$^{-/-}$ EBs at 9 days of differentiation (Fig. 5A, right). Western blots presented in Fig. 5C further confirm this result and show that Oct4 and Nanog protein levels are indeed still expressed in *Suz12*$^{-/-}$ differentiated ES cells but not in the control cells. Similar expression results were also obtained for other genes like *Fgf4*, *Fgf17*, and *Pou2F3* that are expressed in ES cells and repressed upon differentiation (Fig. 4A).

In addition, due to the inability of *Suz12*$^{-/-}$ ES cells to terminally differentiate into neurons, we tested whether *Suz12*$^{-/-}$ ES cells are also impaired in the ability to differentiate into early neuronal precursors. We analyzed the expression of the early neuronal markers *Sox1*, *Nestin*, *Musashi*, and *Calb2* during *Suz12*$^{+/-}$ and *Suz12*$^{-/-}$ ES cell differentiation. Consistent with the lack of terminal differentiation, the activation of the expression of *Sox1*, *Nestin*, *Musashi*, and *Calb2* during ES cell differentiation was observed in only *Suz12*$^{+/-}$ and not *Suz12*$^{-/-}$ ES cells. This result showed that *Suz12*$^{-/-}$ ES cells are impaired in the proper activation of the expression of neuronal precursor markers and suggest that the differentiation defects of *Suz12*$^{-/-}$ ES cells occur during the early commitment phase of differentiation. Taken together, these results demonstrate that Suz12 is required for ES cell differentiation and that the lack of Suz12 results in both deregulation of ES cell-specific genes and the inactivation of genes required for early embryogenesis.

**Mechanisms for PRC-mediated transcriptional regulation during differentiation.** Recently, we have identified genes targeted by PRC2 and PRC1 in a human diploid fibroblast cell line, TIG3 (5). Interestingly, the genes presented in Fig. 5A and B were identified as direct targets of both PRCs in TIG3 cells, suggesting that the PRCs remain associated with the promoters after their recruitment during early embryogenesis. To determine if the binding of the PRCs is conserved between human and mouse, we performed ChIP analysis in MEFs (see Fig. S3A and B in the supplemental material). While Ezh2, Suz12, and histone H3K27me3 were present on all promoters, the PRC1 proteins Cbx8 and Bmi1 were not. Significant Cbx8 binding was detected at the *Brachyury*, *Pax3*, *Pax7*, *Fgf4*, and *Oct4* promoters, while significant Bmi1 binding was detected at all promoters except for the *Pou2f3*. The differential binding could be due to cell type differences and suggests that the composition of PRC1 may differ from one cell type to another. However, the results show that PRC2 binding at these genes is fully conserved between mouse and human.

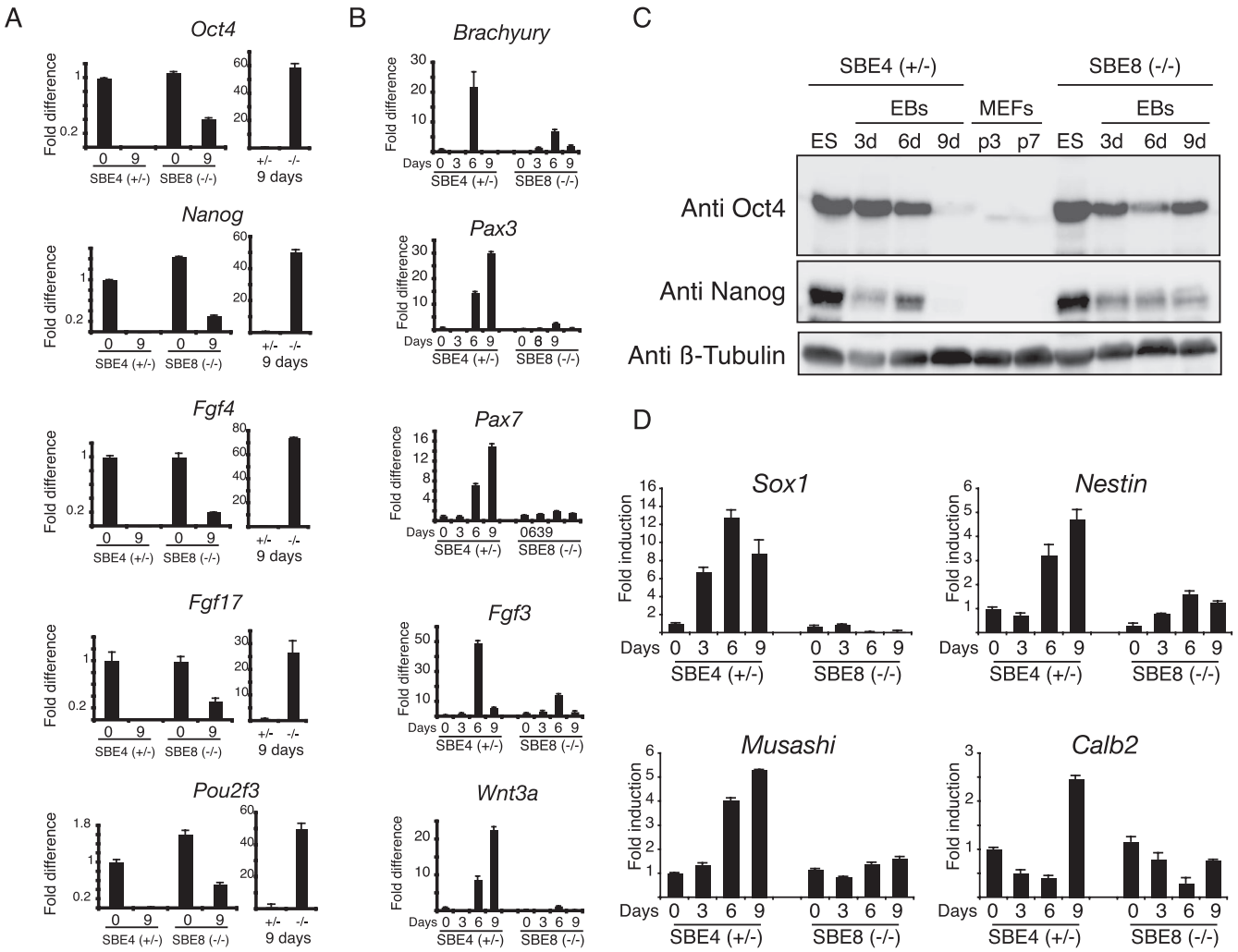


FIG. 5. *Suz12*$^{-/-}$ ES cells fail to repress ES cells markers and to activate differentiation-specific genes upon induction of differentiation. (A) Expression levels of *Oct4*, *Nanog*, *Fgf4*, *Fgf17*, and *Pou2f3* in ES cells and in 9-day differentiated EBs determined by real-time qPCR. Right panels highlight the expression differences between Suz12$^{+/-}$ and Suz12$^{-/-}$ in 9-day differentiated EBs. (B) Expression levels of gastrulation markers in ES cells and in EBs at 3, 6, and 9 days after induction of differentiation. (C) Immunoblotting for Oct4 and Nanog during *Suz12*$^{+/-}$ and *Suz12*$^{-/-}$ ES cell differentiation showing repression of Oct4 and Nanog expression in *Suz12*$^{+/-}$ but not *Suz12*$^{-/-}$ ES cells. (D) Expression levels of *Sox1*, *Nestin*, *Mausashi*, and *Calib2* during *Suz12*$^{+/-}$ and *Suz12*$^{-/-}$ ES cell differentiation showing the lack of activation of the neuronal precursor marker in *Suz12*$^{-/-}$ cells.

Interestingly, while *Brachyury*, *Pax3*, *Pax7*, *Wnt3A*, and *Fgf3* are expressed at low levels in ES cells and become activated upon differentiation (Fig. 4 and 5B), *Fgf4*, *Pou2F3*, *Fgf17*, *Nanog*, and *Oct4* are expressed in ES cells and become repressed upon differentiation (Fig. 5A). Nanog and Oct4 are essential transcription factors for ES cell self-renewal (17, 25) and function both as activators and repressors of important developmental regulators (3, 23). Pou2f3 is a member of the Oct transcription factor family, but its biological role is poorly characterized. Instead, Fgf4 and Fgf17 function as signaling molecules that play a crucial role in the control of development. Fgf4, for example, is required for the proliferation of trophoblast stem cells (14).

To start addressing the mechanisms regulating the expression of the PcG target genes during early differentiation, we analyzed the recruitment of Ezh2, Suz12, and H3K27me3 in both *Suz12*$^{+/-}$ and *Suz12*$^{-/-}$ ES cells induced to undergo differentiation. Surprisingly, we found that even though these two groups of genes are inversely expressed during ES cell differentiation (Fig. 5A and B), increased amounts of H3K27me3 associate with all the promoters in a Suz12-dependent manner during ES cell differentiation (Fig. 6 and 7A). In fact, consistent with the requirement of Suz12 for Ezh2 histone methyl transferase activity, Ezh2 binding and H3K27me3 association were abolished in *Suz12*$^{-/-}$ differentiated ES cells (Fig. 6 and 7A). This result demonstrates that increased association of histone H3K27me3 does not prevent transcription and indicates that this modification could have a role in transcriptional activation. Despite the binding of PRC2 to the *Oct4* and *Nanog* promoters in MEFs (see Fig. S3 in the supplemental material), we were unable to detect any significant PRC2 binding and H3K27me3 association to these promoters during ES cell differentiation (see Fig. S4 in the supplemental material), suggesting that

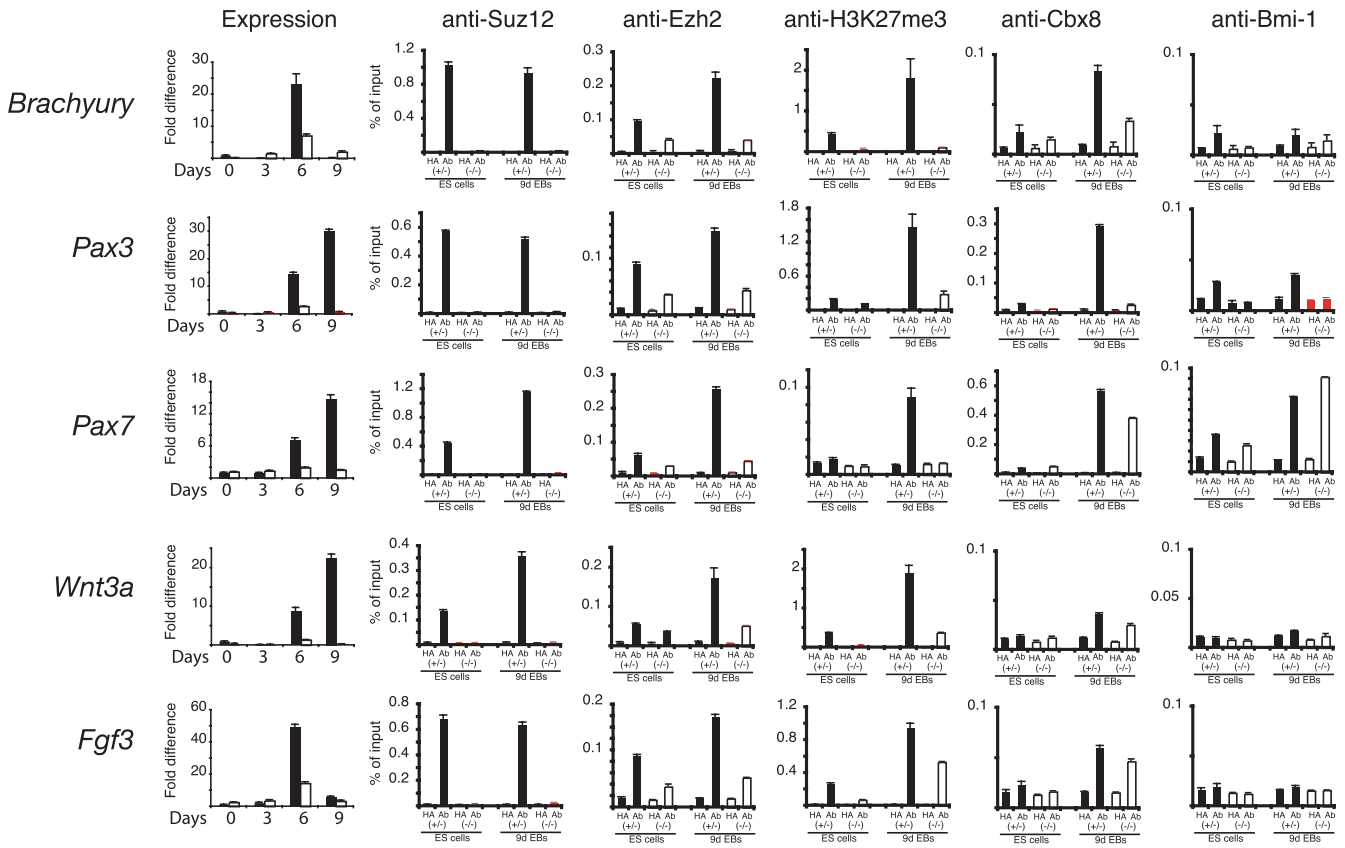


FIG. 6. PcG binding does not prevent transcriptional activation. ChIP analyses performed on promoters of genes that are activated during differentiation of ES cells. Real-time qPCR was used to determine the expression levels of the genes, and values were normalized as described in Materials and Methods. Antibodies specific for Ezh2, Suz12, Cbx8, Bmi1, H3K27me3, and the hemagglutinin (HA) epitope (negative control) were used for ChIPs. Enrichment is given as a percentage of input. Black bars, *Suz12*$^{+/-}$ cells; red bars, *Suz12*$^{-/-}$ cells; d, day; Ab, antibody.

PRC2 indirectly regulates these promoters during the early events of ES cell differentiation.

To understand if PRC1 is also recruited to these PRC2 target promoters, we analyzed the binding of two subunits of PRC1, Cbx8 and Bmi1, in *Suz12*$^{+/-}$ and *Suz12*$^{-/-}$ ES cells. Consistent with the idea that both PRC1 and PRC2 are required for the regulation of the expression of common targets (4, 5, 21), we found that five of these promoters (*Brachyury*, *Pax3*, *Pax7*, *Fgf3*, and *Fgf4*) showed significant binding of Cbx8 in differentiated EBs, while only one promoter (*Pax7*) showed binding of Bmi1 (Fig. 6 and 7A). However, in contrast to the model that has PRC1 recruitment dependent on its ability to specifically bind H3K27me3 (7, 8, 19), we found that PRC1 recruitment is independent of H3K27me3 on three of the five Cbx8 targets (*Pax7*, *Fgf3*, and *Fgf4*) and on the Bmi1 target (*Pax7*). This result, demonstrates that PRC1 can be recruited to target genes in the absence of a functional PRC2 complex and independently of H3K27me3.

Interestingly, Cbx8 and Bmi1 are not present on PcG target genes in nondifferentiated ES cells (Fig. 6 and 7A and data not shown). To obtain a potential explanation for this, we prepared cell lysates from *Suz12*$^{+/-}$ and *Suz12*$^{-/-}$ ES cells at different stages of differentiation, and from proliferating (passage 3 [P3]) and senescent (P7) MEFs (Fig. 5B). Remarkably, Cbx8 is not detectably expressed in undifferentiated ES cells but accumulates during differentiation (Fig. 7B). Interestingly, Cbx8 expression is not fully induced in *Suz12*$^{-/-}$ differentiated ES cells, which could be a result of the lack of proper differentiation of these cells. Unlike Cbx8, Bmi1 is expressed in ES cells and further accumulates 3 days after induction of differentiation. However, since Bmi1 is not recruited to PRC1 target genes in nondifferentiated cells, this may suggest that Bmi1 recruitment is dependent on Cbx8 expression. In addition, slower-migrating forms, which might result from phosphorylations, as suggested by previously published findings, (35), appear during the differentiation of the ES cells with kinetics similar to Cbx8 accumulation. These forms become prominent in MEFs, which may suggest that posttranslation modifications of Bmi1 could be a regulatory mechanism for the recruitment of PRC1 to target genes.

## DISCUSSION

The development of the mammalian organism requires the specification of more than 200 different cell types in a process that takes place prior to and immediately after the implantation of the embryo. Cell type specification is controlled by gradients of tissue-specific transcription factors in concert with a host of epigenetic regulators. Histone modifying enzymes, like the PRCs, are believed to play a critical role in this pro-

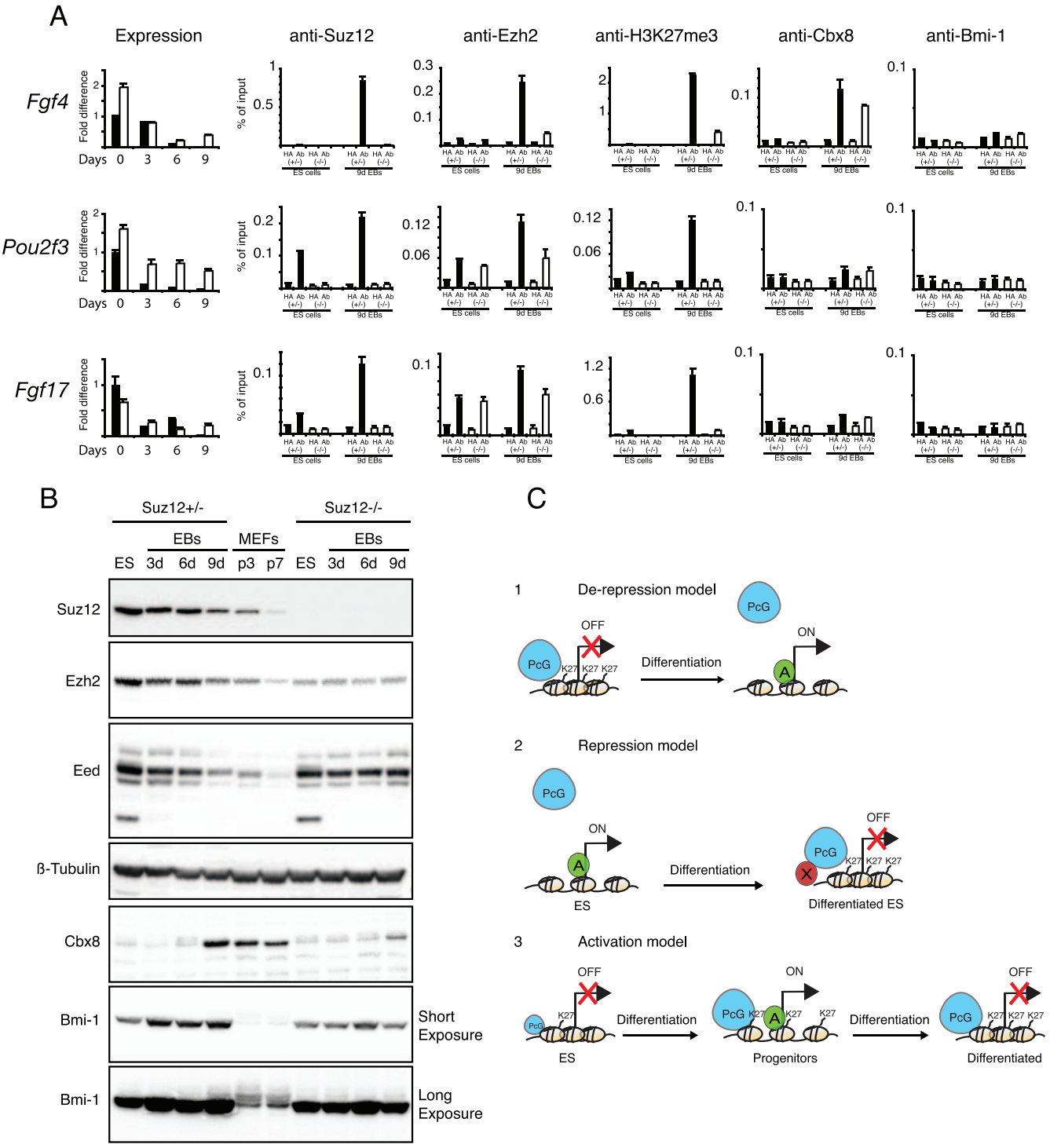


FIG. 7. PRC2 is actively recruited to repress gene transcription during ES cell differentiation. (A) ChIP analysis of the promoter regions of repressed genes during ES cell differentiation using antibodies against Ezh2, Suz12, Cbx8, Bmi1, and histone H3K27me3. Results of ChIPs and qPRCs in *Suz12*$^{+/-}$ (black bars) and *Suz12*$^{-/-}$ ES cells (red bars) are shown. The expression profile for each gene during differentiation is also presented (far left). HA, hemagglutinin; Ab, antibody. (B) Immunoblots of Suz12, Ezh2, Eed, Cbx8, and Bmi1 in *Suz12*$^{+/-}$ and *Suz12*$^{-/-}$ ES cells and in proliferating (P3) and senescent (P7) MEFs. β-Tubulin served as a loading control. (C) Different models for how the PcG proteins regulate transcription during differentiation. In the derepression model (1) the PcGs repress the expression of differentiation-specific genes in proliferating ES cells. The loss of PcG binding during differentiation leads to the activation or derepression of transcription. In the repression model (2) the PcGs are specifically recruited to target genes that undergo transcriptional repression during differentiation. In the activation model (3) PcGs (PRC2) accumulate on a subset of target genes during differentiation despite their transcriptional activation. In this model we propose that the binding of transcriptional activators is sufficient to overcome the PcGs. The binding of the PcGs could be important for the repression of the target genes during terminal differentiation and in this way preprogram the target genes during early development.

cess. PRC2 is required for correct expression of the *HOX* genes in later development (1, 20, 34), but defects in embryos lacking functional PRC2 arise earlier than *HOX* expression, suggesting additional functions of the complex (16, 30, 32).

In this work we have shown that even though *Suz12*$^{-/-}$ ES cells proliferate and appear normal, their ability to undergo proper differentiation is compromised. We provide evidence that this is due to the failure to establish the correct transcription programs during early embryogenesis. This assumption is in agreement both with the fact that *Suz12*$^{-/-}$ and *Eed* mutant ES cells expression patterns are altered toward a more differentiated phenotype and with the finding that Ezh2 activity is required for correct development before the four-cell stage during embryogenesis (4, 13). Similarly to *Suz12*$^{-/-}$ ES cells, Eed mutant ES cells can be maintained in culture, and, despite their tendency to differentiate (4), they can efficiently form EBs in vitro and contribute to gastrulation in vivo in chimeric embryos (27). Interestingly, the fact that Ezh2 (30), but not Eed and Suz12, is required for ES cell proliferation indicates that Ezh2 has additional functions independent of H3K27 methylation. This notion is supported by data obtained in *Drosophila*, where mutants of the *Ezh2* homologue *E(z)* have additional phenotypic defects to those observed in *Esc* (dEed) and *Su(z)12* (dSuz12) mutant flies (1, 20, 34).

The effects of the lack of PRC2 transcriptional control are more evident when *Suz12*$^{-/-}$ ES cells are induced to differentiate. We found that *Suz12* is required for proper ES cell differentiation. Key developmental transcription factors such as *Brachyury*, *Pax3*, and *Pax7* as well as early neuronal markers such as *Sox1*, *Nestin*, *Musashi*, and *Calb2* are not activated by differentiation signals in the absence of Suz12. Moreover, during differentiation Suz12 appears to be required for the repression of genes essential for ES cell self-renewal, such as *Nanog* and *Oct4*. This could be a consequence of the differentiation failure of the *Suz12*$^{-/-}$ ES cells, but since ectopic expression of *Nanog* can block ES cell differentiation in vitro (11), sustained *Nanog* expression in the *Suz12*$^{-/-}$ ES cells could also contribute to the impairment of proper differentiation of the *Suz12*$^{-/-}$ ES cells. Our results showing that PcGs bind directly to the *Nanog* and *Oct4* promoters in MEFs and that they are associated with H3K27me3 support the notion that the expression of these promoters is controlled by the PcGs. In any event, it would be extremely interesting to know if the differentiation defects of *Suz12*$^{-/-}$ ES cells depend on lack of Nanog and/or Oct4 repression; however, this experiment is unfortunately not feasible because ES cells do not grow in the absence of the two transcription factors (25, 29).

Our results demonstrating the accumulation of the PRCs and H3K27me3 on promoters for differentiation-induced genes is very exciting and support our previous findings that PcG-binding and H3K27me3 accumulation might not be sufficient to prevent transcription (5). In our previous work (5), we proposed that PcG binding to active promoters in progenitor cells preprograms these genes for repression during terminal differentiation. Here, we have provided evidence that the PcGs are indeed specifically recruited to active promoters in progenitor cells. Moreover, our results could further suggest that the recruitment of the PcGs to these target genes may be required for their transcriptional activation.

In addition to demonstrating the essential role of Suz12 in ES cell differentiation, our analysis of PcG target genes suggests a number of mechanisms by which the PcGs control transcription. So far the only mechanism that is backed up by experimental data is outlined in the derepression model (Fig. 7C, model 1). This model involves the dissociation of PcG proteins upon induction of differentiation and allows activation of target genes and subsequent differentiation. Several experimental results using myoblasts (10), ES cells (4, 21), and NTera2 cells (5) support this model. ChIP analysis presented in this work further supports the derepression model showing that differentiation-specific genes are indeed repressed by the PcGs in *Suz12*$^{+/-}$ ES cells and that this repressive activity is lost in *Suz12*$^{-/-}$ ES cells.

In this study, we have shown that the PcGs are also actively recruited to target genes upon induction of differentiation. Surprisingly, we found that this recruitment can correlate with either transcriptional repression or activation of genes during differentiation.

In the first case, we found that differentiation signals lead to recruitment of PRC2 to target genes and that this recruitment correlates with repression of transcription. Importantly, this repression is dependent on Suz12, suggesting that direct binding of PRC2 plays a critical role in the repression of these genes. Based on these results we propose a mechanism, summarized in the repression model presented in Fig. 7C, whereby the PcGs regulate transcription of specific target genes during differentiation. If this model is accurate, the identification of the signaling pathways and the factors that trigger the specific recruitment of the PRCs will be important for understanding how differentiation is regulated.

In the second case, we found increased levels of PRC2 on the promoters of genes activated during differentiation. Since PRC2 is believed to function as a repressor of transcription, this is an intriguing result. Furthermore, the striking observation that the genes were not activated in *Suz12*$^{-/-}$ ES cells, i.e., in the absence of PRC2 recruitment, could suggest that PRC2 has a direct role in the transcriptional activation of these targets. However, it is important to stress that the lack of transcriptional activation could be a consequence of the failure of *Suz12*$^{-/-}$ ES cells to undergo proper differentiation. In this case, the early recruitment of the PRCs could simply epigenetically predispose these genes for later repression. A model for how PcGs may participate in the activation of target genes is depicted in Fig. 7C.

In conclusion, our results and those from other laboratories demonstrate that Suz12 and PRC2 are required for ES cell differentiation, most likely by directly controlling specific gene expression during cellular commitment. ES cells become an attractive tool for regenerative therapy due to their in vitro differentiation potential. Embryo cloning by somatic cell nuclear transfer (SCNT) could give important therapeutic advantages as a source of ES-like cells that can be in vitro differentiated and used for autologous implants. Low efficiency in therapeutic cloning is the major obstacle to overcome because epigenetic commitment of somatic cells is a major problem in successful embryo cloning. The failure to achieve correct reactivation of genes like *Nanog* and *Oct4* has been linked to the failure of embryos obtained by SCNT to implant and successfully develop. Manipulation of PRC2 activity could therefore

contribute to the reprogramming of somatic cells and increase the efficiency of successful SCNT embryo cloning.


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