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DISSERTATION

The Role of Polycomb Group Proteins in X Chromosome Inactivation and Embryonic Stem Cell Biology

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Doktor der Naturwissenschaften (Dr. rer. nat.)

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Contents

Zusammenfassung

Polycomb Proteine haben eine wichtige Bedeutung für die Regulation von zellulären Differenzierungsprozessen, Tumorigenese und Imprinting in Säugetieren. Sie sind evolutionär konserviert und dafür verantwortlich die Transkription von Genen stabil über mehrere Zellteilungen epigenetisch zu hemmen. In genomweiten Studien wurde nachgewiesen, dass Polycomb Proteine in embryonalen Stammzellen an mehr als 2500 Promotoren binden. Gene, die von Polycomb Proteinen gebunden werden, haben eine zentrale Steuerfunktion in der embryonalen Entwicklung. Es wurde die Hypothese aufgestellt, dass in frühen embryonalen Zellen durch Polycomb Proteine epigenetische Muster gesetzt werden, die für Differenzierungsprozesse notwendige dynamische Änderungen im Transkriptionsprofil ermöglichen. Zwei unterschiedliche Polycomb Komplexe wurden biochemisch nachgewiesen. Beide besitzen enzymatische Aktivität, welche gegen Histone gerichtet ist. Der „Polycomb repressive complex 1“ (PRC1) vermittelt die Mono-Ubiquitinierung von Histon H2A, wogegen PRC2 für die Histon H3 Tri-Methylierung an Lysin 27 verantwortlich ist. Beide Histon Modifikationen sind charakteristisch für transkriptionell inaktives Chromatin. In Knockout Studien wurde gezeigt dass beide Komplexe essentiell für die embryonale Entwicklung sind und dass das Fehlen eines der beiden Komplexe zum Entwicklungsstopp in der Gastrulation führt.

In meiner Dissertation beschreibe ich zuerst die Funktion des PRC1 Komplexes in embryonalen Stammzellen bezüglich der Regulation von Differenzierungs-Kontrollgenen sowie der X Chromosom Inaktivierung. Meine Resultate belegen, dass der Verlust eines funktionellen PRC1 Komplexes zur Expression von Differenzierungs-Kontrollgenen führt, welche in Wiltdtyp ES Zellen inaktiv sind. Im Gegensatz dazu bleibt die transkriptionelle Repression des inaktiven X Chromosoms aufrecht. Meine Resultate und Ergebnisse aus früheren Studien werfen die Frage auf, in welchem Ausmaß die PRC1 and PRC2 Komplexe überlappende Funktionen im Bezug auf die Regulation von Polycomb gebundenen Genen sowie in der frühen Differenzierung haben. Um eine mögliche Redundanz zwischen den beiden Polycomb Komplexen zu analysieren wurden die zentralen Proteine beider Komplexe durch homologe

Rekombination zerstört. In diesen doppelt defizienten (dKO) ES Zellen wurden Gene identifiziert, die von PRC1 und PRC2 redundant inhibiert werden. Im Gegensatz zu *Eed*$^{-/-}$ oder *Ring1B*$^{-/-}$ ES Zellen, resultiert der Verlust von beiden PcG Komplexen darin, dass keine Teratome gebildet werden können, was darauf hindeutet, dass beide PcG Komplexe eine redundante Funktion in der Differenzierung ausüben. Meine Resultate weisen darauf hin, dass eine Anzahl von PcG gebundenen Genen von beiden Polycomb Komplexen redundant reguliert wird. Die transkriptionelle Regulation durch PcG Proteine ist jedoch nicht auf Gene beschränkt. Meine Ergebnisse zeigen, dass endogene Retroviren von Polycomb Proteinen gebunden und reguliert werden.

Zusammenfassend zeigen meine Resultate eine unerwartete Redundanz zwischen PRC1 und PRC2 in der Regulation der Genexpression und in Differenzierungsprozessen, sowie eine essentielle Funktion von Polycomb Proteinen im Schutz der genomischen Integrität durch die Repression von parasitären DNA Sequenzen.

Abstract

Polycomb complexes establish epigenetic patterns for maintaining gene repression by modifying histone tails. Polycomb complex mediated epigenetic marks are thought to be important for establishing and maintaining cellular identity. In mammals, PcG proteins regulate tumorigenesis, imprinting and dosage compensation. A role in the regulation of self renewal and pluripotency of embryonic stem (ES) cells has been proposed. In order to delineate the function of PcG complexes their genomic binding sites have been determined. In mouse ES cells approximately 2500 PcG target genes have been identified, many of which have central functions in embryonic development. PcG proteins have been proposed to maintain pluripotency by inhibiting the transcription of lineage specifying genes. Developmental plasticity is conferred by the lineage specific activation of PcG target genes in the course of differentiation. Two catalytically active PcG complexes have been biochemically characterized. Polycomb repressive complex 1 (PRC1) contains the ubiquitin E3 ligase Ring1B and catalyses mono-ubiquitination of Lysine 119 on histone H2A (ubH2A). PRC2 contains the PcG proteins Eed, Suz12 and Ezh2, which catalyses tri-methylation of Lysine 27 on histone H3 (H3K27me3). The catalytic functions of PRC1 and PRC2 are essential for development and mutations in either *Eed* or *Ring1B* lead to developmental arrest soon after implantation.

In my thesis I investigated the function of the PcG system in embryonic stem cell biology and X chromosome inactivation. For this, I first generated *Ring1B* deficient ES cells by homologous recombination. I found that the deletion of *Ring1B* disrupts the PRC1 complex, and several PRC1 member proteins as well as ubH2A are absent in *Ring1B*$^{-/-}$ cells. Furthermore, the deletion of PRC1 function results in derepression of lineage control genes which are normally not expressed in ES cells. Despite the strong perturbance of epigenetic gene repression and the destabilization of the ES cell state, X chromosome inactivation was unaffected by the loss of PRC1 activity. The activity of PRC2 remained unaffected and H3K27me3 was recruited to the inactive X chromosome. Vice versa, PRC2 deficiency leads to the absence of H3K27me3 genome wide and on the Xi, whereas PRC1 recruitment to the Xi and genomic ubH2A levels remain largely unaffected. This indicates that parallel modes of

PRC1 and PRC2 recruitment exist and that these marks are, to a large extent, independent of each other. To address the redundancy of PRC1 and PRC2 and the role of the PcG system in maintaining pluripotency, I have established an ES cell line deficient for PRC1 and PRC2 by concomitant deletion of *Ring1B* and *Eed*.

My data provide genetic evidence for an unexpected redundancy between PRC1 and PRC2 and show that the PcG system is essential for the maintenance of differentiated cell types. Furthermore, endogenous retroelements are identified as novel targets for PcG regulation and are activated in the absence of PcG repression. This indicates that apart from its function in gene regulation, the PcG system plays a role in maintaining genome integrity by the repression of parasitic DNA sequences.

Introduction

Polycomb Group proteins are conserved transcriptional repressors

Polycomb Group (PcG) and Trithorax Group (trxG) proteins were first described in *Drosophila melanogaster* as repressors or activators of Hox genes, respectively (Paro, 1995; Ringrose and Paro, 2007). Hox genes are transcription factors that define cell identity along the anterior–posterior axis (Kennison, 1995; Lewis, 1978). PcG and trxG proteins are evolutionary conserved and homologues have been identified throughout the animal kingdom and even in plants (Jacobs et al., 1999; Schuettengruber et al., 2007; Schwartz and Pirrotta, 2007; van Lohuizen et al., 1991) (Figure1a). Notably, in flies PcG or trxG proteins do maintain but not initiate silencing. The initial signal for activation or repression of a target gene is provided by transcription factors like Pho, Pipsqueak and GAGA factor. To maintain repression, the initial signal for silencing is no longer required. In *Drosophila* the genomic binding sites for PcG and trxG proteins, the Polycomb response elements (PRE) can be predicted by sequence analysis (Ringrose et al., 2003). So far the mammalian counterparts of PREs have not been defined. The quest for mammalian PREs is made difficult by the absence of homologues of most of the DNA binding transcription factors that define a PRE in Drosophila.

PcG complexes have enzymatic functions directed at histones

Two principal multi-protein PcG complexes have been characterized which possess catalytic activity directed against histones. Polycomb repressive complex 1 (PRC1) contains the RING finger domain proteins Ring1A and Ring1B, which mediate the monoubiquitination of histone H2A at lysine 119 (ubH2A) via an E3 ubiquitin ligase activity (Alkema et al., 1997; Shao et al., 1999; Wang et al., 2004a). PRC2 consists of the PcG proteins Eed, Suz12, and Ezh2 and catalyzes histone H3 lysine 27 di- and trimethylation (H3K27me3) (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2004) as well as the methylation of histone H1 lysine 26 (Kuzmichev et al., 2004). PcG complex mediated histone modifications are associated with silent chromatin. Presently, 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 (Schwartz and Pirrotta, 2007; Wang et al., 2004b). In turn, PRC1 sets up

ubH2A and mediates transcriptional silencing (Stock et al., 2007) by chromatin compaction (Francis et al., 2001) (Figure 1b). In line with this model, H3K27me3 has been shown to increase the affinity for binding of chromodomain-containing Polycomb proteins, such as Cbx7, which are components of PRC1 (Bernstein et al., 2006b). Furthermore, the binding of Ring1B to PRC2 target promoters is compromised in embryonic stem cells carrying a mutation in the PRC2 gene *Eed* which causes a loss of H3K27me3 (Boyer et al., 2006). In addition, the loss of PRC2 components results in the disruption of PRC1 binding at *Hox* genes (Cao et al., 2005; Cao et al., 2002).

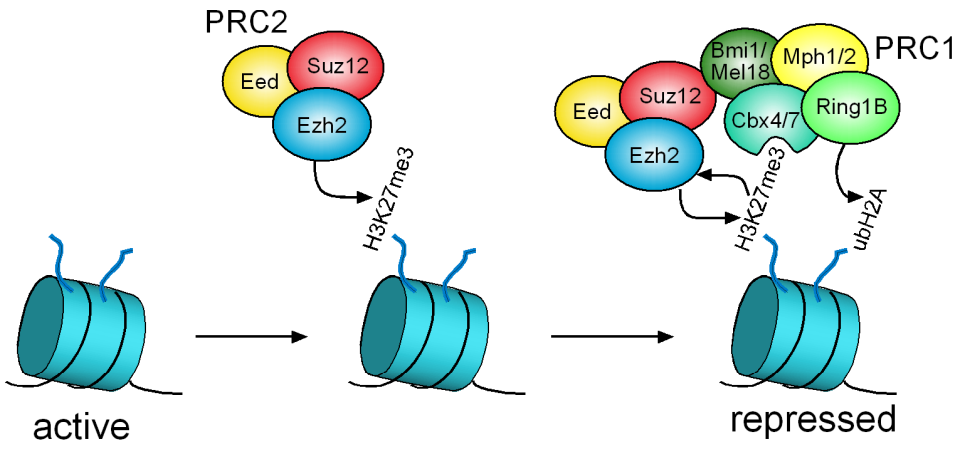
Figure 1 Polycomb Group proteins are conserved transcriptional repressors

(**a**) Evolutionary conservation of the PRC1 and PRC2 complexes between fly and mouse. (**b**) Sequential recruitment model for PcG mediated transcriptional repression. PRC2 sets up H3K27me3 which in turn recruits the PRC1 complex. PRC1 ubiquitinates H2A and leads to transcriptional repression of target genes. (**c**) In ES cells, the repressive H3K27me3 mark coincides with the activating H3K4me3 histone modification, maintaining a poised state. In the course of differentiation into a specified lineage, the bivalent state resolves to either H3K27me3 or H3K4me3 alone, resulting in stable repression or activation, respectively.

a

	Drosophila PcG protein	Mouse homologues
PRC1	PC	M33, Cbx4, Cbx6, Cbx7, Cbx8
PRC1	PH	Mph1, Mph2
PRC1	PSC	Bmi1, Mel18
PRC1	SCE	Ring1B, Ring1A
PRC2	E(Z)	Ezh2, Ezh1
PRC2	ESC	Eed
PRC2	SU(Z)12	Suz12
DNA binding	PHO(L)	YY1

b



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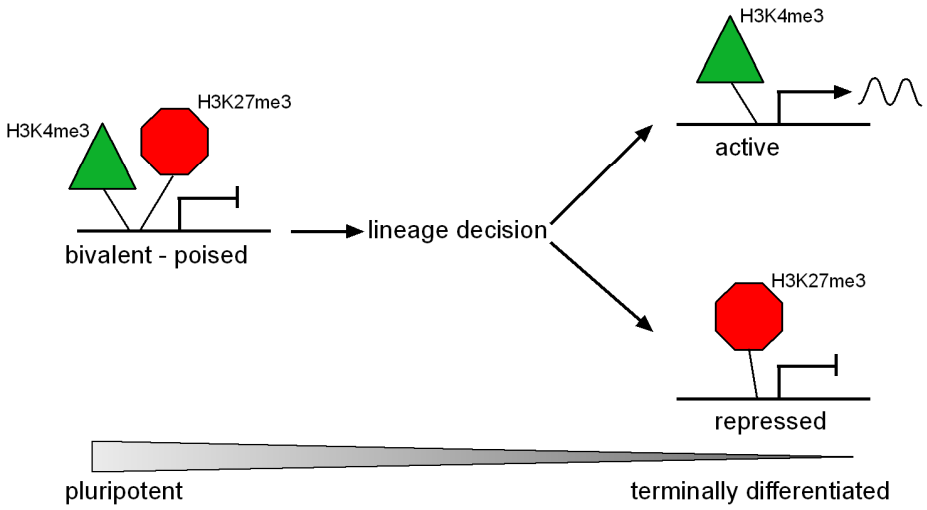

Polycomb function in ES cells

Embryonic stem cells are pluripotent cells derived from the inner cell mass of the mouse blastocyst (Evans and Kaufman, 1981; Martin, 1981). ES cell self renew in vitro and retain the ability to differentiate into cell types of all three germ layers in vitro (Figure 2a). When ES cells are reintroduced into the blastocyst they restart their differentiation program and contribute to all tissues and also to the germ line of adult mice. ES cells have been used as a model system to study early developmental processes (Keller, 2005; Niwa, 2001; Silva and Smith, 2008).

The pluripotent ES cell state is defined by the activity of a transcription factor network around the core components Oct4, Nanog and Sox2 (Figure 2b). Binding of these factors to promoters of target genes can result in activation or repression. How the differential regulation of pluripotency TF target genes is achieved is unclear. Interestingly, many repressed pluripotency TF targets are also bound by PcG proteins in ES cells (Mikkelsen et al., 2007). It was proposed that Polycomb proteins could have a role in maintaining pluripotency by repression of developmental control genes (Pietersen and van Lohuizen, 2008) and that during differentiation repression is selectively removed to allow for the expression of lineage defining genes (Bracken et al., 2006) (Figure 2b). However, ES cells deficient for PRC1 or PRC2 are viable and PcG deficient embryos develop until they arrest at the gastrulation stage (Faust et al., 1998; O'Carroll et al., 2001; Pasini et al., 2004; Voncken et al., 2003). In addition, also other epigenetic mechanisms like DNA methylation are not required for ES cell self renewal (Tsumura et al., 2006). Yet, the extent to which epigenetic regulation is required to maintain pluripotency and early cell fate decisions remains largely unclear.

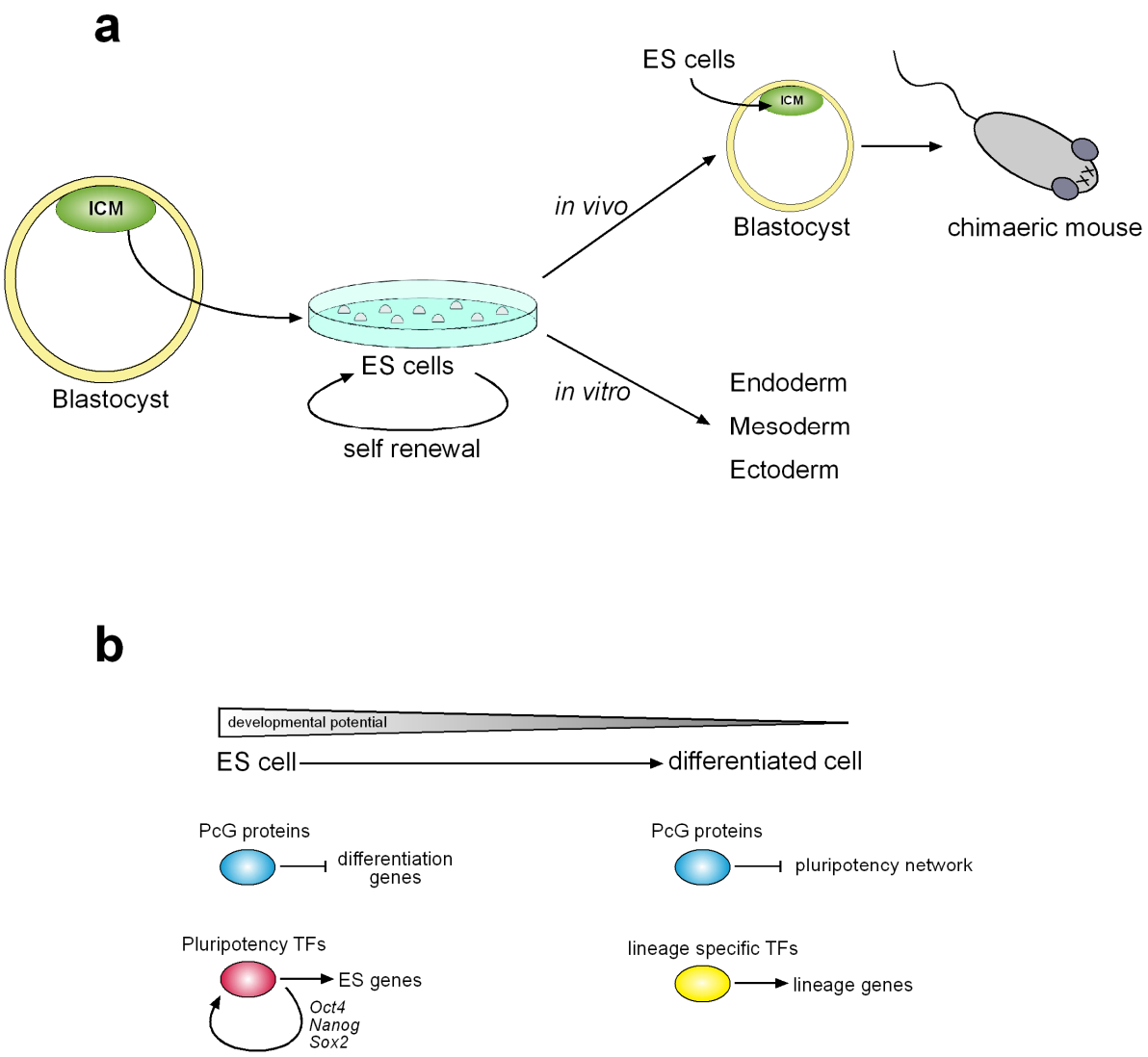

Figure 2 ES cells are pluripotent cells from the inner cell mass

(**a**) ES cells are derived from the inner cell mass (ICM) and can self renew in culture. Upon reintroduction of ES cells into the ICM they contribute to all tissues and the germ line of chimaeric mice. (**b**) The pluripotency transcription factor network around the core components Oct4, Nanog and Sox2 maintains the pluripotent state of ES cells. This requires potent mechanisms to silence genes with a lineage specific transcription profile. The silencing system must convey sufficient plasticity to enable the activation of developmental regulator genes in a lineage specific manner. The PcG system has been hypothesized to perform such a function in ES cells.

Polycomb Group proteins regulate developmental control genes in mammals

Genome wide ChIP on chip and ChIP sequencing studies have revealed a set of more than 2500 promoters which are bound by PcG proteins in mouse and human ES cells (Boyer et al., 2006; Ku et al., 2008; Lee et al., 2006; Mikkelsen et al., 2007). PcG target genes show a strong enrichment for transcription factors and developmental control genes. These genes need to be maintained in an inactive state in ES cells in order to preserve pluripotency. However, the transcriptional silencing of lineage specific transcription factors needs to be reversible, thereby ensuring developmental plasticity. In differentiation, developmental control genes can be selectively derepressed by diminishing PcG enrichment on their promoters, (Bracken et al., 2006; Pietersen and van Lohuizen, 2008). Interestingly, in ES cells the silencing mark H3K27me3 which is deposited by the PcG system largely coexists with histone H3 lysine four tri-methylation (H3K4me3), a histone mark associated with active transcription. This bivalent chromatin state has been proposed to maintain PcG target genes in a state poised for activation (Figure 1c). Indeed, upon differentiation the bivalent state of many PcG target genes resolves to either H3K27me3 of H3K4me3 alone in a lineage specific manner, depending on repression or activation, respectively (Bracken et al., 2006; Mikkelsen et al., 2007).

Virtually all promoters bound by H3K4me3, and consequently most PcG target genes, are characterized by a high CpG content (Bernstein et al., 2006a; Ku et al., 2008). Interestingly, housekeeping genes as well as genes which are conserved between mammals and invertebrates are mostly CpG rich (Mohn and Schubeler, 2009). This indicates that the evolutionary context might define the dependence of repression on PcG proteins. Importantly, the mode of transcriptional initiation differs between CpG rich and CpG poor promoters. Whereas CpG poor promoters are inactive in the absence of activation, CpG rich promotors retain residual activity and RNA polymerase II (RNAP II) is bound to their promoters and low levels of transcription can be observed. In line with this, PcG target genes are not completely silent in ES cells. Bivalent genes assemble active RNAP II complexes and are transcribed at a low level (Stock et al., 2007). This poised RNAP II configuration is maintained by ubH2A,

as the deletion of *Ring1A* and *Ring1B* leads to the release of poised RNAP and subsequent gene derepression (Stock et al., 2007). These observations make it tempting to speculate if PcG regulation of genes has evolved from a system to repress genomic transcriptional noise (Chi and Bernstein, 2009).

PcG complexes are essential for embryonic development in mammals

Genetic deletion studies have shown that PcG proteins are essential for embryonic development. Disruption of either of the PRC2 core components results in embryonic lethality at the time of gastrulation (Faust et al., 1998; O'Carroll et al., 2001; Pasini et al., 2004). Mice deficient for the PRC1 members Bmi1 or Mel18 are viable but show skeletal abnormalities associated with misregulation of homeotic genes (Akasaka et al., 1996; van der Lugt et al., 1994). The generally milder phenotype in PRC1 mutant mice can be explained by the presence of multiple homologues with overlapping functions for each PRC1 member (Figure 1a). In line with this, the deletion of both homologues of the *Drosophila* PSC, *Bmi1* and *Mel18*, results in a severe exacerbation of the phenotype and in embryonic lethality (Akasaka et al., 2001). However, the deletion of the catalytic subunit of PRC1, Ring1B, results in gastrulation arrest similar to PRC2 deficiency. Deletion of Ring1A, a close homologue of Ring1B, is compatible with embryonic development and Ring1A deficient mice show only a mild homeotic phenotype. This indicates that Ring1A cannot compensate for the loss of Ring1B, and that Ring1B function is essential for early embryonic development. This is in line with the opposing transcription patterns of Ring1A and Ring1B. Ring1B is highly expressed in embryonic stem (ES) cells and protein levels decrease during development. In contrast, Ring1A levels are low in ES cells and increase in the course of differentiation. Notably, the early embryonic lethality observed in *Ring1B* deficient mice can be partially rescued by concomitant deletion of the $p16^{Ink4a}$/$p19^{Arf}$ locus. The $p16^{Ink4a}$/$p19^{Arf}$ tumor suppressor gene has been shown to be directly regulated by the PRC1 complex (Jacobs et al., 1999; Kotake et al., 2007). These observations link PcG function to cell cycle control and tumorigenesis (Sparmann and van Lohuizen, 2006).

In the epidermis the PRC2 member Ezh2 is required for differentiation in a lineage specific manner by temporally and spatially controlling gene expression (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 (Chen et al., 2009; Dhawan et al., 2009; Voncken et al., 2003). Recently, *Bmi1*, a member of the PRC1 complex has been proposed to have a function in directly controlling mitochondrial function, DNA damage response and antioxidant defense in mice (Chatoo et al., 2009; Liu et al., 2009). It is conceivable that the embryonic lethality observed in PcG deficient embryos is not solely defined by an intranuclear function of PcG complexes. It has been shown that the activation of signaling pathways can result in the export of PRC2 into the cytoplasm, where it interacts with and reorganizes the actin cytoskeleton (Su et al., 2005). Based on these results, a role for *Ezh2* in extranuclear signalling has been proposed

Polycomb function in X chromosome inactivation

Mammals adjust the genetic imbalance that arises from the different number of X chromosomes between the sexes by transcriptional silencing of one of the two X chromosomes in females (Figure 3a). This dosage-compensation mechanism is specific for placental mammals and requires the long non-coding *Xist* RNA (Leeb et al., 2009; Ng et al., 2007; Payer and Lee, 2008). The chromosome wide localization of *Xist* RNA establishes a repressive nuclear compartment which excludes the transcription machinery (Chaumeil et al., 2006) and recruits repressive chromatin modifications like histone H3K27me3 and histone ubH2A to the inactive X chromosome (Xi) (Ng et al., 2007) (Figure 3b). PcG mediated histone modifications are early marks of the Xi and are recruited during the initiation phase at the onset of X chromosome inactivation (Kohlmaier et al., 2004; Plath et al., 2004; Schoeftner et al., 2006) (Figure 3b). The mechanism of PcG recruitment to the Xi is unknown. Moreover, the exact function of PcG proteins in X inactivation remains unclear as the loss of PRC2 does not result in disruption of *Xist* mediated silencing in embryonic cells (Kalantry and Magnuson, 2006; Schoeftner et al., 2006). However, a point mutation in the *Eed* gene perturbs the maintenance of X chromosomal silencing in certain extraembryonic lineages (Kalantry et al., 2006), indicating that, at

least to some extent, the maintenance of chromosome wide silencing requires the presence of the PcG system. Importantly, PcG recruitment to the Xi is strictly dependent on *Xist* (Kohlmaier et al., 2004; Schoeftner et al., 2006). Furthermore, a repeat A deficient *Xist* RNA, which localizes to the Xi in cis, but cannot induce silencing is still able to recruit PcG proteins. Thus, the recruitment of PcG proteins to the Xi is not sufficient to induce chromosome wide silencing.

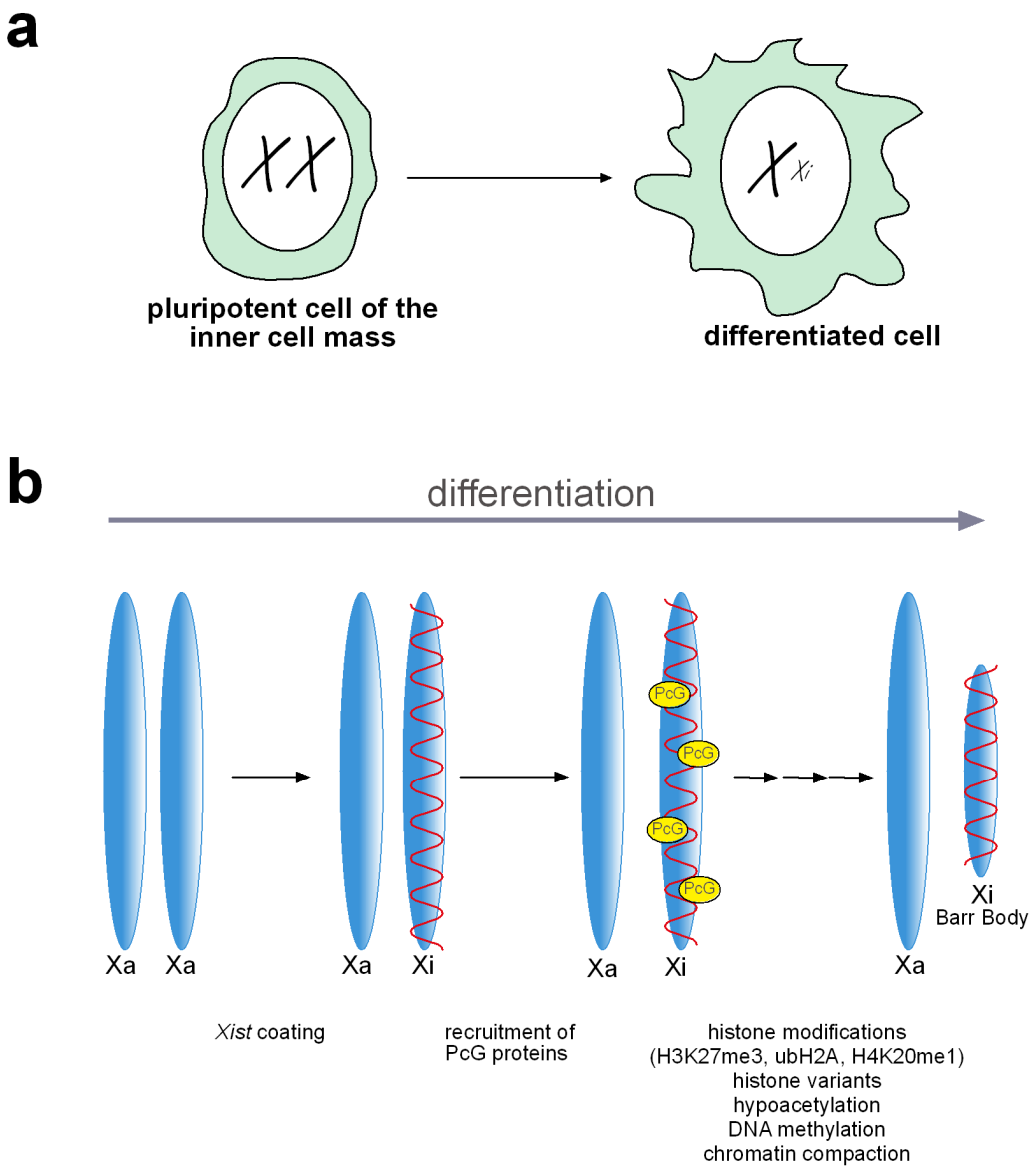

Figure 3 PcG proteins associate with the inactive X chromosome

(**a**) X chromosome inactivation equalizes the gene dosage between XY males and XX females. (**b**) X chromosome inactivation is a stepwise process involving the recruitment of PcG proteins by *Xist* RNA.

Research question

In development, all somatic cells are derived from a single fertilized zygote. The differential readout of the genetic information and the stable execution of developmental programs which define cellular identity require potent regulatory mechanisms. Based on the binding of PcG proteins to developmental regulator genes in pluripotent ES cells and the early embryonic lethality observed in PcG deficient mice, my hypothesis was that PcG proteins perform this master regulator function, thereby governing lineage decisions. Specifically, I was interested in the extent to which PcG proteins are required to maintain the pluripotent ES cell state and how they contribute to the epigenetic regulation of early embryonic development.

In order to answer these questions I followed a reverse genetic approach and generated ES cells deficient for PRC1 activity by deleting the *Ring1B* gene. Subsequently, I extended my analysis by generating ES cells deficient for both PcG complexes.

Results

Part I: *Ring1B* is crucial for the regulation of developmental control genes and PRC1 proteins but not X inactivation in embryonic cells

To study the function of the PRC1 complex in pluripotency and X chromosome inactivation I have analysed *Ring1B*$^{-/-}$ ES cells which were generated as a part of my diploma work (Leeb, 2006). These ES cells were analysed in detail concerning their transcriptional state and X chromosome inactivation, and the results were published in the Journal of Cell Biology (Leeb and Wutz, 2007). In this publication we report that disruption of the *Ring1B* gene disrupts the PRC1 complex and results in either absence or strong reduction of protein levels of PRC1 members. Consequently, the enzymatic activity of the PRC1 complex, histone H2A mono-ubiquitination, is not detectable in *Ring1B*$^{-/-}$ ES cells. *Ring1B* deficient ES cells were difficult to establish due to a strong tendency to differentiate upon acute loss of PRC1 function. However, self renewal is not affected and in optimal culture conditions *Ring1B*$^{-/-}$ ES cells can be maintained over multiple passages and express the pluripotency marker Oct4. Loss of *Ring1B* leads to derepression of developmental control genes which are normally not expressed in ES cells. The differentiation potential is compromised as shown by aberrant embryoid body formation, possibly due to the inability of *Ring1B*$^{-/-}$ cells to establish the appropriate epigenetic patterns to progress through differentiation normally. Despite the pronounced transcriptional effect of loss of PRC1 function, neither initiation nor maintenance of X chromosome inactivation is affected by deletion of *Ring1B*. Ring1A, a homologue of Ring1B, is not expressed in ES cells and cannot be responsible for initiating *Xist* mediated silencing in the absence of Ring1B. In contrast, differentiated cells express Ring1A but genome-wide ubH2A cannot be restored, possibly due to the absence of several PRC1 members. However, ubH2A on the *Xist* silenced chromosome is specifically re-established in differentiated *Ring1B*$^{-/-}$ cells, indicating a function for Ring1A in decorating the Xi in a Ring1B independent manner.

I performed and analysed all the experiments in this paper and prepared the manuscript together with my supervisor Dr. Anton Wutz.

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Ring1B is crucial for the regulation of developmental control genes and PRC1 proteins but not X inactivation in embryonic cells

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The Polycomb group (PcG) gene *Ring1B* has been implicated in the repression of developmental control genes and X inactivation and is essential for embryogenesis. Ring1B protein contains a RING finger domain and functions as an E3 ubiquitin ligase that is crucial for the monoubiquitination of histone H2A (H2AK119ub1). Here, we study the function of *Ring1B* in mouse embryonic stem (ES) cells. The deletion of *Ring1B* causes the loss of several PcG proteins, showing an unanticipated function in the regulation of PcG protein levels. Derepression of lineage genes and an aberrant differentiation potential is observed in Ring1B-deficient ES cells. Despite a crucial function of *Ring1B* in establishing the chromosome-wide ubiquitination of histone H2A lysine 119 (H2AK119ub1) upon *Xist* expression in ES cells, the initiation of silencing by *Xist* is independent of *Ring1B*. Other chromatin marks associated with the initiation of X inactivation are not affected in *Ring1B*-deficient cells, suggesting compensation for the loss of *Ring1B* in X inactivation in contrast to the repression of lineage genes.

Introduction

Polycomb group (PcG) proteins are conserved transcriptional regulators with roles in cell identity, lineage specification, cell cycle control, and X inactivation (Rice et al., 2002; Ringrose and Paro, 2004; Lucchesi et al., 2005; Heard and Disteche, 2006). Their function in regulating homeotic genes has been established in many organisms, including flies and mammals. Several PcG genes are essential for development. PcG proteins exert their function, in part, via histone-modifying activities. Two biochemically distinct complexes have been isolated and possess catalytic activity. Polycomb repressive complex 1 (PRC1) contains the RING finger domain proteins Ring1A and Ring1B, which mediate the monoubiquitination of histone H2A lysine 119 (H2AK119ub1) via an E3 ubiquitin ligase activity. PRC2 consists of the PcG proteins Eed, Suz12, and Ezh2 and catalyzes histone H3 lysine 27 di- and trimethylation (H3K27me3; Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002) as well as the methylation of histone H1 lysine 26 (Kuzmichev et al., 2004). PcG complex–mediated histone modifications have been associated with silent chromatin. H3K27me3 has been shown to increase the affinity for binding of chromodomain-containing Polycomb proteins such as Cbx7, which are also components of PRC1 (Fischle et al., 2003; Bernstein et al., 2006). Based on this, PRC2-mediated H3K27me3 has been proposed to act as a recruitment signal for PRC1, which, in turn, would catalyze H2AK119ub1. Consistently, Ring1B binding is compromised in embryonic stem (ES) cells carrying a mutation in the PRC2 gene *Eed*, which causes a loss of H3K27me3 (Boyer et al., 2006). Furthermore, loss of PRC1 components results in the disruption of PRC1 binding at *Hox* genes (Cao et al., 2002, 2005).

Mammals achieve dosage compensation between XX females and XY males by the inactivation of one of the two X chromosomes in female cells. X inactivation is initiated by *Xist* RNA, which associates with the inactive X chromosome (Xi) and initiates chromosome-wide silencing. *Xist* is crucial for the initiation of X inactivation but is dispensable for maintaining the Xi at later stages of differentiation, when other epigenetic mechanisms, including DNA methylation, ensure stable silencing (Brown and Willard, 1994; Csankovszki et al., 1999; Wutz and Jaenisch, 2000). PcG proteins are recruited by *Xist* and contribute to the establishment of histone modifications along the Xi (Plath et al., 2004). The initiation of X inactivation is characterized by chromosome-wide histone modifications, including H3K27me3, H2AK119ub1, and monomethylation of histone H4 lysine 20 (H4K20me1; Plath et al., 2003; de Napoles et al., 2004;

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Abbreviations used in this paper: EB, embryoid body; ES, embryonic stem; PcG, Polycomb group; PRC, Polycomb repressive complex; RYBP, Ring1 and YY1-binding protein.

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Fang et al., 2004; Kohlmaier et al., 2004). A mutant *Xist* RNA, which lacks the *Xist* repeat A sequence and, thus, cannot cause transcriptional repression, is still able to recruit PcG proteins and establish chromosome-wide histone modifications. This indicates that PcG recruitment occurs independently of the initiation of silencing (Plath et al., 2003; Kohlmaier et al., 2004; Schoeftner et al., 2006) and that PcG recruitment is not sufficient for the initiation of chromosome-wide silencing.

An involvement of PcG proteins in the maintenance of X inactivation has been proposed based on their function in maintaining the repression of homeotic genes. However, *Xist* is required for the recruitment of PcG proteins and histone modifications throughout ES cell differentiation and in differentiated cell types. This suggests that in X chromosome inactivation, PcG complexes have a function in the establishment of the maintenance of stable silencing rather than being silencing factors themselves. Thus, recruitment of PcG complexes in X inactivation might differ from recruitment to developmental control genes. Consistent with an involvement in the maintenance of X inactivation, the PRC2 gene *Eed* is required for maintenance of the Xi in differentiating trophoblast stem cells (Kalantry et al., 2006). In contrast, PRC2 function is dispensable for X inactivation in embryonic cells (Kalantry and Magnuson, 2006; Schoeftner et al., 2006), and *Ring1B* and H2AK119ub1 can be recruited to the *Xist*-expressing chromosome in cells lacking PRC2 function caused by disruption of the *Eed* gene (Schoeftner et al., 2006). This suggests a PRC2-independent mode of Ring1B recruitment in X inactivation. The ability of *Eed*-deficient ES cells to initiate chromosome-wide silencing could either be explained by a potential redundancy of PRC1 and PRC2 or, alternatively, Ring1B could be of primary functional importance for X inactivation in embryonic cells. Previously, it has been shown that both Ring1A and Ring1B mediate H2AK119ub1 on the Xi in mouse embryonic fibroblasts (de Napoles et al., 2004).

Ring1B is an essential gene in the mouse, and its mutation leads to gastrulation arrest and cell cycle inhibition

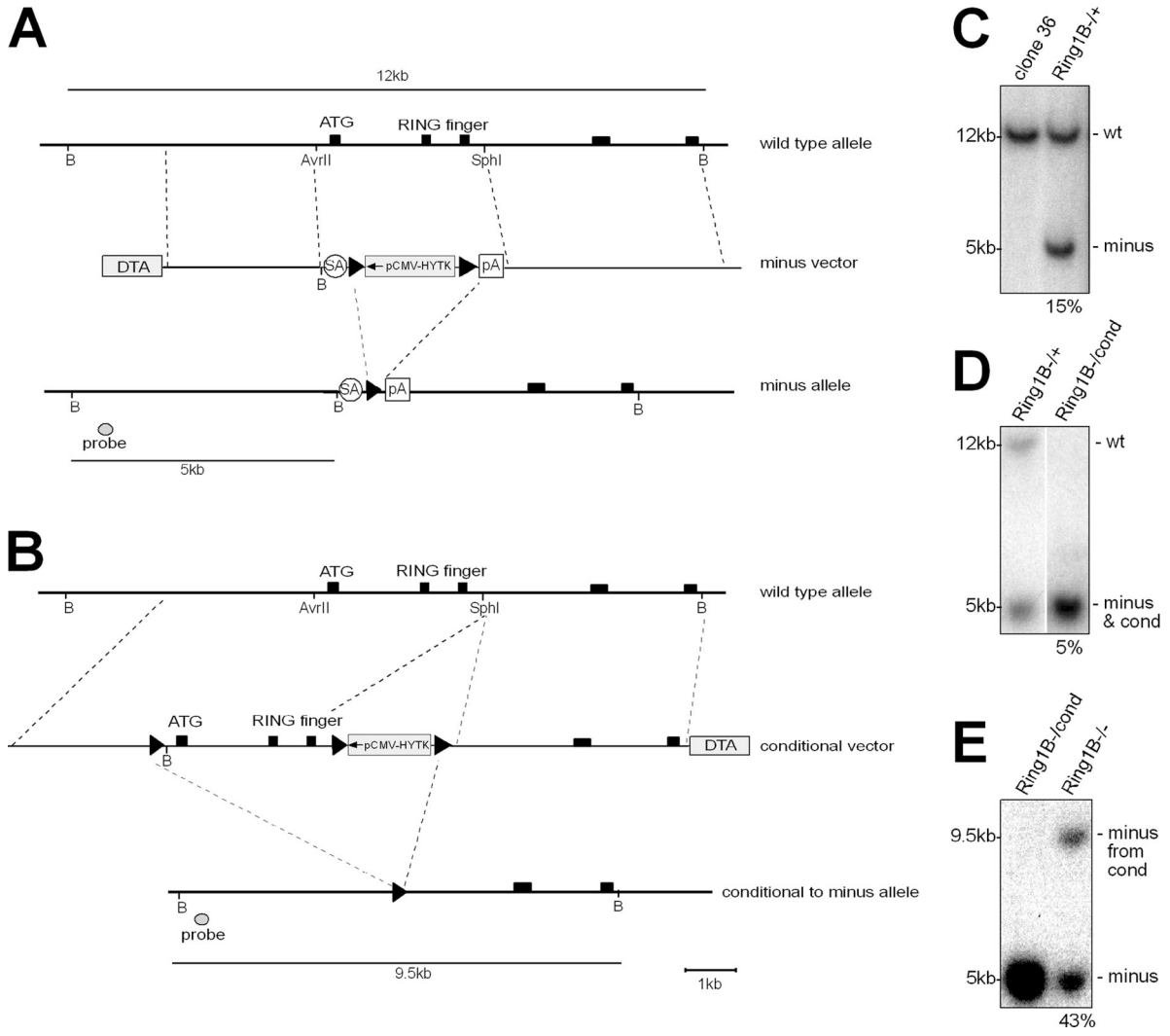

Figure 1. **Generation of *Ring1B*-deficient ES cells.** (A) Schematic representation of the *Ring1B* locus and the minus targeting vector replacing the start codon and the RING finger domain with a stop cassette. (B) *Ring1B* conditional targeting vector allowing for deletion of the *Ring1B* locus after Cre-mediated excision. (C–E) Southern analyses of $36^{Ring1B-/+}$ (C), $36^{Ring1B-/cond}$ (D), and $36^{Ring1B-/-}$ (E) ES cells. Lanes were grouped where necessary. The white line indicates that intervening lanes have been spliced out. wt, wild type.

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(Voncken et al., 2003). An involvement in embryonic axis specification and regulation of homeotic genes has also been demonstrated (Suzuki et al., 2002). Ring1B appears to be associated with several distinct complexes. Apart from its function as a catalytic E3 ubiquitin ligase in the PRC1 complex, recruitment of Ring1 proteins by the transcriptional repressor E2F6 (Trimarchi et al., 2001) and the spliceosomal component Sf3b1 (Isono et al., 2005) has been observed. It is conceivable that histones are not the only targets to be modified by PcG proteins. Recent results indicate a function for Ring1B in ubiquitination of the PcG-associated protein Ring1 and YY1-binding protein (RYBP; Arrigoni et al., 2006). In the present study, we address the function of Ring1B in the regulation of developmental control genes, PRC1 protein levels, and the initiation of X inactivation in mouse ES cells.

Results

Loss of PRC1 proteins in *Ring1B*-deficient ES cells

To investigate the function of *Ring1B* in clone 36 ES cells (Wutz and Jaenisch, 2000), we generated a targeting vector that replaced the start codon and the catalytically active RING finger domain with a floxed hygromycin selection marker (Fig. 1 A). A splice acceptor site and an SV40 polyA sequence flanking the selection marker were inserted to avoid production of truncated protein products. Targeting of the first allele was efficient with a frequency of 15% and was confirmed by Southern analysis (Fig. 1 C). The second allele could only be targeted with an efficiency of 0.3%, and $Ring1B^{-/-}$ clones could not be isolated as a result of a strong tendency to differentiate. Following a conditional targeting strategy (Fig. 1 B), $Ring1B^{-/cond}$ ES cells were obtained with a frequency of 5% (Fig. 1 D). Cre-mediated recombination established $36^{Ring1B-/-}$ clones with a frequency of 43% as confirmed by Southern analysis (Fig. 1 E). About half of these clones were lost as a result of spontaneous differentiation, but the other half could be recovered and cultured for >20 passages. However, $36^{Ring1B-/-}$ ES cells appeared to have a strong propensity to differentiate, were extremely sensitive to stress, especially upon freezing and thawing, and could only be maintained under pristine culture conditions.

The absence of Ring1B protein was confirmed by Western analysis in two independently derived $36^{Ring1B-/-}$ ES clones (Fig. 2 A). In $36^{Ring1B-/cond}$ ES cells, Ring1B protein levels were reduced, indicating that the conditional targeting vector yielded a hypomorphic *Ring1B* allele before Cre-mediated recombination (Fig. 2, A and E), which is similar to a hypomorphic *Ring1B* allele reported previously (Suzuki et al., 2002). Notably, the abundance of the PRC1 proteins Mph1, Mel18, and Rybp was reduced to undetectable levels in *Ring1B*-deficient $36^{Ring1B-/-}$ ES cells (Fig. 2 A). The levels of Mph2 and Mpc2 were strongly

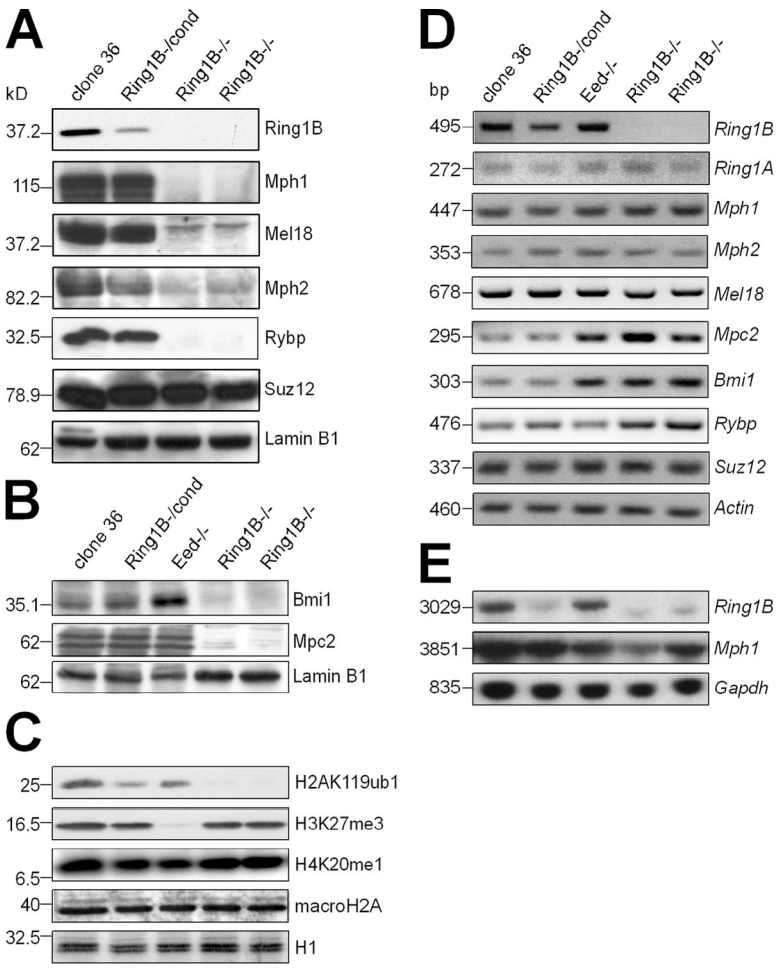

Figure 2. **Analysis of PcG expression in clone $36^{Ring1B-/-}$ cells.** (A) Western analysis of PcG proteins in nuclear extracts from control clone 36, $36^{Ring1B-/cond}$, and $36^{Ring1B-/-}$ ES cells. A representative lamin B1 loading control is shown. (B) Western analysis of Bmi1 and Mpc2 in nuclear extracts of clone 36, $36^{Ring1B-/cond}$, $36^{Eed-/-}$, and $36^{Ring1B-/-}$ ES cells. (C) Western analysis of global levels of histone modifications associated with the initiation of X inactivation in clone 36, $36^{Ring1B-/cond}$, $36^{Eed-/-}$, and $36^{Ring1B-/-}$ ES cells. Ponceau-stained histone H1 bands show loading. (D) Expression analysis of PcG trancription by semiquantitative RT-PCR. (E) Northern analysis of *Ring1B* and *Mph1* expression in ES cells. *Glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) was used as a loading control.

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reduced (Fig. 2, A and B). All PRC1 proteins were abundantly detected in control clone 36 ES cells. We conclude that disruption of *Ring1B* leads to the reduction of several PRC1 proteins in *Ring1B*-deficient ES cells.

Ring1B is required for the repression of developmental control genes

PcG proteins have been implicated in the repression of developmental control genes in ES cells (Boyer et al., 2006). To investigate whether the derepression of such genes occurs in *Ring1B*-deficient ES cells and could contribute to the instability of stem cell identity, we performed an expression analysis of lineage-specific genes, including the trophoblast stem cell markers *Cdx2* and *Eomes* and the markers for extraembryonic endoderm *Foxa2*, *Hand1*, and *Hnf4*, which are normally not expressed during ES cell differentiation. All trophoblast stem cell and extraembryonic endoderm markers were repressed in control clone 36 ES cells but were up-regulated in $36^{Ring1B-/-}$ ES cells (Fig. 3 A). In $36^{Eed-/-}$ ES cells, which are deficient for PRC2 function as a result of a null mutation in *Eed* (Schoeftner et al., 2006), a substantial up-regulation of *Cdx2*, *Eomes*, and *Hand1* but only a weak derepression of *Foxa2* and *Hnf4* was observed (Fig. 3 A). The pattern of derepression of lineage-specific genes in *Ring1B*- and *Eed*-deficient ES cells is largely consistent with the previously reported binding of *Eed* and *Ring1B* to the respective chromosomal loci in mouse ES cells (Boyer et al., 2006). *Hnf4* has not been reported as a PcG target, and derepression could be an indirect effect of the loss of *Ring1B*. Deregulation of developmental control genes is not limited to markers for extraembryonic development, as *Nestin*, a marker for neuronal differentiation, is slightly up-regulated in *Ring1B*- and *Eed*-deficient ES cells. Expression of the pluripotency-associated gene *Oct4* was observed in *Ring1B*-deficient, *Eed*-deficient, and control ES cell lines at comparable levels (Fig. 3 A and Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200612127/DC1). We conclude that *Ring1B*-deficient ES cells can be isolated and maintained but show the derepression of lineage genes, which contributes to a predisposition to differentiation and compromises stem cell maintenance.

To analyze the differentiation potential of *Ring1B*-deficient ES cells, we investigated their ability to form embryoid bodies (EBs; Fig. 3, B and C). After 7 d in suspension culture, a portion of $36^{Ring1B-/-}$ EBs formed large, hollow spheres. In contrast, EBs derived from control clone 36 ES cells formed compact aggregates (Fig. 3 C). When these EBs were plated on gelatine-coated dishes, they attached and formed beating structures indicative of the development of contractile cardiomyocytes. EBs derived from $36^{Ring1B-/-}$ ES cells neither attached nor formed contractile cardiomyocytes after 7 d in suspension culture but continued to grow in suspension as hollow spheres, reaching a diameter of up to 5 mm after 3 wk (Fig. 3 B). $36^{Ring1B-/cond}$ EBs, which have reduced Ring1B protein levels, did not attach efficiently but formed contractile structures in suspension culture after 1 wk (Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200612127/DC1). These peculiar beating spheres were not observed in control clone 36 EBs and could indicate cardiomyocyte development at reduced Ring1B protein levels.

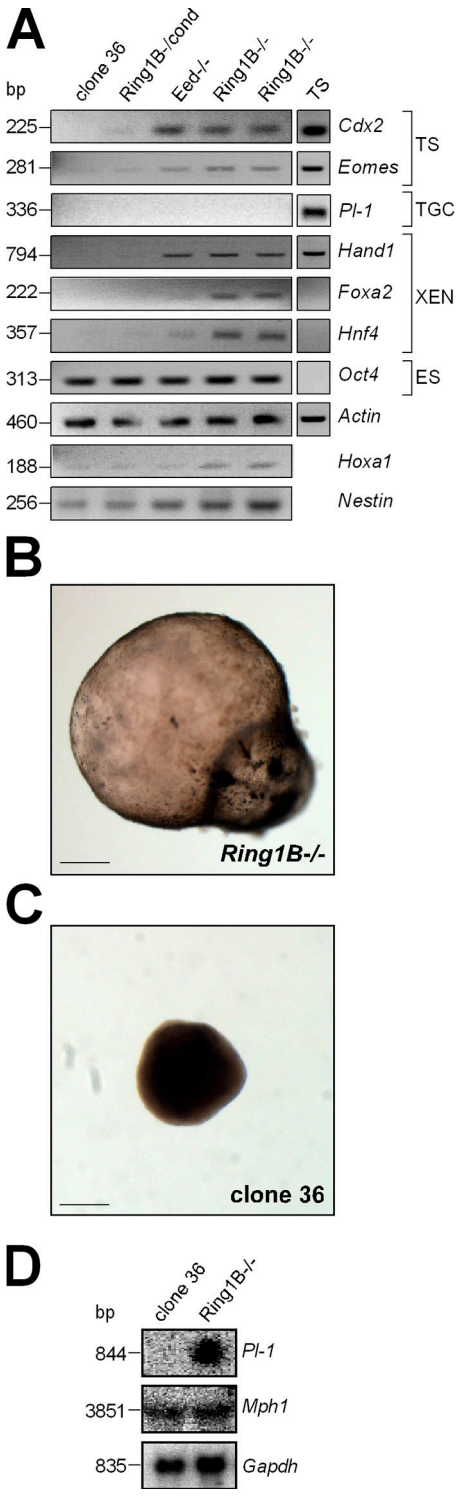

Figure 3. **Deregulation of developmental control genes upon the loss of *Ring1B*.** (A) Expression analysis of *Cdx2*, *Eomes*, *Pl-1*, *Hand1*, *Foxa2*, *Hnf4*, *Oct4*, *Hoxa1*, *Nestin*, and the loading control *β-actin* using RNA from ES cells as indicated by RT-PCR. (B and C) EBs derived from clone 36 and $36^{Ring1B-/-}$ ES cells after 3 wk of suspension culture. Images were obtained at 20× magnification. (D) Northern analysis of *Pl-1* and *Mph1* expression in clone 36 and $36^{Ring1B-/-}$ EBs. Bars, 1 mm.

A deregulation of lineage gene expression was observed in $36^{Ring1B-/-}$ EBs after 2 wk of differentiation, which is consistent with the aberrant differentiation potential (Fig. S1 D). When

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$36^{Ring1B-/-}$ EBs were plated on gelatine after 48 h, some attached, and, after 3 wk, cells with a morphology reminiscent of trophoblast giant cells developed (unpublished data). Consistent with this, we observed the expression of *Pl-1*, which is normally exclusively expressed in trophoblast giant cells (Fig. 3 D).

Ring1B is critical for the regulation of PRC1 protein abundance

To investigate whether Ring1B controls the expression of other PcG genes, we performed expression analysis of the PRC1 genes *Ring1B*, *Ring1A*, *Mph1*, *Mph2*, *Mel18*, *Mpc2*, *Bmi1*, *Rybp*, and the PRC2 member *Suz12* (Fig. 2 D). As expected, we observed a loss of *Ring1B* expression in $36^{Ring1B-/-}$ ES cells (Fig. 2, D and E). Transcription of the PcG genes *Ring1A*, *Mph1*, *Mph2*, *Mel18*, and *Suz12* was unaffected by the loss of either *Ring1B* or *Eed* (Fig. 2, D and E). However, the levels of *Bmi1* and *Mpc2* transcript were up-regulated in $36^{Ring1B-/-}$ and $36^{Eed-/-}$ ES cells (Fig. 2 D), which is consistent with the reported binding of Ring1B and Eed to the *Bmi1* and *Mpc2* promoters in mouse ES cells (Boyer et al., 2006). Transcription of *Rybp* was found to be slightly up-regulated in *Ring1B*- but not *Eed*-deficient ES cells. We conclude that in general, PcG genes are not regulated by Ring1B at the transcriptional level, but we find that *Bmi1*, *Mpc2*, and *Rybp* transcription is negatively regulated by *Ring1B*.

This showed that the loss of PRC1 proteins in *Ring1B*-deficient ES cells was not mediated by transcriptional repression but occurred at the level of protein stability or translation. Compared with clone 36 ES cells, Bmi1 protein levels were reduced to undetectable levels in $36^{Ring1B-/-}$ ES cells but were more abundant in $36^{Eed-/-}$ ES cells (Fig. 2 B). Thus, the up-regulation of *Bmi1* transcription in $36^{Ring1B-/-}$ and $36^{Eed-/-}$ ES cells resulted in an accumulation of Bmi1 protein in *Eed*-deficient but not *Ring1B*-deficient ES cells. This could be explained by a critical role of Ring1B in stabilization of the PRC1 complex. Consistent with this, several PRC1 proteins could not be detected in *Ring1B*-deficient ES cells (Fig. 2, A and B) despite unaffected transcription (Fig. 2, D and E). The PRC2 protein Suz12 was unaffected by the loss of *Ring1B* in $36^{Ring1B-/-}$ ES cells (Fig. 2 A). We conclude that Ring1B is critical for PRC1 but not PRC2 protein levels in ES cells, possibly by the regulation of translation or protein stabilization.

Ring1B is essential for *Xist*-mediated H2AK119ub1 in ES cells but not in differentiated cells

To characterize the effect of PRC1 disruption on histone modifications associated with X inactivation, we performed Western analysis of ES cells lacking *Ring1B*. H2AK119ub1 was absent in $36^{Ring1B-/-}$ ES cells compared with clone 36 and $36^{Eed-/-}$ ES cells (Fig. 2 C), which is consistent with a previous report of a crucial function of Ring1B in the ubiquitination of histone H2A (de Napoles et al., 2004). H3K27me3 was unaffected in $36^{Ring1B-/-}$ ES cells but was absent in $36^{Eed-/-}$ ES cells, which lack PRC2 (Schoeftner et al., 2006). Global levels of H4K20me1 as well as macroH2A were unchanged in *Ring1B*- and *Eed*-deficient ES cells compared with control clone 36 ES cells (Fig. 2 C).

To analyze the recruitment of PcG proteins by *Xist* and the establishment of histone marks, we performed immunofluorescence analysis combined with *Xist* RNA FISH. In clone 36 and $36^{Ring1B-/-}$ ES cells, *Xist* expression can be induced from a transgene inserted into chromosome 11 by the addition of doxycycline (Fig. 4 A). Upon the addition of doxycycline for 3 d, *Xist* was induced efficiently in $36^{Ring1B-/-}$ ES cells, and a focal *Xist* cluster was observed in 57 ± 5% of the nuclei compared with 62 ± 5% in control clone 36 ES cells. In $36^{Ring1B-/-}$ ES cells, colocalization of focal H2AK119ub1 staining with *Xist* was reduced and observed in 7 ± 4% of the nuclei compared with 90 ± 6% in control clone 36 ES cells after 3 d of induction with doxycycline (Fig. 4, B and D). Colocalization of H3K27me3 with *Xist* was unaffected by the loss of *Ring1B* with 92 ± 5% and 95 ± 3% of the nuclei showing focal staining in wild-type and $36^{Ring1B-/-}$ ES cells, respectively (Fig. 4 D and Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200612127/DC1). Similarly, the establishment of H4K20me1 on the *Xist*-expressing chromosome was not impaired by the loss of *Ring1B*, and focal staining was observed in 51 ± 5% and 46 ± 6% of wild-type and $36^{Ring1B-/-}$ ES cells, respectively (Fig. 4 F).

We next characterized the recruitment of PcG proteins by *Xist* (Fig. 4 D). Mph1 was recruited in 30% of control 36 but not in $36^{Ring1B-/-}$ ES cells. In addition, the immunofluorescence signal for Mph1 was weaker in $36^{Ring1B-/-}$ ES cells compared with wild-type ES cells (not depicted), which is consistent with our observation that the levels of several PRC1 proteins were strongly reduced in *Ring1B*-deficient ES cells (Fig. 2, A and B). In contrast, recruitment of the PRC2 members Ezh2 and Suz12 was not affected by the loss of *Ring1B* in ES cells (Fig. 4 D). Colocalization of Ezh2 with *Xist* was observed in 96 ± 1% and 91 ± 2% in wild-type and *Ring1B*-deficient ES cells, respectively. Similarly, Suz12 colocalized with *Xist* in 89 ± 4% of wild-type clone 36 and 90 ± 6% of $36^{Ring1B-/-}$ ES cells. To demonstrate the specificity of the effect of the *Ring1B* deletion on H2AK119ub1 and PcG recruitment in $36^{Ring1B-/-}$ ES cells, a knockin strategy was used to rescue the *Ring1B* disruption after attempts to transiently or stably express *Ring1B* transgenes were unsuccessful. For this, we used the conditional vector to establish $36^{Ring1B-/knockin}$ ES cells. In $36^{Ring1B-/knockin}$ ES cells, H2AK119ub1 is observed on the *Xist*-expressing chromosome in 75% of analyzed nuclei (Fig. 4 D). Furthermore, Mph1 protein levels and recruitment by *Xist* in $36^{Ring1B-/knockin}$ ES cells were comparable to clone 36 ES cells (unpublished data). This demonstrated that the loss of *Ring1B* specifically disrupts PRC1 function and H2AK119ub1 in ES cells. However, PRC2 function as well as H4K20me1 is recruited by *Xist* independent of PRC1 in $36^{Ring1B-/-}$ ES cells.

After 3 d of retinoic acid–induced differentiation in the presence of doxycycline, the colocalization of H2AK119ub1 with *Xist* became evident in $36^{Ring1B-/-}$ ES cells, and, after 8 d, 72 ± 6% of the cells showed the colocalization of focal H2AK119ub1 staining with *Xist* compared with 90 ± 2% of control clone 36 cells. We found that Ring1A protein levels were strongly up-regulated upon the differentiation of $36^{Ring1B-/-}$ and control ES cells (Fig. S1, A and B), and we observed Ring1A colocalization with *Xist* on day 8 of differentiation (Fig. S2 C).

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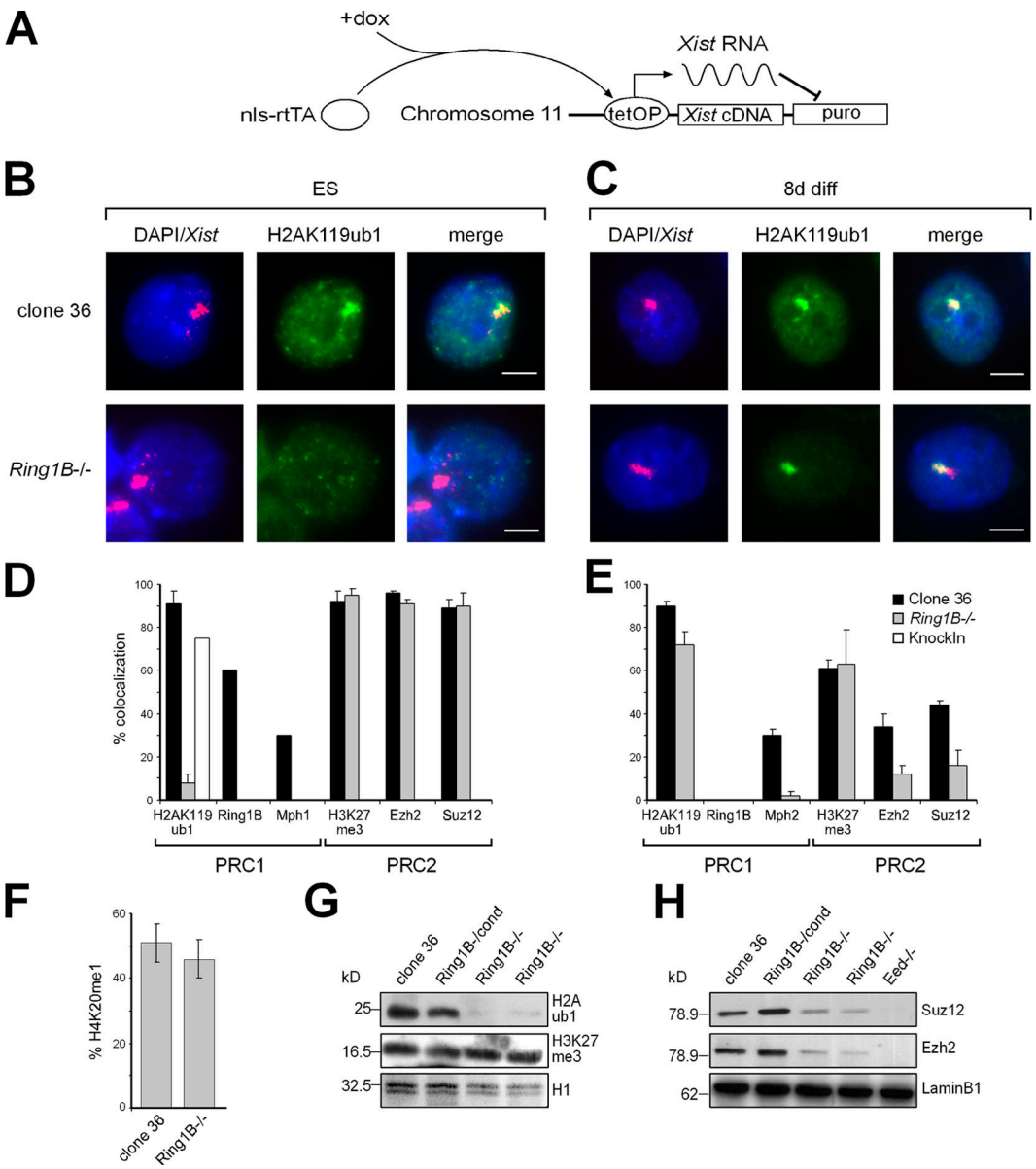

Figure 4. **Recruitment of histone modifications and PcG proteins by *Xist* in *Ring1B*-deficient cells.** (A) Scheme showing the inducible *Xist* expression system in clone 36 ES cells. In the presence of the inducer doxycycline, the tetracycline-regulated transactivator (nls-rtTA) binds to the inducible promoter (tetOP) and activates *Xist*, which then causes the repression of a puromycine selection marker gene (puro). (B and C) H2AK119ub1 immunofluorescence analysis combined with *Xist* RNA FISH of clone 36 and $36^{Ring1B-/-}$ ES (B) and differentiated cells (C). Bars, 5 μm. (D) Statistical analysis of the recruitment of PcG proteins and histone modifications by *Xist* in clone 36 and $36^{Ring1B-/-}$ ES cells. Error bars represent SD ($n > 100$). Results for Ring1B, Mph1, and H2AK119ub1 in $36^{Ring1B-/knockin}$ were counted once ($n > 100$). (E) Analysis of PcG protein recruitment and histone modifications by *Xist* in differentiated clone 36 and $36^{Ring1B-/-}$ cells as in D. (F) Percentage of nuclei showing focal H4K20me1 staining in undifferentiated clone 36 and $36^{Ring1B-/-}$ ES cells ($n > 100$). (G) Western analysis of global H3K27me3 and H2AK119ub1 in ES cells differentiated for 8 d as indicated. (H) Western analysis of the PRC2 proteins Suz12 and Ezh2 in nuclear extracts from clone 36, $36^{Eed-/-}$, and $36^{Ring1B-/-}$ cells that were differentiated for 8 d. Lamin B1 was used as a loading control.

This suggested that Ring1A could possibly contribute to H2AK119ub1 in differentiated *Ring1B*-deficient cells, which is consistent with a previous report that *Ring1A* can compensate for the disruption of *Ring1B* in embryonic fibroblasts (de Napoles et al., 2004). Furthermore, the establishment of H2AK119ub1 early in the differentiation of $36^{Ring1B-/-}$ cells could explain the small proportion of *Ring1B*-deficient ES cells showing the colocalization of H2AK119ub1 and *Xist*. Nonetheless, Western analysis demonstrated that global H2AK119ub1 levels were not restored upon differentiation in $36^{Ring1B-/-}$ cells (Fig. 4 G). In addition, *Xist* was unable to recruit Mph2 efficiently in *Ring1B*-deficient cells despite the recovery of H2AK119ub1. On day 8 of differentiation, 30% of control clone 36 but only 2 ± 2% of $36^{Ring1B-/-}$ cells showed the colocalization of Mph2 with *Xist* (Fig. 4 E). This could be explained by reduced Mph2, Bmi1, and Mel18 protein levels in differentiated $36^{Ring1B-/-}$ cells compared with controls (Fig. S1 E).

H3K27me3 colocalization with Xist was unaffected and was observed in 63 ± 16% of differentiated $36^{Ring1B-/-}$ cells comparable with 61 ± 1% in controls (Fig. 4 E). Furthermore, macroH2A

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recruitment by Xist was not affected in Ring1B-deficient cells after 8 d of differentiation, and 78 ± 5% of H3K27me3-positive cells showed colocalizing macroH2A signals compared with 76% of control clone 36 cells (Fig. S3 A, available at http://www.jcb.org/cgi/content/full/jcb.200612127/DC1). Control 36 cells showed a 34 ± 6% colocalization of Ezh2 and a 44 ± 2% colocalization of Suz12 with Xist. In $36^{Ring1B-/-}$ cells, the percentages decreased to 12 ± 4% and 16 ± 7% for Ezh2 and Suz12, respectively, after 8 d of differentiation (Fig. 4 E). This is consistent with Western analysis showing a reduction of the PRC2 protein levels of Suz12 and Ezh2 (Fig. 4 H), possibly as a result of the heterogeneous expression of PRC2 proteins in a subset of cells (not depicted). However, the reduction in the abundance of PRC2 proteins in $36^{Ring1B-/-}$ was not as severe as in Eed-deficient cells (Fig. 4 H) and did not lead to a measurable difference in H3K27me3; thus, this might not be of functional relevance. We conclude that despite a recovery of H2AK119ub1 colocalization with Xist upon the differentiation of Ring1B-deficient ES cells, the stability of the PcG system critically depends on the presence of Ring1B. A redundant E3 ligase activity can remedy defects in ubiquitination in X inactivation but not in global histone H2A ubiquitination.

Ring1B is dispensable for the initiation and maintenance of X inactivation

We next assessed the ability of *Xist* to initiate gene silencing in the absence of Ring1B and PRC1. The induction of *Xist* expression in clone 36 ES cells causes repression of a puromycin marker gene (puro), which is cointegrated with the *Xist* transgene. Thus, *Xist*-mediated silencing can be analyzed by Northern analysis of puro expression. After the induction of *Xist* for 3 d, repression of the puro marker in $36^{Ring1B-/-}$ ES cells was comparable with control 36 ES cells (Fig. 5 A). We further confirmed this result by analysis of cell growth under puromycin selection. *Ring1B*-deficient as well as control 36 ES cells became puromycin sensitive upon the addition of doxycycline to the medium (Fig. S3 B). A control heterozygous $36^{Ring1B+/-}$ ES cell clone that had lost the ability to express *Xist* remained puromycin resistant upon exposure to doxycycline. We conclude that initiation of silencing by *Xist* is independent of *Ring1B* and H2AK119ub1.

To investigate whether *Ring1B* is essential for the maintenance of silencing, ES cell differentiation was induced with all-trans–retinoic acid. *Xist* was either turned on from the beginning of differentiation, for 4 d followed by 4 d without induction or cells were differentiated without doxycycline for 8 d in parallel cultures (Fig. 5, B and C). Expression of the puro marker gene was quantified on day 8 of differentiation by Northern analysis. Repression of the puro marker was observed in *Ring1B*-deficient $36^{Ring1B-/-}$ cells comparable with control 36 ES cells after 8 d of differentiation in the presence of doxycycline (Fig. 5 B). Furthermore, silencing was efficiently maintained independent of *Xist* expression in *Ring1B*-deficient cells, which were differentiated in the presence of doxycycline for 4 d followed by 4 d without. To confirm that the maintenance of *Xist*-mediated silencing is not limited to the cointegrated puro marker, we performed Northern analysis of the imprinted *Meg1* gene that is expressed

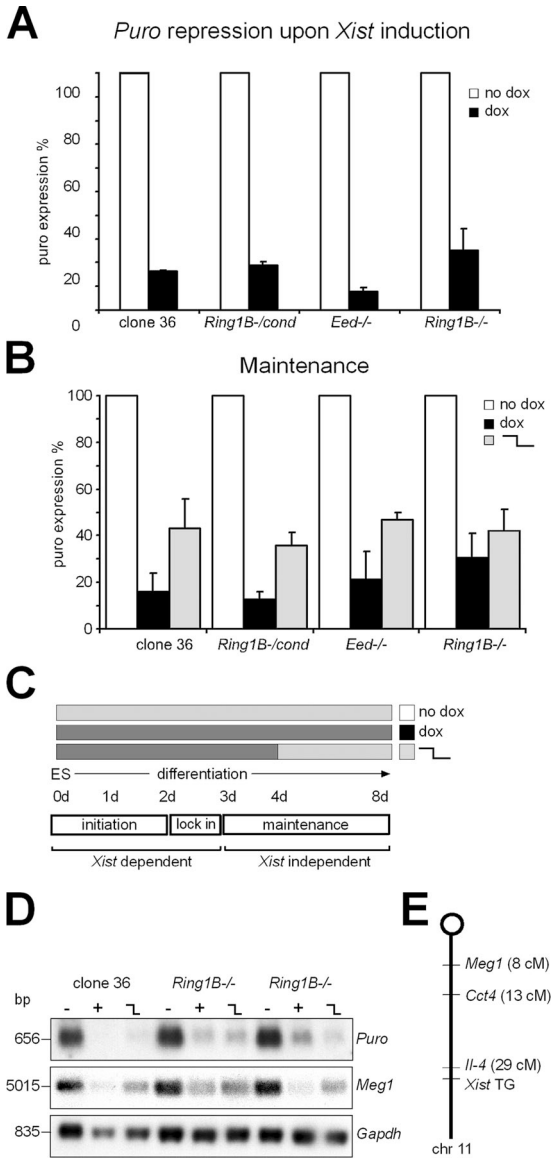

Figure 5. **Initiation and maintenance of *Xist*-mediated silencing in *Ring1B*-deficient cells.** (A) Quantification of puro repression upon *Xist* induction with doxycycline (dox) in clone 36, $36^{Ring1B-/cond}$, $36^{Eed-/-}$, and $36^{Ring1B-/-}$ ES cells by Northern analysis. (B) Maintenance of puro repression in differentiated ES cells of indicated genotypes quantified by Northern analysis. Error bars represent SD. (C) Schematic representation of the doxycycline induction scheme (light gray, no dox; dark gray, +dox) used for the experiment in B. The phases of X inactivation are indicated below. (D) Stable maintenance of chromosome-wide silencing in the absence of *Xist* expression as shown for *Meg1* and *Puro* by Northern analysis. (E) Scheme showing the location of *Meg1*, *Cct4*, and the *Xist* transgene on chromosome 11.

from the maternal chromosome 11, into which the *Xist* transgene was integrated (Fig. 5 E; Wutz and Jaenisch, 2000). We found that *Meg1* is repressed by *Xist* expression in clone 36 control and $36^{Ring1B-/-}$ cells after day 8 of differentiation in the presence of doxycycline. Repression was further stably maintained if *Xist* was turned off after 4 d of differentiation (Fig. 5 D). We further confirmed these results by real-time PCR analysis of *Cct4* expression, a nonimprinted gene on chromosome 11 (Fig. S3 C). This demonstrated that *Ring1B* is dispensable for the chromosome-wide maintenance of silencing in differentiated cells.

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We next assessed the ability of *Ring1B*-deficient cells to establish a chromosomal memory that is set up by the expression of *Xist* early in differentiation and allows for the efficient recruitment of H3K27me3 by *Xist* in differentiated cells (Kohlmaier et al., 2004). We found that the establishment of memory is independent of *Ring1B* (Fig. S3 D). After 15 d of doxycycline treatment, 58% of clone 36 and 71% of $36^{Ring1B-/-}$ cells with a *Xist* focus also showed colocalizing H3K27me3. The delayed induction of *Xist* after 4 d of differentiation without doxycycline resulted in reduced H3K27me3 recruitment, with 37% of clone 36 and 35% of $36^{Ring1B-/-}$ cells showing focal H3K27me3 colocalizing with *Xist*. When *Xist* was turned on for the first 4 d of differentiation followed by 4 d without doxycycline and reinduction for 7 d more, H3K27me3 recruitment was observed in 58% of control clone 36 and 65% of $36^{Ring1B-/-}$ cells comparable with differentiation in the continuous presence of doxycycline. This shows that a chromosomal memory regulating H3K27me3 in differentiated cells can be established by *Xist* independent of *Ring1B*.

Discussion

A dual role for *Ring1B* in the regulation of lineage genes and PRC1 proteins

We find that a null mutation in *Ring1B* leads to a reduction of PRC1 proteins, including Mph1, Bmi1, and Mel18, and a loss of H2AK119ub1 in ES cells. Consequently, the loss of PRC1 causes the derepression of lineage-restricted genes in ES cells and leads to aberrant differentiation. The genes *Cdx2*, *Eomes*, *Hand1*, and *Foxa2* are derepressed in *Ring1B*-deficient ES cells, which is consistent with a previous report of Ring1B binding to their promoters (Boyer et al., 2006). Moreover, *Eomes*, *Hand1*, and *Cdx2*, which are bound by Ring1B and PRC2, are derepressed in either *Ring1B*-deficient or *Eed*-deficient ES cells. This demonstrates that both *Ring1B* and PRC2 are essential for the repression of developmental genes, which is consistent with reports that PRC2 is required for PRC1 recruitment to the *Ultrabithorax* locus in *Drosophila melanogaster* cells (Cao et al., 2002; Muller et al., 2002). Notably, *Foxa2*, a target of PRC1 but not PRC2, is derepressed strongly in *Ring1B*-deficient but only weakly in *Eed*-deficient ES cells. This indicates that PRC2-dependent and independent modes of PRC1 recruitment to developmental control genes exist, similar to our previous observation in X inactivation (Schoeftner et al., 2006).

Loss of the repression of lineage-specific genes in *Ring1B*-deficient ES cells contributes to a marked predisposition to differentiation. Nonetheless, if *Ring1B*-deficient ES cells are cultured under optimal conditions, they proliferate normally and express the pluripotency-associated marker *Oct4* comparable with wild-type ES cells. Differentiation of *Ring1B*-deficient ES cells leads to abnormal EB formation, which is possibly the result of a failure to generate the normal spectrum of cell types. This results in the inability of the EB to form contractile cardiomyocytes but does not impair the proliferation of differentiating cells. Aberrant differentiation is consistent with the observation that disruption of the *Ring1B* gene in mice results in gastrulation arrest (Voncken et al., 2003). Notably, we find the expression of *Pl-1*, a gene that is specific for terminally differentiated trophoblast cells, upon the differentiation of *Ring1B*-deficient ES cells. This could indicate an aberrant differentiation potential toward extraembryonic lineages, which is not observed in normal mouse ES cells. The effect of *Ring1B* on lineage specification is dosage sensitive, as we observe a partial phenotype in $36^{Ring1B-/cond}$ ES cells, which show reduced levels of Ring1B protein as a result of a hypomorphic *Ring1B* allele. These cells can form contractile cell types but attach to culture plates only inefficiently, resulting in the formation of peculiar contracting spherical structures.

Several PcG proteins were present in reduced amounts in *Ring1B*-deficient cells. By Western and immunofluorescence analyses, we found that Rybp, Mel18, Mpc2, and Mph1 are virtually absent in *Ring1B*-deficient ES cells. The finding that these PRC1 transcripts were detected in $36^{Ring1B-/-}$ ES cells suggests regulation at the protein level. The *Bmi1* promoter has been reported as a target of both PRC1 and PRC2 (Boyer et al., 2006). Consistent with this, we found elevated *Bmi1* transcript levels in *Ring1B*- and *Eed*-deficient cells. However, Bmi1 protein accumulates in *Eed*-deficient but is virtually absent in *Ring1B*-deficient ES cells despite elevated mRNA levels. This suggests that Ring1B is needed for Bmi1 protein translation or stabilization, possibly by complex formation. This is in line with a recent report that Ring1B and Bmi1 are required for mutual stabilization (Ben-Saadon et al., 2006). Notably, Ring1B and PRC2 regulate *Bmi1* expression at the transcriptional and protein levels. The requirement of Ring1B for the regulation of protein levels of other PRC1 members is somewhat reminiscent of the situation in PRC2, in which Eed controls the abundance of Ezh2 protein but *Ezh2* transcription is unaltered in *Eed*-deficient cells (Schoeftner et al., 2006). This suggests that PcG proteins in general might be regulated at the protein level to achieve proper complex composition. We conclude that *Ring1B* has a dual function in the regulation of PRC1 protein levels and in the maintenance of transcriptional repression of developmental control genes in ES cells.

Ring1B is crucial for the recruitment of H2AK119ub1 by *Xist* in ES cells

Xist expression cannot establish chromosome-wide H2AK119ub1 in *Ring1B*-deficient ES cells. This is in contrast to the situation in mouse embryonic fibroblasts, in which the disruption of *Ring1B* has no effect on H2AK119ub1 on the Xi, but only the double deficiency of *Ring1A* and *Ring1B* leads to a loss of H2AK119ub1 (de Napoles et al., 2004). Likewise, we find that H2AK119ub1 colocalization with *Xist* is restored upon the differentiation of *Ring1B*-deficient ES cells. This indicates the presence of a redundantly acting E3 ligase activity similar to that of Ring1A in embryonic fibroblasts. Consistent with this, we observe Ring1A colocalization with *Xist* in differentiated ES cells. We conclude that in ES cells, the establishment of H2AK119ub1 on the *Xist*-expressing chromosome as well as on developmental control genes requires the specific recruitment of Ring1B.

In differentiated $36^{Ring1B-/-}$ ES cells, H2AK119ub1 is observed on the *Xist*-expressing chromosome despite the absence of Ring1B and several PRC1 proteins. H2A ubiquitination

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activity is specifically recruited by *Xist*, but global levels of H2AK119ub1 are not restored upon the differentiation of $36^{Ring1B-/-}$ cells. Similar results were reported in mouse embryonic fibroblasts, in which global H2AK119ub1 was lost, but H2AK119ub1 on the Xi was unaffected upon the deletion of *Ring1B* (de Napoles et al., 2004). Ring1A E3 ligase activity in the absence of Mph2 has been shown in vitro (Buchwald et al., 2006; Li et al., 2006). Additionally, our previous observation that Ring1B can catalyze H2AK119ub1 without Mph1 recruitment in *Eed*-deficient ES cells (Schoeftner et al., 2006) supports the idea of the PRC1-independent recruitment of Ring1A to the *Xist*-expressing chromosome in differentiating $36^{Ring1B-/-}$ cells. Bmi1 is sufficient for the H2A ubiquitination activity of Ring1A when a Ring1A–Bmi1 complex is reconstituted in vitro (Buchwald et al., 2006). In contrast, our data suggest that Bmi1 and Mel18 are not essential for the recruitment of E3 ligase activity by *Xist*. Our findings indicate that H2A ubiquitination in X inactivation depends on a special mode of PcG recruitment by *Xist*, and Ring1B appears to be critical for global H2AK119ub1 in ES cells and differentiated cells.

H2AK119ub1 is not required for the initiation of *Xist*-mediated silencing

We have previously shown that H2AK119ub1 can be recruited by a mutant *Xist* RNA, which lacks the 5′ repeat A and does not initiate gene silencing in ES cells (Schoeftner et al., 2006). Thus, H2AK119ub1 is not sufficient for gene silencing in X inactivation. However, it remained conceivable that H2AK119ub1 could be a prerequisite for silencing. In this study, we find that *Xist* initiates silencing in the absence of H2AK119ub1 in *Ring1B*-deficient ES cells. From this and from our previous data (Schoeftner et al., 2006), we conclude that neither H2AK119ub1 nor H3K27me3 are essential for silencing in X inactivation. This is in contrast to the finding that developmentally regulated genes are derepressed in *Ring1B*-deficient ES cells. Thus, we conclude that the requirement for PcG recruitment differs between the silencing of developmental genes and X inactivation. The reason for this discrepancy could be that PRC1 and PRC2 are recruited in parallel by *Xist* RNA and, thus, could compensate for each other's loss of function. Consistent with this notion, the other initiation marks of X inactivation, namely H3K27me3 and H4K20me1, are efficiently recruited by *Xist* in *Ring1B*-deficient cells.

Maintenance of X inactivation in *Ring1B*-deficient cells

Xist expression in ES cells initiates reversible chromosome-wide gene repression. Therefore, a potential repressive activity of *Ring1B* might be masked by active repression by *Xist*. Upon differentiation, *Xist* loses its ability to initiate silencing, and repression is maintained independently of *Xist*. The PcG system appears severely compromised in differentiating $36^{Ring1B-/-}$ cells, as the abundance of several PRC1 and PRC2 proteins is strongly reduced. However, we observe that chromosome-wide histone modifications characteristic of the Xi are not affected by the absence of *Ring1B* in differentiated cells. Moreover, chromosomal silencing is stably maintained independently of *Xist* in differentiated *Ring1B*-deficient cells. This is in stark contrast to the regulation of developmental control genes, which are derepressed in ES cells carrying mutations in either *Eed* or *Ring1B*. We note that the chromosome-wide silencing of X inactivation is more robust in the face of a loss of PcG proteins than the repression of developmental regulators. This might suggest that in X inactivation, several levels of control act synergistically, and the loss of Ring1B causes only a minor destabilization, which we could not detect by our assays. In the future, it will be imperative to study the simultaneous loss of PRC1 and PRC2 function and examine whether such a mutant background is compatible with stem cell maintenance. Thus, X inactivation can provide a sophisticated model system for studying aspects of PcG protein recruitment and to dissect their effect on chromatin and gene expression.

Materials and methods

Cell culture and generation of ES cell lines

ES cell culture was described previously (Wutz and Jaenisch, 2000). *Xist* expression was induced with 1 μg/ml doxycycline. Differentiation medium contained 100 nM of all-trans–retinoic acid and no Leukemia inhibitory factor (LIF). EBs were generated by the hanging drop method in medium without LIF for 2 d. Then, aggregates were cultured in suspension and subsequently plated on gelatin-coated dishes for up to 3 wk. Cells were counted with a Casy 1 cell counter (Schaerfe System GmbH). For *Ring1B* targeting, a 10-kb HindIII–BamHI genomic fragment was isolated from a bacterial artificial chromosome clone (RP22-287N19) from the RPCI22 129 mouse bacterial artificial chromosome library (Children's Hospital Oakland Research Institute). For the minus targeting vector, a 3-kb AvrII–SphI fragment containing three exons, including the start codon and RING domain, was replaced by a stop cassette containing the adenoviral splice acceptor, a *loxP*-flanked hygromycin-thymidine kinase cassette, and a polyadenylation signal. For counter selection, a diphtheria toxin A chain cassette was added (Fig. 1 A). Clone 36 ES cells (Wutz and Jaenisch, 2000) were electroporated with 50 μg of linearized targeting vector. After selection with 130 μg/ml hygromycin B, targeted clones were identified by Southern analysis of BamHI-digested DNA by a 5-kb band (wild type at 12 kb). The targeting frequency was 15%. The selection cassette was removed by electroporation of 30 μg Cre recombinase expression vector followed by 2 μM gancyclovir selection. For the conditional targeting vector, a *loxP*-flanked hygromycin-thymidine kinase cassette was integrated into the SphI restriction site in intron 4. A *loxP* and a BamHI site were inserted into an AvrII site in intron 1 (Fig. 1 B). $36^{Ring1B-/cond}$ ES cells were obtained with a frequency of 5%, and, after Cre-mediated recombination, $36^{Ring1B-/-}$ ES cells were established with a frequency of 43%.

Immunostaining and RNA FISH

ES cells were preplated twice for 30 min to remove feeder cells and were spun onto poly-L-lysine–coated slides (Sigma-Aldrich) using a centrifuge (Cytospin 3; Thermo Shandon). Differentiated cells were grown on Roboz slides (CellPoint Scientific). Immunostaining was performed as described previously (Kohlmaier et al., 2004). In brief, cells were fixed for 10 min in 4% PFA in PBS, permeabilized for 5 min in 0.1% Na citrate/0.5% Triton X-100, and blocked for 30 min in PBS containing 5% BSA and 0.1% Tween 20. For H2AK119ub1 immunostaining, cells were preextracted in 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 10 mM Pipes, pH 6.8, and 0.5% Triton X-100 for 2 min before fixation, and washes after incubation with primary and secondary antibody were performed in KCM buffer (120 mM KCl, 20 mM NaCl, 10 mM Tris, pH 8.0, and 0.5 mM EDTA)/0.1% Tween 20.

RNA FISH probes were generated by random priming (Stratagene) using Cy3-dCTP (GE Healthcare). After immunostaining, cells were fixed in 4% PFA in PBS for 10 min, dehydrated, hybridized, and washed as described previously (Wutz and Jaenisch, 2000). Vectashield (Vector Laboratories) was used as imaging medium. Images were obtained at room temperature with a fluorescence microscope (Axioplan; Carl Zeiss MicroImaging, Inc.) at a magnification of 100× using a plan Neofluar NA 1.3 objective, a CCD camera (CoolSNAP *fx*; Photometrics), and MetaMorph image analysis software (Universal Imaging Corp.). Color levels were adjusted in Photoshop 7.0 (Adobe). For colocalization analysis, at least two

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independently derived *Ring1B*$^{-/-}$ ES cell lines were analyzed, and the means and SDs of at least two experiments were calculated and normalized to the number of *Xist*-expressing cells unless stated differently.

RNA and protein analysis

Northern analysis was performed using 15 μg RNA (TRIzol; Invitrogen) as described previously (Wutz and Jaenisch, 2000). Quantification was performed using a scanner (STORM 860; Molecular Dynamics) and ImageQuant TL software v2003.03 (GE Healthcare). Mean and SD was calculated from at least two 36$^{Ring1B-/-}$ cell lines and from at least two independent experiments. Histones were acid extracted in 0.2 N HCl. Nuclear proteins were extracted in 10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 25% glycerol, and 0.4 M NaCl after the cytoplasm had been separated. Protein concentration was measured by the Bradford assay. Loading was controlled by Ponceau S staining and lamin B1.

The following antibodies were used for immunofluorescence/Western analysis (Antisera dilutions are given in immunofluorescence/Western blot pairs. "−/…" identifies that the antisera was not used for immunofluorescence; "…/−" was not used in Western blot): α-Ring1B (1:100/1:100; Atsuta et al., 2001), α-Ring1A (1:100/1:100; Schoorlemmer et al., 1997), α-MPc2 (−/1:300; Santa Cruz Biotechnology, Inc.), α-Bmi1 (−/1:500; Abcam), α-Mph1 (1:5/1:2; Isono et al., 2005), α-Mph2 (1:100/1:50; Isono et al., 2005), α-Mel18 (1:300/1:500; Santa Cruz Biotechnology, Inc.), α-Suz12 (1:1,000/1:1,000; Upstate Biotechnology), α-Ezh2 (1:500/1:500; Schoeftner et al., 2006), α-H3K27me3 (1:1,000/1:1,000; Kohlmaier et al., 2004), α-H4K20me1 (1:1,000/1:1,000; Kohlmaier et al., 2004), α-H2AK119ub1 (1:50/1:500; Upstate Biotechnology), α-RYBP (−/1:1,000; Chemicon), α-histone macroH2A–containing antiserum (1:500/−), α-histone macroH2A (−/1:500; Upstate Biotechnology), and α-lamin B1 (−/1:5,000; Abcam). Secondary antibodies used are as follows: AlexaFluor488 goat anti–rabbit IgG (1:500/−), AlexaFluor488 goat anti–mouse IgG (1:500/−), and AlexaFluor568 rabbit anti–goat IgG (1:500/−); and HRP-conjugated Affinipure goat α-rabbit IgG (−/1:10,000), HRP-conjugated Affinipure goat α-mouse IgG (−/1:5,000), HRP-conjugated donkey α-goat IgG (−/1:2,000), and HRP-conjugated donkey α-human IgG (−/1:2,000) from Jackson ImmunoResearch Laboratories.

Semiquantitative and quantitative PCR expression analysis

cDNA was generated from 400 ng of total RNA from clone 36, 36$^{Ring1B-/cond}$, 36$^{Ring1B-/-}$, 36$^{Eed-/-}$ ES cells, and female trophoblast stem cells using the Superscript II Reverse transcription kit (Invitrogen) and dT_{12-18} primers. Expression of the genes *Cdx2*, *Eomes*, *Pl-1*, *Hand1*, *Foxa2*, *Hnf4*, *Oct4*, *Hoxa1*, *Ring1A*, *Ring1B*, *Bmi1*, *Mph1*, *Mph2*, *Mpc2*, *Mel18*, *Rybp*, *Suz12*, and *β-actin* was analyzed by PCR (for primer sequences and conditions, see Table S1, available at http://www.jcb.org/cgi/content/full/jcb.200612127/DC1). Real-time PCR analysis was performed as described previously (Schoeftner et al., 2006).

Online supplemental material

Fig. S1 describes the expression analysis of differentiated *Ring1B*-deficient ES cells. Fig. S2 presents immunofluorescence analysis of H3K27me3 and Ring1A recruitment in clone 36 and 36$^{Ring1B-/-}$ cells. In Fig. S3, we present the analysis of chromosome-wide silencing in clone 36 and 36$^{Ring1B-/-}$ cells. Video 1 shows contractile spheres formed by differentiating Ring1B$^{-/cond}$ ES cells. Table S1 provides PCR primer sequences for semiquantitative expression analysis. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200612127/DC1.

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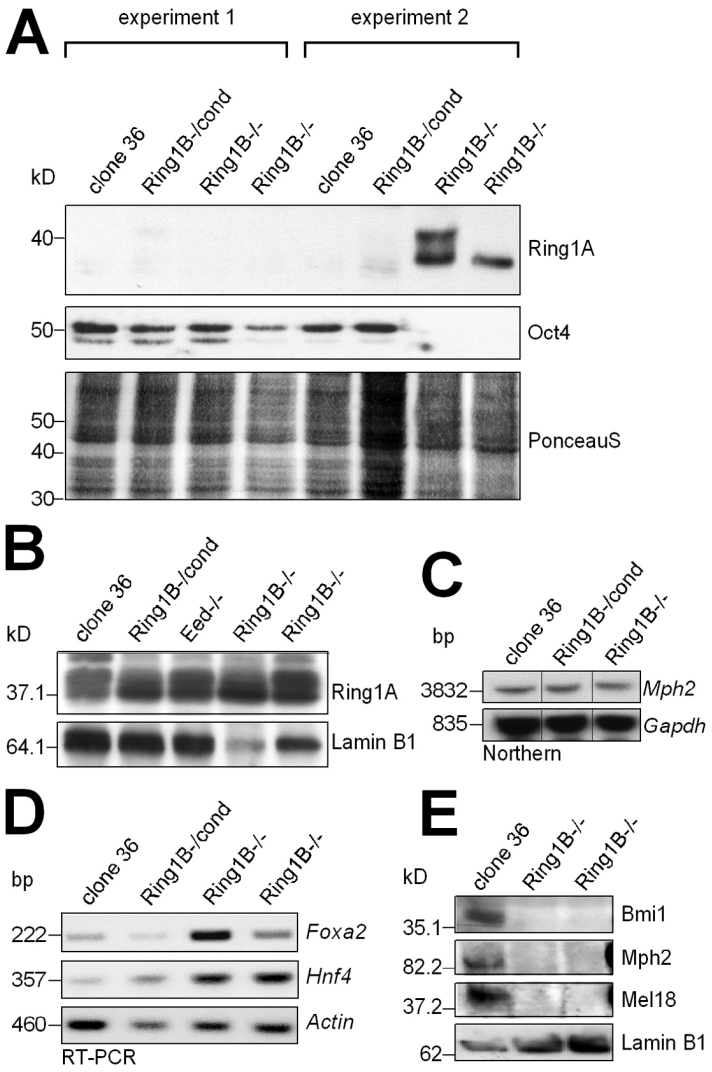
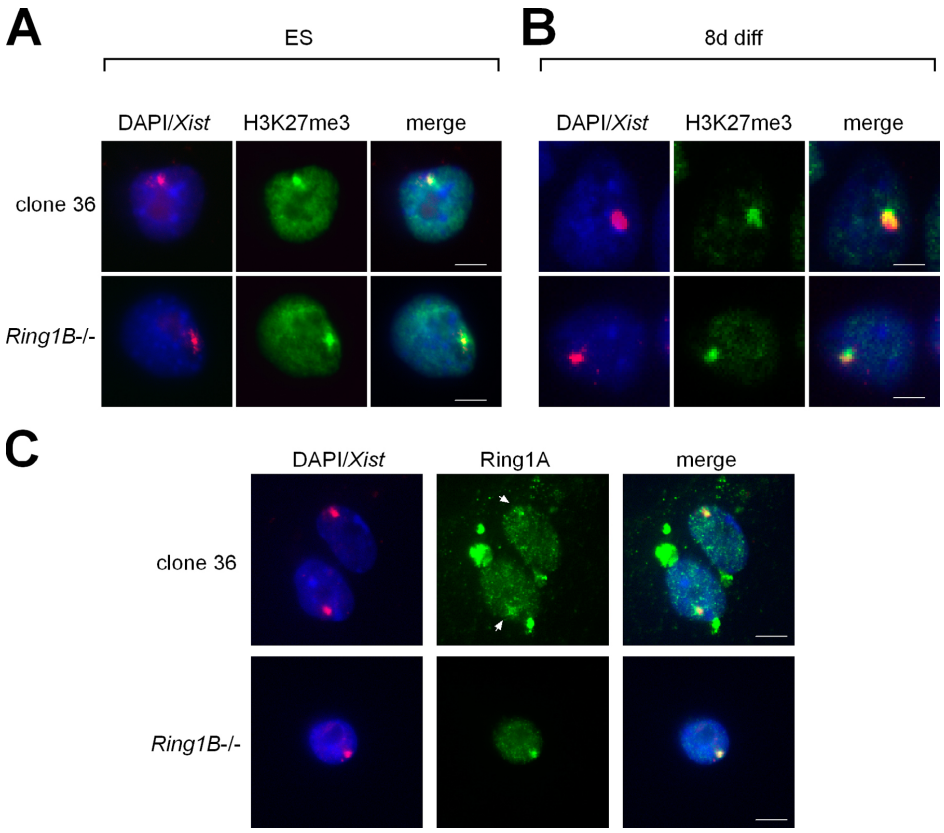
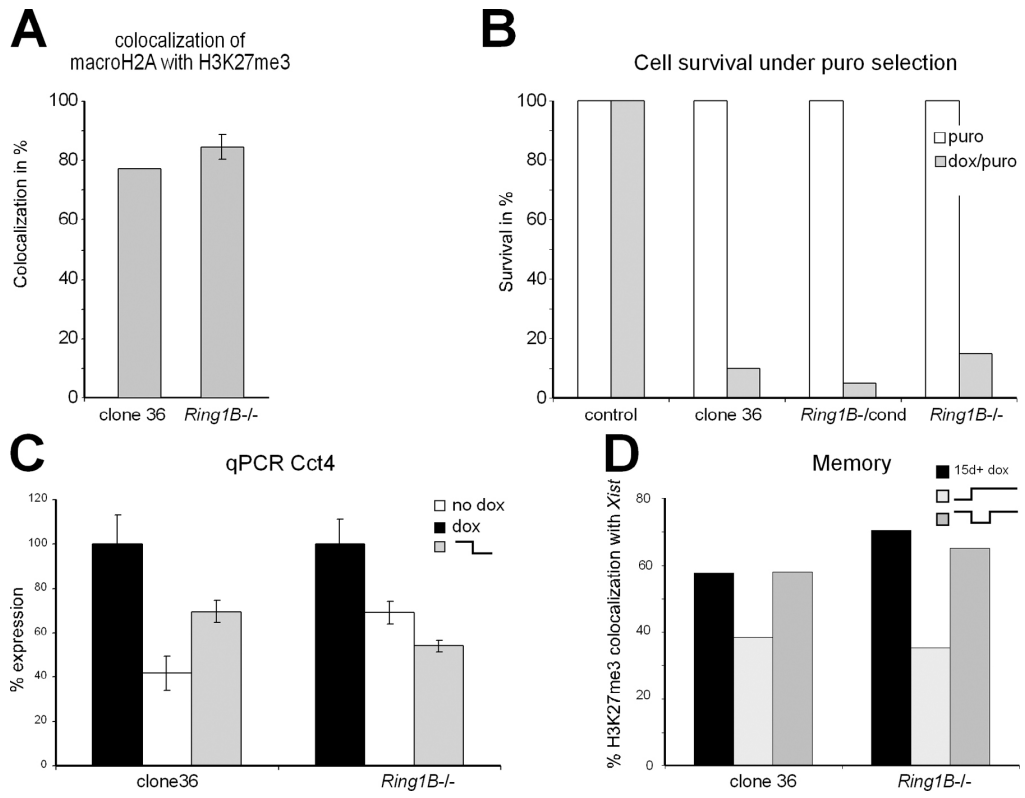


JCB Figure S1. Analysis of the differentiation of *Ring1B*-deficient ES cells. (A) Western analysis of Ring1A protein levels in control clone 36, $36^{Ring1B-/cond}$, and $36^{Ring1B-/-}$ cells. Two independent experiments are shown. Ring1A is up-regulated in $36^{Ring1B-/-}$ cultures in experiment 2, which have lost Oct4 expression as a result of spontaneous entry into differentiation. Ponceau S staining shows protein loading. (B) Western analysis of Ring1A protein levels in control clone 36, $36^{Ring1B-/cond}$, $36^{Eed-/-}$, and $36^{Ring1B-/-}$ after 8 d of differentiation. Lamin B1 was used as a loading control. (C) Northern analysis of *Mph2* expression in wild-type and *Ring1B* mutant cells after 8 d of differentiation. *Gapdh* was used as a loading control. Lanes were grouped. (D) Expression analysis of lineage genes and the loading control *β-actin* using RNA from EBs as indicated by RT-PCR after 2 wk in suspension culture. (E) Western analysis of Bmi1, Mel18, and Mph2 in nuclear extracts from 8-d differentiated clone 36, $36^{Eed-/-}$, and $36^{Ring1B-/-}$ cells.



JCB Figure S2. Immunofluorescence analysis of H3K27me3 and Ring1A recruitment in clone 36 and 36$^{Ring1B-/-}$ cells. (A and B) Localization of H3K27me3 to the *Xist*-expressing chromosome is independent of Ring1B in ES (A) and differentiated cells (B). (C) Combined immunofluorescence with *Xist* RNA FISH showing that Ring1A protein is recruited by *Xist* in differentiated control clone 36 and *Ring1B*-deficient ES cells. Arrows indicate Ring1A foci. Pictures were obtained as described at 63x magnification using an NA 1.4 plan Apochromat objective (Carl Zeiss MicroImaging, Inc.). Bars (A and B), 5 µm; (C) 2 µm.



JCB Figure S3. Analysis of chromosome-wide silencing and memory formation in $36^{Ring1B-/-}$ cells. (A) MacroH2A colocalization with H3K27me3 in clone36 and $36^{Ring1B-/-}$ cells induced to differentiate with retinoic acid for 8 d (*n* = 100). Error bars represent SD of measurements of two independent *Ring1B*-deficient clones, and controls were counted once. (B) Cell survival under puromycin selection of control, clone 36, $36^{Ring1B-/cond}$, and $36^{Ring1B-/-}$ ES cells with and without *Xist* induction by the addition of doxycycline (dox). Control ES cells have lost their ability to induce *Xist* expression (see text). (C) Real-time PCR analysis of Cct4 repression in the *Xist*-independent maintenance phase of X inactivation (see Fig. 5 B). Repression of one allele of Cct4 on chromosome 11 by *Xist* results in a reduction of transcript abundance to 50% in control clone 36 ES cells at day 8 of differentiation (dox) compared with control cultures, in which *Xist* was not induced (no dox). Similarly, repression was observed in *Ring1B*-deficient cells. Furthermore, silencing was stably maintained if *Xist* was turned after 4 d of differentiation (¯|_). (D) *Xist* expression early in ES differentiation regulates H3K27me3 in differentiated cells independent of *Ring1B*. The percentage of cells with focal H3K27me3 staining colocalizing with *Xist* is shown for cells that were differentiated for 15 d in the presence of doxycycline (black bars), for 4 d without followed by 11 d with doxycycline (light gray bars), or for 4 d with doxycycline followed by 4 d without and subsequent induction for 7 d with doxycycline (dark gray bars).

Part II: Polycomb group complexes act redundantly in embryonic stem cell differentiation and repress endogenous retroviruses

Despite showing a strong effect on expression of developmental control genes and overall gene expression profiles, *Ring1B*$^{-/-}$ ES cells self renewed in culture and X chromosome inactivation was unaffected by the loss of PRC1 activity. Furthermore, the genome wide activity of PRC2 remained unaffected and H3K27me3 was recruited to the inactive X chromosome. Vice versa, PRC2 deficiency leads to the absence of global H3K27me3, whereas genomic ubH2A levels and PRC1 recruitment to the Xi remain largely unaffected (Schoeftner et al., 2006). This indicates that parallel modes of PRC1 and PRC2 recruitment exist and that these marks are, at least to some extent, independent of each other. In order to investigate if redundant functions of PRC1 and PRC2 might have masked a critical function of PcG complexes in pluripotency and cellular differentiation, I aimed for generating ES cells deficient for PRC1 and PRC2 function by concomitant deletion of *Ring1B* and *Eed*.

Generation of *Eed* / *Ring1B* double deficient ES cells

I generated ES cells lacking both PRC1 and PRC2 activity following a conditional gene targeting strategy to disrupt *Ring1B* in *Eed* deficient ES cells (Leeb and Wutz, 2007; Schoeftner et al., 2006). The first allele of *Ring1B* was disrupted using a deletion vector (Leeb and Wutz, 2007). After excision of the Hygromycin-HSV thymidine kinase selection cassette, the second allele was targeted with a conditional targeting vector. At first, deletion of the second *Ring1B* allele was induced by transient transfection with a CMV-Cre recombinase vector followed by selection with Ganciclovir. Using this strategy the efficiency of deletion after subcloning was less than 1% and no viable PRC1 / PRC2 double deficient (dKO) ES cells could be obtained (Figure 4a). The efficiency of *Ring1B* disruption could be improved to about 60% by using an Adenovirus carrying the Cre recombinase gene (Figure 4b). However, no proliferating ES cell clones could be established due to massive differentiation of ES cell cultures, possibly induced by the Adenovirus infection. This strong

tendency to differentiate upon Adenoviral infection I could not observe in wt ES cells and to a lesser degree in PcG single KO ES cells, indicating that PcG double deficiency results in a weakened ES cell state that is not compatible with viral infection. To overcome the negative side-effects of Adenovirus infection, I transfected *Eed*$^{-/-}$*:Ring1B*$^{-/fl}$ ES cells with a Cre recombinase / estrogen receptor (CreER:EF1α-BSD) fusion construct. Clonal ES cell lines were established by selection with Blasticidine. Expression of CreER and translocation to the nucleus upon addition of 4-Hydroxy-Tamoxifen (4OHT) was confirmed by immunofluorescence (IF) analysis (Figure 4c). Addition of 4OHT resulted in very efficient deletion of the *Ring1B* gene, and in 85-95% of the cells a nuclear Ring1B IF signal was not detectable.

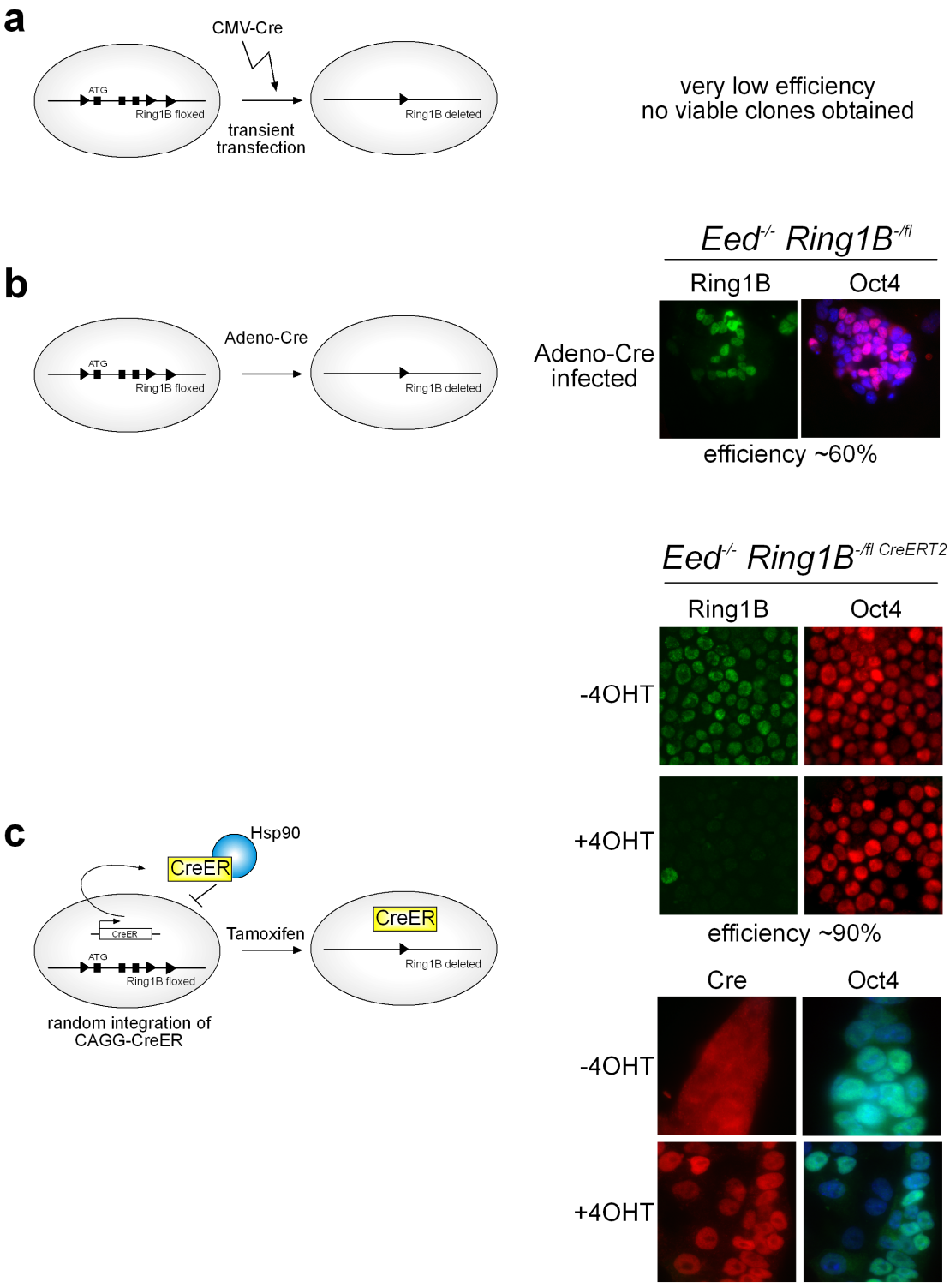


Figure 4 Establishment of PcG dKO ES cells using different strategies for Ring1B deletion

(**a**) Deletion of *Ring1B* in *Eed*$^{-/-}$*:Ring1B*$^{-/fl}$ ES cells by transfection with a CMV-Cre recombinase vector was not successful. (**b**) Infection with an Adenovirus carrying the Cre recombinase gene efficiently deleted the loxP flanked *Ring1B* allele and established dKO ES cells, but resulted in increased differentiation. (**c**) Using a Tamoxifen inducible CreER fusion construct, dKO ES cells could successfully be established and maintained in culture.

PcG dKO ES cells express markers of pluripotency and self renew in culture

PcG proteins have been proposed to have a function in maintaining the pluripotent state of ES cells by repression of lineage control genes. In contrast, I found that *Ring1B*$^{-/-}$ */ Eed*$^{-/-}$ double deficient ES cell lines maintained a typical ES cell morphology for at least 20 passages (Figure 5a). However, the growth of dKO ES cells was slightly retarded compared to wt or single PcG deficient ES cells (Figure 5b). Protein levels of Oct4 and Nanog were largely unchanged in dKO compared to wt and PcG single KO ES cells as determined by IF and Western blotting (Figures 5c and 6a). Furthermore dKO ES cells also expressed *Oct4*, *Rex1* and *Klf4*, and other transcription factors known to be important for pluripotency of ES cells at levels comparable to wt or single PRC deficient ES cells (Figure 5d). However, dKO ES cells were extremely sensitive to suboptimal culture conditions and showed a strong inclination to differentiate upon stress. I also observed a pronounced dependence of dKO ES cells on fibroblast feeder cells, which was not overcome by increasing the dose of LIF. Apparently, the lack of PRC1 and PRC2 activity caused an instable ES cell phenotype but did not block ES cell self renewal and expression of pluripotency markers.

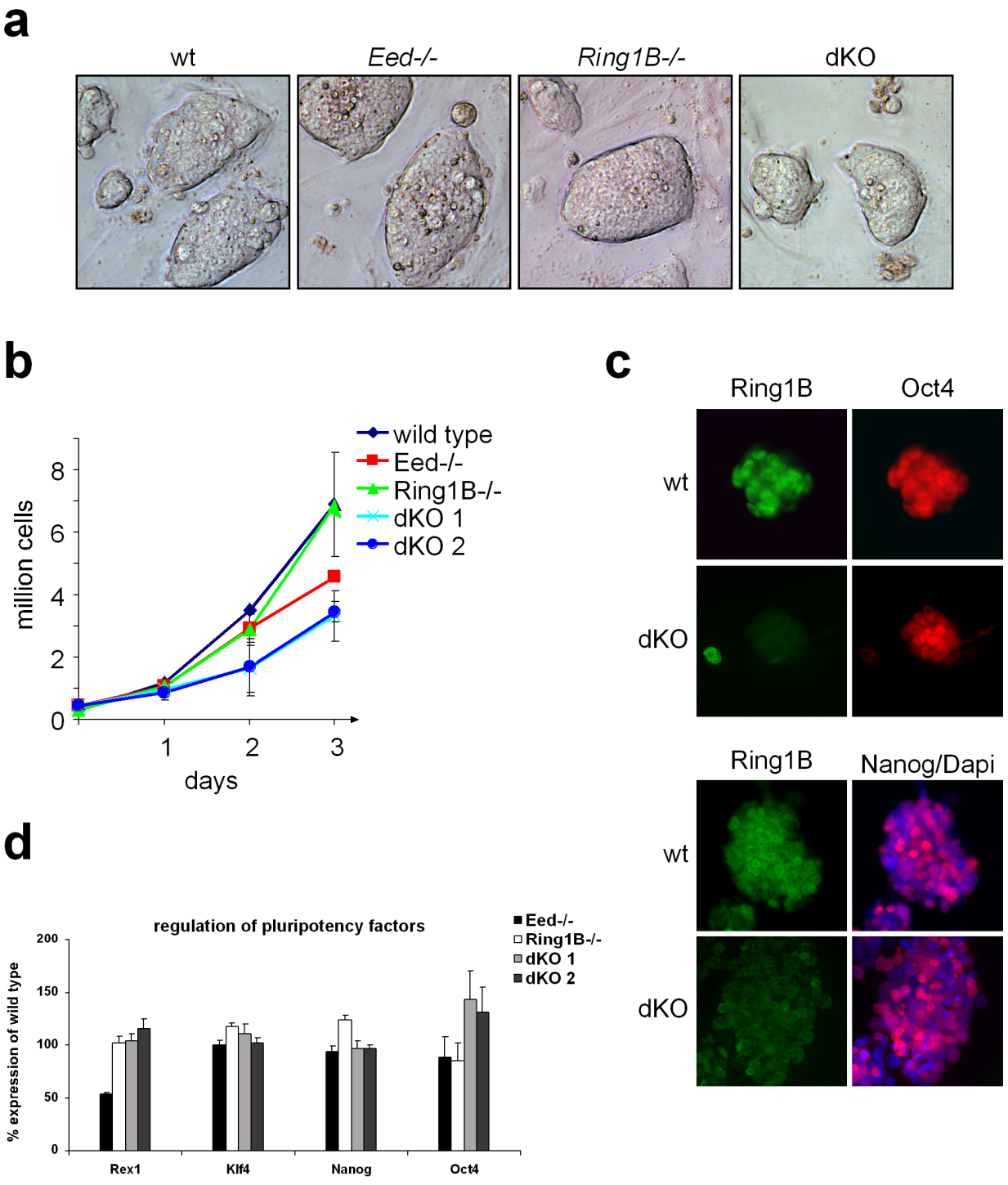


Figure 5 PcG dKO ES cells can be maintained in culture

(**a**) Morphology of wild type (wt), *Ring1B*$^{-/-}$, *Eed*$^{-/-}$ and dKO ES cell colonies. (**b**) Growth curves of wt, *Ring1B*$^{-/-}$, *Eed*$^{-/-}$ and dKO ES recorded over three days (**c**) Immunofluorescence staining showing Oct4 and Nanog expression in dKO and wild type ES cell colonies. (**d**) Quantitative expression analysis of PcG deficient ES cells. Expression of ES cell markers is largely unchanged in all PcG mutant ES cells compared to wild type ES cells.

Concomitant deletion of *Ring1B* and *Eed* disrupts the PRC1 and PRC2 complexes

In dKO ES cells the PRC1 proteins Ring1B, Mel18 and Mph2 were undetectable by Western analysis (Figure 6a). Similarly, Eed was absent and the PRC2 proteins Suz12 and Ezh2 were reduced (Figure 6a). These data are in line with the results obtained in PcG single KO ES cells (Leeb and Wutz, 2007; Schoeftner et al., 2006). The loss of genomic H3K27me3 and ubH2A confirmed the absence of PRC1 and PRC2 activity in dKO ES cells (Figure 6b). These data show that deletion of *Eed* and *Ring1B* disrupts the PRC1 and PRC2 complexes and leads to decreased protein levels of complex members and the absence of the respective histone modifying activities.

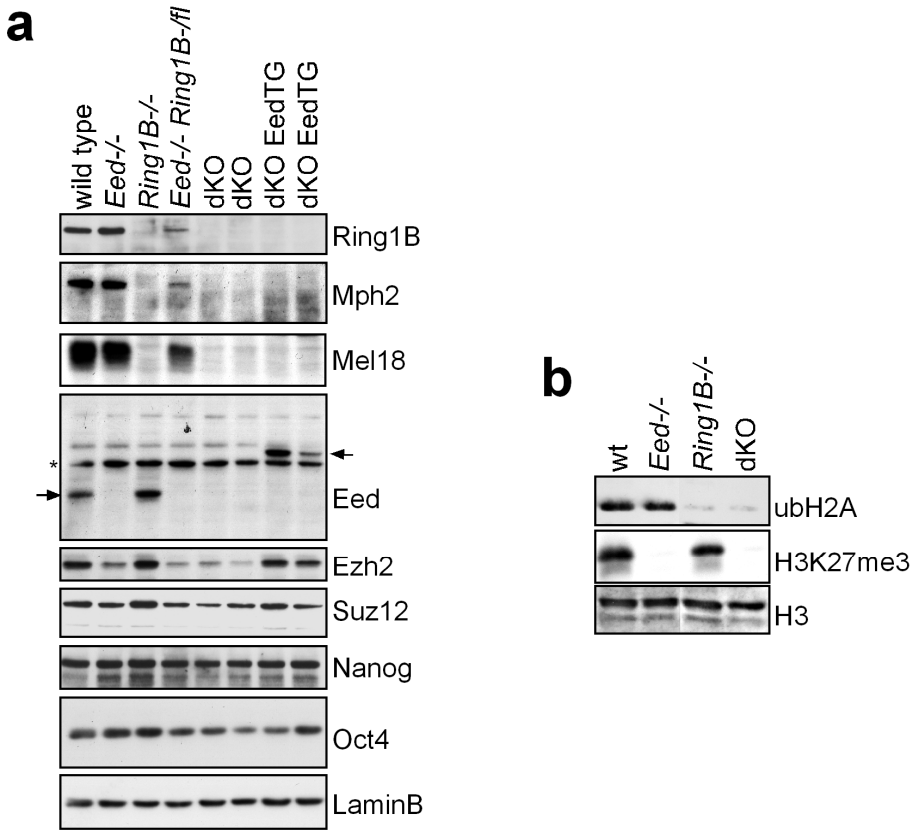


Figure 6 Analysis of PRC stability and function in the absence of PRC1 and PRC2

(**a**) 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 in dKO ES cells. Arrows indicate Eed (asterisk; non specific band). The pluripotency markers Nanog and Oct4 are largely unaffected. Lamin B was used as loading control. (**b**) Western analysis of acid extracted histones shows that ubH2A and H3K27me3 are absent in dKO ES cells.

The PcG system is essential for regulated ES cell differentiation

To investigate the function of the PcG system in differentiation I analyzed the ability of wild type, *Eed*$^{-/-}$, *Ring1B*$^{-/-}$ and dKO ES cells to form teratomas (Figure 7a). For this I injected 1x10^6 ES cells into the flanks of immune deficient recipient mice (nu/nu) using matrigel high concentration as a carrier. Teratomas were excised after three weeks. Wild type, *Eed* and *Ring1B* deficient ES cells formed teratomas, which contained differentiated structures. Immonohistochemical analysis showed that the tumors consisted of cells from the three germ layers, demonstrating that neither PRC1 nor PRC2 activity is essential for differentiation and making lineage choices (Figure 7b). However, teratomas from *Eed* and *Ring1B* deficient ES cells were significantly smaller (Figure 7c) and I noted a larger proportion of cells with an endodermal or ectodermal origin, respectively, when compared to wild type teratomas. In contrast, dKO ES cells did never form teratomas. When dKO grafts were excised after 6 month only Matrigel with a negligible amount of cells remained. This indicates that at least one PcG complex is required for tumor formation. Importantly, the tumor formation potential of dKO ES cells could be restored by the expression of an *Eed*GFP transgene and injection of dKO$^{Eed\text{GFP}}$ ES cells resulted in efficient formation of teratomas in all injection sites. This showed that dKO ES cells retain pluripotency but are not able to generate differentiated structures in vivo, possibly due to the inability to establish and maintain epigenetic patterns required for progression through development.

To further test the differentiation potential of dKO ES cells, I used a protocol for directed differentiation into the neuronal lineage (Pollard et al., 2006). Nestin positive neural stem (NS) cells could readily be established from *Eed* deficient ES cells that were either wildtype for *Ring1B* or contained a conditional *Ring1B* allele over a null allele (*Eed*$^{-/-}$ *Ring1B*$^{-/fl}$), which resulted in strongly hypomorphic *Ring1B* expression (Figures 8a). NS cells could not be obtained from dKO ES cells. Furthermore, three to four days after deletion of *Ring1B* in *Eed*$^{-/-}$:*Ring1B*$^{-/fl}$ NS cells utilizing a Tamoxifen (4OHT) inducible CreERT2 recombinase massive cell death was observed in the NS cell cultures (Figures 8c and d). Control *Eed* deficient NS cells were not affected by the addition of 4OHT. Thus, in contrast to ES cells, the combined loss of PRC1 and PRC2 activity is not compatible

with NS cell survival. In addition, when differentiation was induced with retinoic acid in monolayer culture massive cell death was observed after 4 days in dKO, but not in wild type, *Ring1B* or *Eed* deficient ES cells. In conclusion, these data demonstrate that although ES cells deficient for PRC1 and PRC2 can differentiate, differentiated cells deficient for both PcG complexes are not viable or do not proliferate.

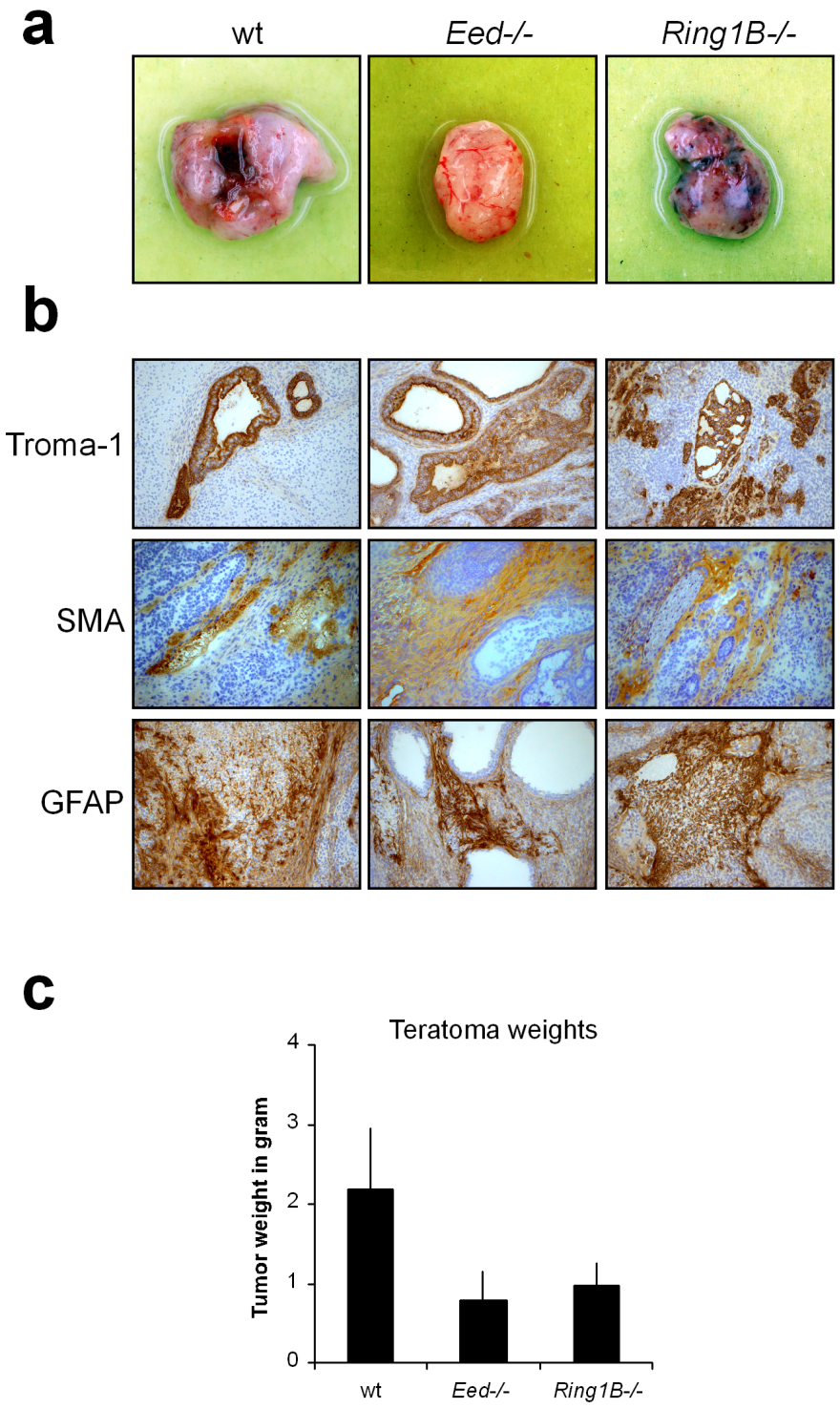


Figure 7 *Eed* and *Ring1B* are redundantly required for differentiation.

(**a**) Teratomas formed by wt, *Eed*$^{-/-}$ and *Ring1B*$^{-/-}$ ES cells three weeks after injection. dKO ES cells did not give rise to teratomas. (**b**) Immunohistochemical analysis of wt, *Ring1B*$^{-/-}$ and *Eed*$^{-/-}$ teratomas using markers for endoderm (Troma1), mesoderm (smooth muscle actin, SMA) and ectoderm (GFAP). (**c**) Graph showing retarded growth in PcG deficient teratomas. Error bars show the standard deviation (n=3).

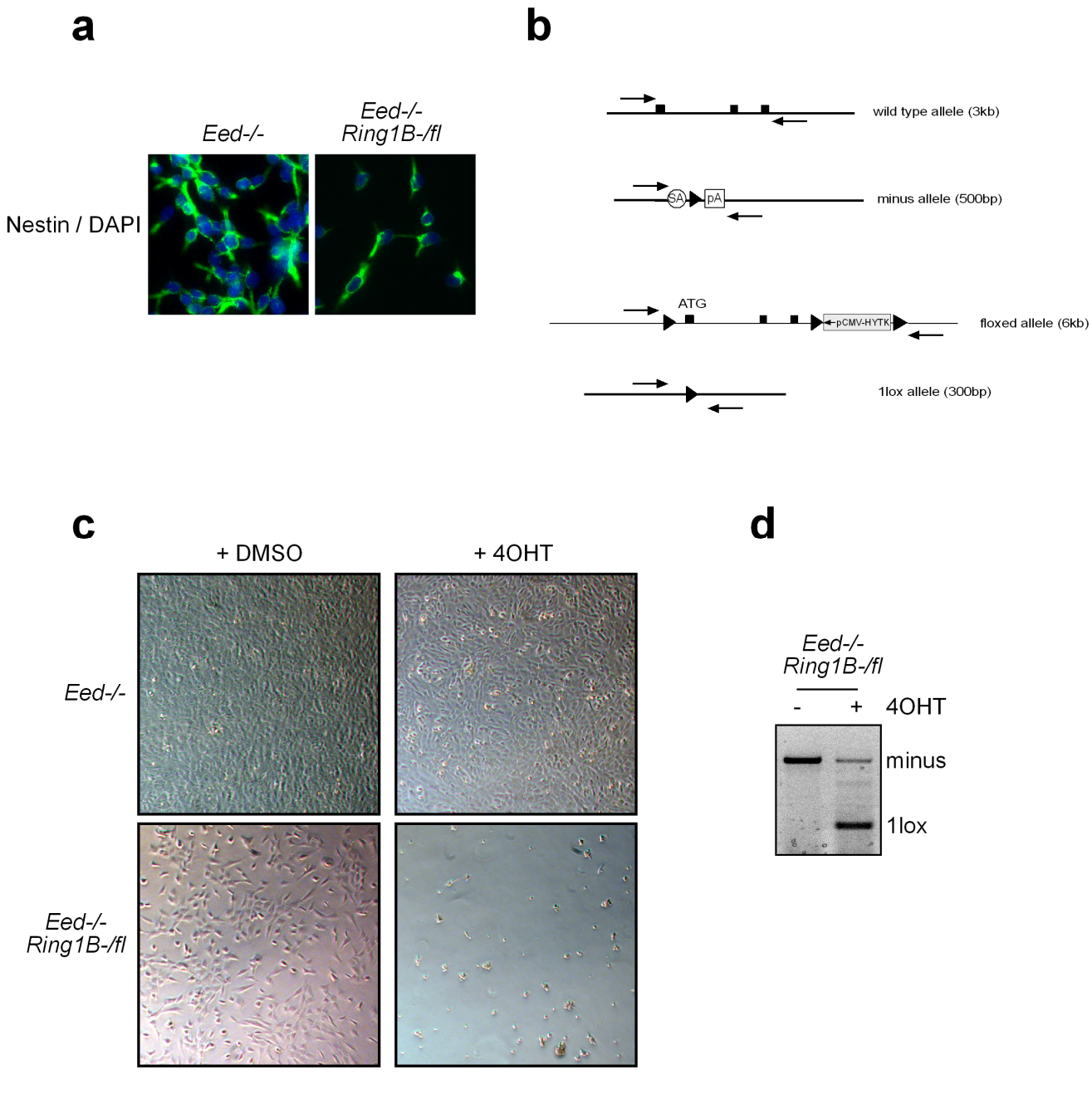


Figure 8 Double deficiency for PRC1 and PRC2 is not compatible with NS cell viability

(**a**) *Eed* deficient and *Eed*$^{-/-}$*:Ring1B*$^{-/fl}$ NS cells are Nestin positive. (**b**) PCR strategy to distinguish between different *Ring1B* alleles. (**c**) *Eed* and *Ring1B* double deficiency for PRC1 and PRC2 is not compatible with NS cell viability. Deletion of *Ring1B* in *Eed*$^{-/-}$*:Ring1B*$^{-/fl}$ 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.

Genome wide gene expression analysis reveals a set of genes which are redundantly silenced by PRC1 and PRC2

The severe phenotype upon a combined loss of PRC1 and PRC2 shows a redundancy for both Polycomb complexes in cell differentiation. To understand the mechanistic basis of this redundancy I analyzed the effect of loss of PcG complex function on gene expression profiles by subjecting wildtype, *Ring1B* and *Eed* deficient, and dKO ES cells to Affymetrics microarray analysis on a GeneChip 430 2.0 array (Figure 9a). Previous studies have already analysed the transcription profiles of *Ring1B* and *Eed* deficient ES cells (Boyer et al., 2006; Endoh et al., 2008; Stock et al., 2007). However, due to the different genetic backgrounds of the analysed cell lines, these data cannot be directly compared and a possible differential contribution of PRC1 and PRC2 to gene silencing could not be addressed. *Ring1B*, *Eed* and dKO ES cells analysed in this study have the same genetic background and were derived from the same wild type ES cell line (Wutz and Jaenisch, 2000), making a direct comparison of transcription profiles possible.

Before subjecting ES cells to transcription analysis, the ES cell identity was carefully confirmed based on colony morphology. A total of 814 and 1151 genes were derepressed more than 2-fold ($p<0.05$) in *Ring1B* and *Eed* deficient ES cells, respectively. In dKO ES cells the number of derepressed genes increased to 2017 (Figure 9b). To assess the direct effect of the loss of PcG activity, I analyzed only genes that were reported to be bound by PcG complexes (Ku et al., 2008). 279 (10% of annotated PcG targets) and 476 (18%) PcG target genes were derepressed more than two-fold ($p<0.05$) in *Ring1B* or *Eed* deficient ES cells, respectively. In dKO ES cells the number of derepressed PcG target genes increased to 757 (29%) (Figure 9c) (Tables 1 and 2). Interestingly, a set of 329 genes was derepressed specifically only after the combined loss of PRC1 and PRC2, but remained repressed in *Ring1B* and *Eed* deficient ES cells (Figure 9c) suggesting that PRC1 and PRC2 function redundantly in repressing these genes. I confirmed the specific derepression of the *Arg2*, *Dlk*, *Nrp1*, *Foxa2*, and *Dkk1* genes in dKO ES cells by quantitative RT-PCR (Figure 9d).This redundantly silenced gene set defines a new class of PcG targets and behaves differently from other genes such as *Hoxd13*,

Hoxd11, *Zic1*, and *Sox7* which were already derepressed in the absence of a single PcG complex (Figure 9e). Importantly, gene repression was restored after introducing an *Eed*GFP transgene into dKO ES cells, indicating that derepression in dKO cells was reversible and that genes remain marked for repression even in the absence of the PcG system. Interestingly, all *Hox* genes belong to the non-redundantly silenced gene set and show full derepression already after loss of a single PcG complex (Figure 9e and Figure 10). Whether silencing of *Hox* genes indeed depends on the presence of both PcG complexes or if other mechanisms like histone deacetylation or DNA methylation provide an additional layer of silencing, masking the redundancy between PRC1 and PRC2, remains an important question for future research.

Loss of PcG function does not only cause upregulation but also results in repression of genes, possibly due to indirect effects. Nevertheless, gene repression upon PcG deficiency could contribute to the destabilized ES cell state. Exclusively in dKO and not in *Ring1B*$^{-/-}$ or *Eed*$^{-/-}$ 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 5-fold downregulated (Tables 3 and 4), possibly contributing to the destabilization of the ES cell phenotype. Based on these data I propose that transcriptional changes including the loss of genes and derepression of redundantly repressed PcG targets lead to a destabilization of the ES cell phenotype and consequently a defect in differentiation of dKO cells.

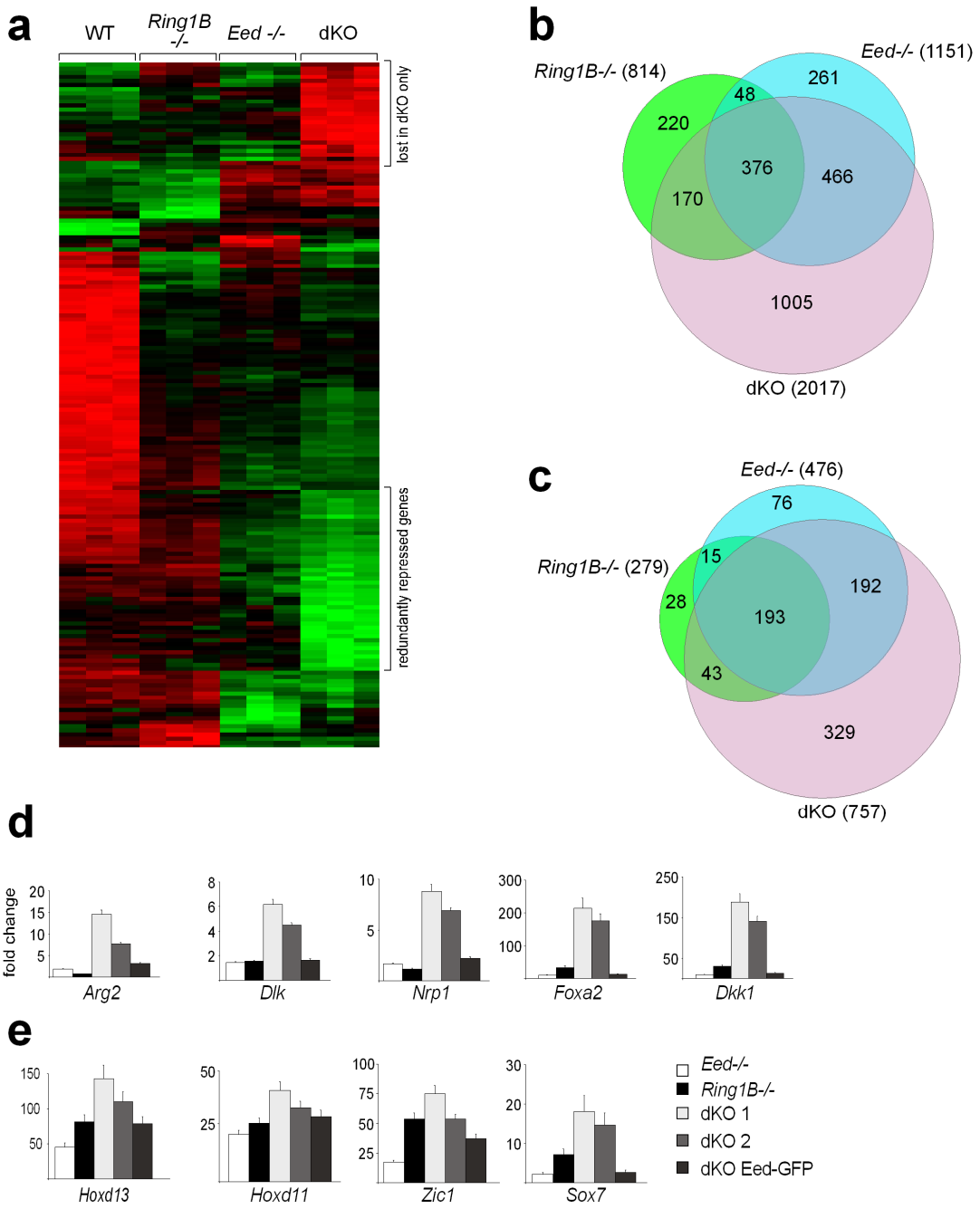


Figure 9 Analysis of global transcription profiles in PcG deficient ES cells

(**a**) A heatmap showing high (green), intermediate (black), and low (red) expression of genes in wild type, *Eed*$^{-/-}$, *Ring1B*$^{-/-}$ and dKO ES cells. (**b**) Venn diagram showing genes that are upregulated (> 2 fold; p<0.05) in *Eed*$^{-/-}$, *Ring1B*$^{-/-}$ and dKO ES cells compared to wt. (**c**) Venn diagram showing upregulated genes in all genotypes (> 2 fold; p<0.05) that have previously been reported as PcG targets (**d** and **e**) 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. (**d**) A redundantly repressed set of PcG target genes is specifically derepressed in dKO ES cells. (**e**) PcG target genes derepressed in all PcG mutant ES cells.

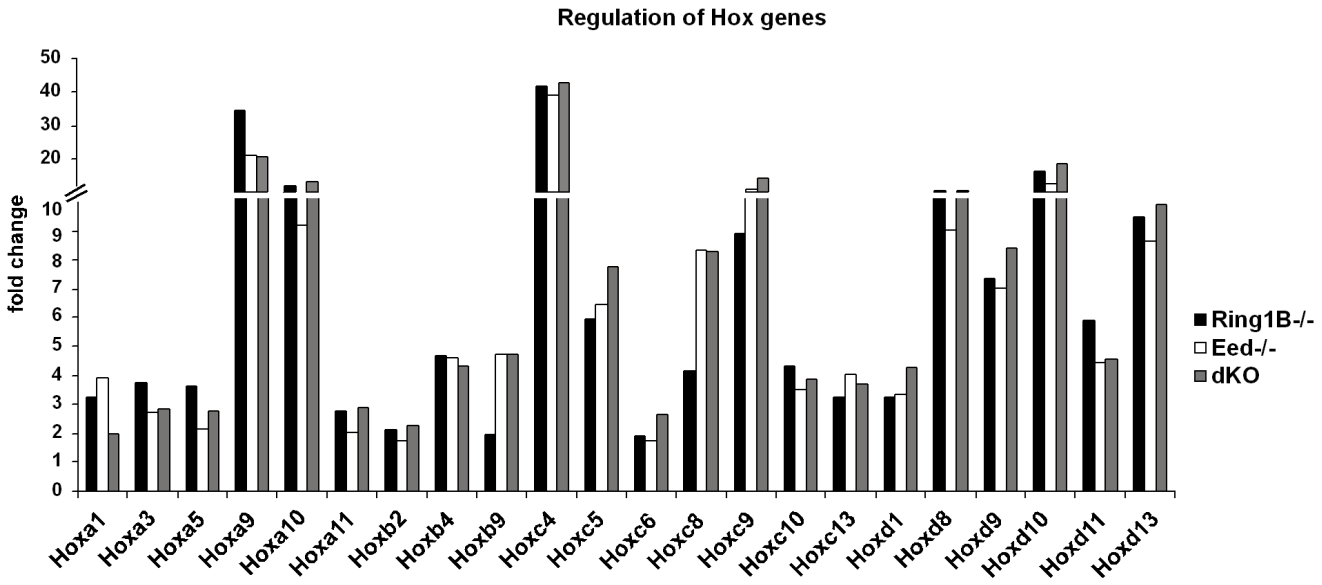


Figure 10 *Hox* genes are non-redundantly silenced and fully derepressed in *Ring1B* or *Eed* deficient ES cells

Graph showing the fold change of expression of *Hox* genes in PcG mutant ES cells versus wt. Only *Hox* genes with a p value of less than 0.05 in all genotypes were plotted.

The molecular environment of PcG target promoters differentially affects the sensitivity of gene repression to loss of PcG function

Recently it has been reported that PcG target promoters can be classified into two subgroups. One group is bound by PRC2 only whereas the other is also bound by the PRC1 component Ring1B (Ku et al., 2008). To test if a functional difference exists between the PRC1 bound and unbound bivalent genes, I investigated if PRC1 positive bivalent genes are more likely to be derepressed in PcG deficient ES cells. Indeed I found them to be preferentially derepressed in *Ring1B*$^{-/-}$ ES cells (Figure 11a). However, the preferential derepression was not limited to PRC1 deficient ES cells, but also in *Eed*$^{-/-}$ and in dKO ES cells, Ring1B positive genes were more likely to be derepressed than expected from the genomic distribution of these two promoter classes. This indicates that silencing of Ring1B positive bivalent genes is more sensitive to loss of PcG proteins than the silencing of other bivalent genes. The reason for this differential regulation is unclear but it indicates a difference in the molecular setup of Ring1B positive PcG target promoters

It has been proposed that the pluripotency TF network cooperates with the PcG system to keep lineage control genes in a silent state in ES cells. I analysed whether genes which are bound by both systems behave differently than other PcG target genes in the face of a loss of PcG function. I found that there is no correlation between the presence of pluripotency associated TFs or core regulators of pluripotency (Oct4, Nanog and Sox2) on PcG target genes (Kim et al., 2008; Ku et al., 2008) with the derepression upon disruption of the PcG system (Figures 11b-e).

Virtually all PcG targets and most H3K4me3 bound genes have CpG rich promoters (Figures 12a-d). Promoters showing an intermediate or low CpG level are depleted for both H3K27me3 and H3K4me3 (Bernstein et al., 2006a; Ku et al., 2008). Overall, genes derepressed in *Ring1B*$^{-/-}$, *Eed*$^{-/-}$ or dKO ES cells are randomly distributed in all three CpG classes (Figure 12e). As expected, derepressed Polycomb target genes have almost exclusively high CpG class promoters. Surprisingly, derepressed non PcG target genes are depleted for high CpG class promoters and show a stronger enrichment of intermediate and

low CpG promoters in all three PcG knockout ES cell lines (Figure 12e). It has been reported that genes with a lineage specific metabolic function tend to have a CpG poor promoter. Based on the function of the PcG system to silence lineage determining transcription factors, the derepression of metabolic lineage genes with a low CpG promoter is an expected secondary consequence of the loss of PcG mediated repression.

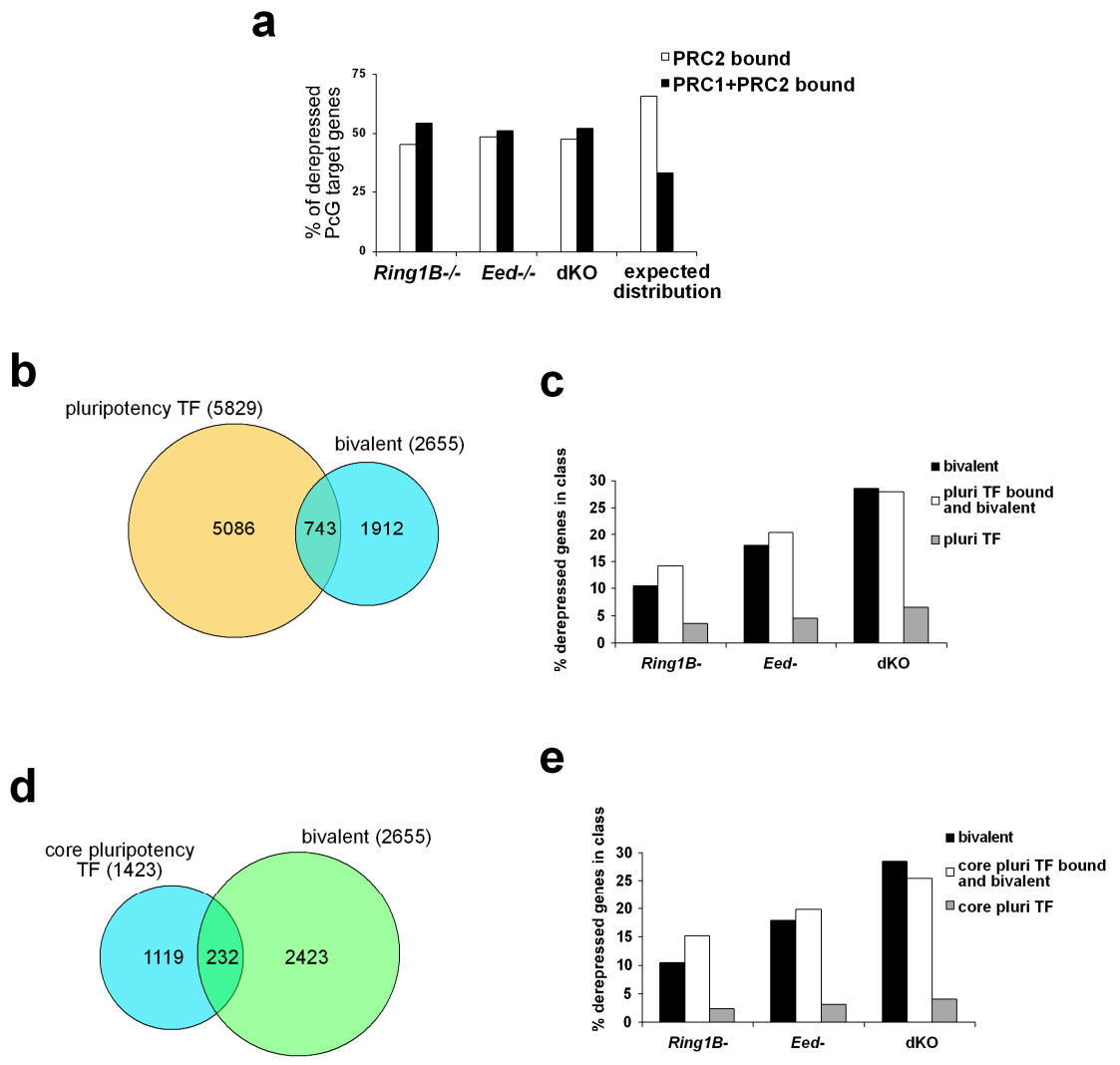


Figure 11 Contribution of the molecular environment of PcG target promoters to PcG mediated repression

(**a**) Ring1B positive bivalent genes are preferentially derepressed in *Eed*$^{-/-}$, *Ring1B*$^{-/-}$ and dKO ES cells. (**b**) Venn diagram showing an overlapping set of genes bound by the PcG system and pluripotency transcription factors (TFs) (**c)** PcG target genes which are also bound by pluripotency TFs are not preferentially activated in PcG KO ES cells (**d**) The presence of pluripotency associated TFs or (**e**) core regulators of pluripotency (Oct4, Nanog and Sox2) on PcG target genes does not increase derepression upon disruption of the PcG system.

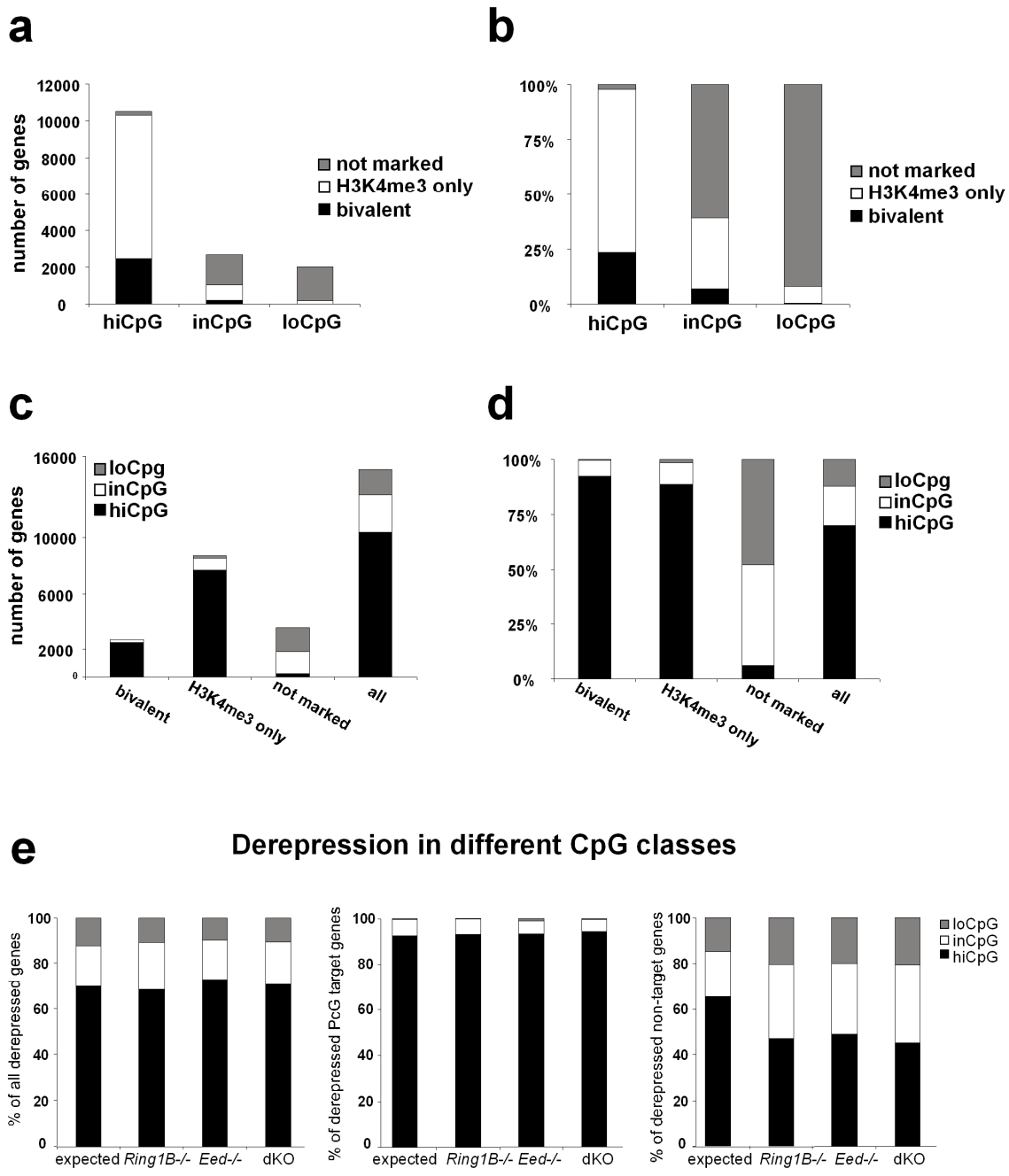


Figure 12 Contribution of the molecular set up of PcG target promoters to PcG mediated repression

(**a** to **d**) Virtually all PcG targets and most H3K4me3 bound genes have CpG rich (hiCpG) promoters. Promoters showing an intermediate (inCpG) or low (loCpG) CpG level are depleted for both H3K27me3 and H3K4me3. Graphs are based on published datasets (Ku et al., 2008; Mikkelsen et al., 2007). (**e**) Overall, genes derepressed in *Ring1B*$^{-/-}$, *Eed*$^{-/-}$ or dKO ES cells are randomly distributed in all three CpG classes. Derepressed Polycomb target genes do almost exclusively have high CpG class promoters. Derepressed non PcG target genes are depleted for high CpG class promoters and show a stronger enrichment of intermediate and low CpG promoters in all three PcG knockout ES cell lines.

Endogenous retroviral elements are novel PcG targets which are redundantly repressed by PRC1 and PRC2

It has previously been estimated that 15% of genomic histone H3 is tri-methylated on lysine 27 (Peters et al., 2003; Schoeftner et al., 2006) and 10% of histone H2A is ubiquitinated (de Napoles et al., 2004) in ES cells. Considering that genes make up approximately 2% of the genome the amount of PcG catalyzed histone modifications cannot solely be attributed to gene regulation. This raised the question if PcG proteins could have a function apart from the regulation of genes. Interestingly, a number of LTR retrotransposons were derepressed in dKO, but not in PRC1 or PRC2 deficient ES cells in the Affymetrix microarray analysis (Figure 13a). I confirmed that intracisternal A-particle (IAP) and murine leukaemia virus (MLV) retroelements were derepressed in dKO but not *Eed* deficient ES cells by Northern analysis (Figure 13b). *Ring1B*$^{-/-}$ ES cells expressed minimal amounts of IAP. IAP repression could be partially restored by introduction of an *Eed*GFP transgene in dKO ES cells. This indicates that genomic repeats could be redundantly repressed by PRC1 and PRC2. To test if retroelements are bound by PcG complexes I performed chromatin immunoprecipitation (ChIP). I found that MLV retroelements were enriched for H3K27me3 in wildtype but not in *Eed* deficient or dKO ES cells (Figure 13c), demonstrating the specificity of the observed H3K27me3 signals. The promoter of Lef1, a reported PcG target gene, was used as a positive control. In order to determine the significance of the enrichment observed on MLVs, the binding of H3K27me3 on negative control promoters of *Gapdh* and *Oct4*, and an intergenic sequence on chromosome 8 were analyzed (Figure 13c). The enrichment of H3K27me3 varies between different regions of MLV and I observed the strongest signals in the 5' *gag* and in the 3' *env* regions of MLV. The reverse transcriptase encoding pol region was depleted for H3K27me3.

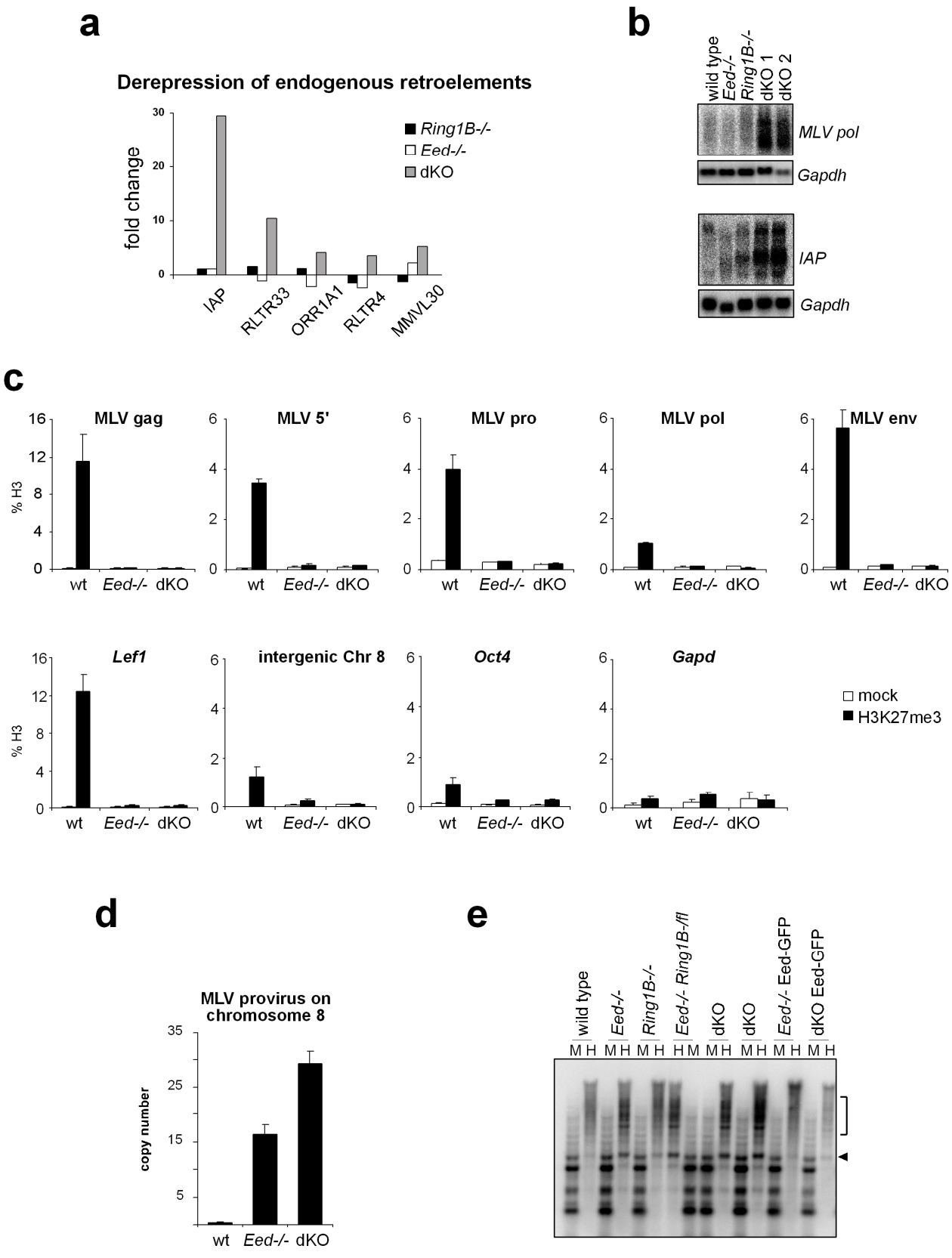
To investigate if the derepression led to mobilization of MLV elements I measured the copy number of a specific proviral integrant on chromosome 8 (Changolkar et al., 2008). I observed a 15 fold increase in the copy number of this MLV provirus in *Eed* deficient and a 30 fold increase in dKO ES cells (Figure 13d). This supports the idea that a subset of MLV repeats is a directly

controlled and functionally repressed by the PcG system. However, for IAPs H3K27me3 enrichment could not be shown. One possible explanation for this failure to detect H3K27me3 enrichment on IAPs could be that due to the high copy number of IAPs, which are present in 1000-2000 copies in the mouse genome (Kuff and Lueders, 1988), the enrichment of a subset of retroelements is diluted and cannot be detected by my analysis.

Previously, it has been shown that DNA methylation is essential for maintaining repression of endogenous IAP retroelements during embryogenesis (Walsh et al., 1998). I investigated whether depletion of the PcG system also affects DNA methylation of IAPs. Using methylation sensitive restriction enzyme analysis I observed a reduction of DNA methylation on IAP elements in *Eed* deficient and in dKO ES cells (Figure 13e). 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 class retroviral sequences, including IAP and MLV elements, belong to the group of PcG targets that are redundantly repressed by PRC1 and PRC2 in ES cells. This indicates that the Polycomb system contributes to host defense in embryonic cells.

Figure 13 Redundant repression of endogenous retroviruses by PRC1 and PRC2

(**a**) Several LTR class retrotransposons were derepressed specifically in dKO ES cells on the Affymetrix array. (**b**) Northern analysis showing derepression of endogenous MLV retroelements and IAPs in dKO ES cells. *Gapdh* was used as a loading control. (**c**) Directed chromatin immunoprecipitation showing enrichment of H3K27me3 over MLV elements in ES cells. Different regions of the MLV provirus sequence were investigated by quantitative PCR (top row). *Lef1* was used as positive and *Oct4*, *Gapdh* and an intergenic sequence on chromosome 8 as negative control (bottom row). H3K27me3 signals are diminished in *Eed* deficient and dKO ES cells demonstrating specificity. (**d**) 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. (**e**) Restriction analysis with methylation sensitive HpaII [H] and methylation resistant MspI [M] restriction enzymes and subsequent Southern analysis shows loss of DNA methylation on IAP elements in *Eed* deficient and dKO ES cells. Demethylated bands in HpaII lanes are indicated by arrow and square bracket.


a
Derepression of endogenous retroelements
fold change
30
20
10
0
Ring1B-/-
Eed-/-
dKO
IAP
RLTR33
ORR1A1
RLTR4
MMVL30
b
wild type
Eed-/-
Ring1B-/-
dKO 1
dKO 2
MLV pol
Gapdh
IAP
Gapdh
c
MLV gag
MLV 5'
MLV pro
MLV pol
MLV env
% H3
wt
Eed-/-
dKO
Lef1
intergenic Chr 8
Oct4
Gapd
mock
H3K27me3
d
MLV provirus on chromosome 8
copy number
35
25
15
5
wt
Eed-/-
dKO
e
wild type
Eed-/-
Ring1B-/-
Eed-/- Ring1B-/fl
dKO
dKO
Eed-/- Eed-GFP
dKO Eed-GFP
M H

Discussion

Ring1B is essential for the repression of lineage control genes but not for silencing of the inactive X chromosome

The disruption of the PRC1 complex severely affects the transcription profile of developmental control genes, which are aberrantly expressed in *Ring1B*$^{-/-}$ ES cells. *Ring1B* deficiency causes the disruption of the PRC1 complex and several of its member proteins are absent in *Ring1B*$^{-/-}$ ES cells. Consequently, ubH2A is not detectable in *Ring1B* deficient ES cells. However, despite the strong defects in transcriptional repression, the silencing of the inactive X chromosome remains unaffected by the loss of PRC1 function. This is not caused by a redundant function of Ring1A, which is not expressed in ES cells. In differentiated *Ring1B*$^{-/-}$ cells, however, global levels of ubH2A remain low but the enrichment of ubH2A on the inactive X chromosome is restored, possibly by the action of Ring1A. Interestingly, protein levels for the PRC1 members Mel18, Mph2 and Bmi1 remain below the detection limit in differentiated *Ring1B* deficient cells. The nature of the H2A ubiquitinating activity in differentiated *Ring1B*$^{-/-}$ cells remains to be resolved. Based on *in vitro* and *in vivo* data (Buchwald et al., 2006; Cao et al., 2005), it is unlikely that the Ring1A E3 ligase function alone is sufficient to establish chromosome wide ubH2A on the inactive X chromosome in the absence of accessory proteins. Therefore, the molecular context in which Ring1A can set up ubH2A needs to be defined. Furthermore, it will be of interest to study if Ring1A is recruited to targets other than the inactive X chromosome in differentiated cells in a manner independent of Ring1B and large parts of the PRC1 complex.

Epigenetic regulation by PcG complexes is not required for self renewal and ES cell identity

My data show that *Ring1B* or *Eed* are not essential for maintaining the pluripotent ES cell state. This interpretation is further supported by previously published results (Chamberlain et al., 2008; Endoh et al., 2008; Schoeftner et al., 2006). *Ring1B* and *Eed* are essential for maintaining the integrity of PcG complexes. Hence, their deletion results in disruption of the PRC1 or PRC2 complex and the absence of ubH2A and H3K27me3, respectively. However, deletion of *Ring1B* leaves the function of the PRC2 unaffected and, vice versa, *Ring1B* deficiency does not result in loss of H3K27me3. To address the

question to which extent both PcG complexes perform redundant functions, I have generated an ES cell line double deficient for *Eed* and *Ring1B*. dKO ES cells were viable and self renewed in culture indicating that the PcG system is not required for the maintenance of a pluripotent ES cell state. Moreover, PcG mutant ES cells expressed markers associated with ES cell identity. This supports the hypothesis, that epigenetic regulation is dispensable for ES cells and that the ES cell state represents a ground state which is defined by the absence of differentiation cues rather than by an active repression of lineage determining expression profiles (Silva and Smith, 2008). However, dKO ES cells were very difficult to establish and strongly inclined to differentiate upon suboptimal culture conditions. This indicates that, despite not being essential for pluripotency, the PcG system contributes to the stability of the ES cell state, possibly by impeding the stochastic activation of master regulators of differentiation.

PRC1 and PRC2 cooperate in gene repression of PcG target genes

My data clearly show that the loss of PcG function results in the derepression of Polycomb target genes and that the silencing of a large set of PcG target genes is established by PRC1 and PRC2 in a redundant manner. This is unexpected, as it is not compatible with the sequential recruitment model (Boyer et al., 2006; Cao et al., 2005; Cao et al., 2002), according to which PRC2 functions upstream of PRC1 and recruitment of PRC1 is dependent on the H3K27me3 mark. Despite being supported by a decreased binding of PRC1 members to PcG targets in *Eed* deficient ES and the ability of Cbx proteins to bind to H3K27me3 (Bernstein et al., 2006b), this model has been challenged recently (Muller and Verrijzer, 2009) based on results obtained in *Drosophila melanogaster*. My data further strengthens the notion that PRC1 and PRC2 perform parallel and partially redundant functions which are not compatible with the sequential recruitment model.

A redundant function of PRC1 and PRC2 is in line with the observation that PRC1 is recruited to the inactive X chromosome independently of a functional PRC2 complex (Schoeftner et al., 2006). It provides an explanation why global ubH2A levels remain largely unaffected upon loss of PRC2 and why H3K27me3

is not reduced by deletion of PRC1. However, how the redundancy is defined in molecular terms remains to be shown in the future. If it is an intrinsic property of PcG regulation, or whether redundant genes employ additional epigenetic layers of silencing which can step in for PRC1 or PRC2 is unclear. For this it would be interesting to study and compare the epigenetic setup of redundantly versus non-redundantly silenced promoters. I show that the introduction of an *Eed*GFP transgene in dKO ES cells re-establishes silencing of previously derepressed PcG target genes. This implies that PcG targets remain marked for repression in the absence of H3K27me3 and ubH2A. The nature of this remaining mark remains unclear but is an interesting and important target for future research.

Interestingly, in ES cells more than half of PcG target genes are not derepressed using our cut off criteria, even in the absence of both PcG complexes. It is conceivable that additional layers of silencing exist to maintain repression of these genes. Furthermore, the maintenance of repression could be determined by the absence of activation signals in the ES cell state. It remains possible that upon differentiation genes which are silent in dKO ES cells become aberrantly activated. However, given the differentiation defect and possibly aberrant lineage choice in dKO ES cells such an effect is difficult to study. Taken together my results show that PRC1 and PRC2 cooperate in silencing of common target genes. The activity of both PcG complexes acts in parallel, and the presence of one complex is sufficient to maintain silencing of a large set of common targets which are redundantly silenced by PRC1 and PRC2. My data demonstrate that the parallel recruitment of PRC1 and PRC2 constitutes a critical function in establishing patterns of gene repression during development.

At present the possibility that residual PcG activity remains in *Eed* and *Ring1B* deficient ES cells cannot by completely eliminated. Albeit I could not detect H3K27me3, Ezh2 protein is still present at a reduced amount and could be functional to an extent below detection. However, 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 homologue of Ring1B, is not expressed in ES cells and does not restore genomic ubH2A levels. In addition, *Ring1B* deletion leads to a loss of several PRC1 proteins including Rybp, Cbx4, Mel18 and Bmi1. These data suggest that depletion of *Eed* and *Ring1B* causes the complete disruption of the PcG system in ES cells.

A redundant activity of PRC1 and PRC2 defines the differentiation potential of ES cells

Surprisingly, PRC1 or PRC2 deficient ES cells can terminally 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. Furthermore, in contrast to *Eed*$^{-/-}$ and the parental *Eed*$^{-/-}$*:Ring1B*$^{-/fl}$ ES cell lines, dKO ES cells could not differentiate to neuronal stem cells in vitro. The ability of an *Eed*GFP transgene to restore the tumor formation potential of dKO ES cells shows that dKO ES cells retain pluripotency but that epigenetic patterns required to progress through differentiation cannot be set up in the absence of PcG regulation. These data are in line with the observation that PcG deficient embryos progress in development until they arrest at the gastrulation stage (Faust et al., 1998; Voncken et al., 2003). This suggests that the differentiation defect of PcG deficient ES cells is not due to a lock in of the ES cell state but rather due to the inability to fine tune lineage choices in the course of differentiation. This idea is supported by the fact that the distribution of different cell types and tissues varies between wt and PcG KO teratomas. These findings establish a critical function for the PcG system in early differentiation processes.

Endogenous retroelements are novel PcG targets which are redundantly repressed by PRC1 and PRC2

Mouse ES cells employ potent mechanisms to restrict retroviral activity (Wolf and Goff, 2007; Wolf and Goff, 2009). However, in the course of evolution the mouse germ line has acquired an enormous viral load. More than 1000 IAPs and about 70 murine leukemia proviruses have been annotated in the mouse genome (Changolkar et al., 2008; Kuff and Lueders, 1988). The sequence

integrity of several endogenous MLVs and of other retroelements has been retained, making reliable silencing necessary in order to maintain genome stability. My data identifies genomic repeat elements as novel Polycomb targets. MLV and IAP retroelements are derepressed in PcG dKO but not in PRC1 or PRC2 deficient ES 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 our previous observations that genome-wide H3K27me3 is maintained in *Ring1B* deficient ES cells (Schoeftner et al., 2006), and genomic ubH2A is maintained in *Eed* deficient ES cells (Leeb and Wutz, 2007). This indicates that by far the greatest proportion of Polycomb mediated chromatin modification is located in the non-genic regions of the genome and not on genes which make up for only 2% of the DNA sequence. These results might have important implications for understanding how Polycomb complexes regulate gene expression in mammals, as it suggests a role of repeat sequences in the gene silencing mechanism. Notably, in mammals Polycomb response elements (PRE), which are central to Polycomb mediated regulation in *Drosophila* (Ringrose et al., 2003; Schuettengruber et al., 2007), have not been identified. PREs are thought to interact with gene promoters to maintain gene repression. It is conceivable that genomic repeat sequences in mammals perform a function similar to PREs in *Drosophila* and provide a platform for PcG complex binding. Loss of both PRC1 and PRC2 leads to derepression of genomic repeats and, thus, interferes with the function of this platform. It will be exciting to investigate if genomic repeats associate with Polycomb regulated genes similar to the clustering of Polycomb regulated genes and PREs in *Drosophila* (Grimaud et al., 2006). Insights into how PcG proteins associate with genomic repeats might come from studying X chromosome inactivation. PRC1 and PRC2 are recruited to the core of the inactive X chromosome, which contains genomic repeats (Chaumeil et al., 2006; Clemson et al., 2006; de Napoles et al., 2004). Both PcG complexes are recruited independently of each other by the non-coding *Xist* RNA (Leeb and Wutz, 2007; Schoeftner et al., 2006). In addition, both PRC1 and PRC2 are independently required to maintain imprinted silencing at the Kcnq1 locus by establishing a three dimensional cluster. Similar to X inactivation, this cluster is deprived of RNA Polymerase II and PcG proteins

colocalize with the non-coding *Kcnqot1* transcript (Terranova et al., 2008). This suggests that Polycomb complexes might have a general role in compartmentalizing the nucleus by establishing chromatin domains for gene repression.

Conclusion and Outlook

My data demonstrate that the deletion of both PRC1 and PRC2 disrupts the function of the PcG system and interferes with the establishment and maintenance of differentiated cell types. In single PRC deficient cells such a defect is also evident, yet less pronounced. This shows that PRC1 and PRC2 have redundant and overlapping functions in determining cellular identity, possibly by modulating the expression of developmental control genes. The observation that endogenous retroviral sequences are controlled by PRC1 and PRC2 shows that the PcG system has an unexpected function beyond the regulation of genes. It is conceivable that the derepression of genomic repeat sequences contributes to the aberrant transcription profiles in PcG mutant ES cells and to the defect in differentiation.

In the future, the ability to maintain ES cell in the absence of PRC1 and PRC2 will provide an opportunity for studying the function of the PcG system in gene repression, chromatin organization and genome stability. Studying PcG deficient ES cells might also help to further delineate the extent to which epigenetic mechanisms are responsible for defining the pluripotent state of embryonic stem cells.

Materials and Methods

Cell culture and generation of ES cell lines

ES cells were cultured as described previously (Wutz and Jaenisch, 2000). All ES cells generated are based on the clone 36 ES cell line carrying an *Xist* transgene which can be induced by addition of 1µg/ml doxycycline to the culture medium (Wutz and Jaenisch, 2000). Differentiation medium contained 100 nM all-*trans*-retinoic acid and no LIF. Embryoid bodies were generated by the hanging drop method. Cells were counted with a Casy cell counter (Schaerfe System GmbH, Germany). Gene targeting of the *Ring1B* locus was performed in *Eed*$^{-/-}$ ES cells following a conditional strategy as previously described (Leeb and Wutz, 2007). In brief, the first allele was targeted using a deletion construct carrying a selectable Hygromycin/HSV-Thymidine Kinase cassette. For targeting the second allele of Ring1B, a conditional targeting vector was used. Here, exons two to four, which encode for the catalytically active Ring finger domain, were flanked by loxP sites. Adenoviral infection was performed at a multiplicity of infection (m.o.i.) of 120. For successful 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 5µg/ml. For establishing dKO cells Ring1B deletion was induced with 1µM 4-hydroxy-tamoxifen (4OHT) for 48 hours. dKO ES cells were established with a frequency of 85-95% and subcloned before further analysis. To reconstitute *Eed* expression dKO ES cells were transfected with an pCAG-EGFP-Eed-IRES-HygpA transgene (Schoeftner 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).

Teratoma formation assay

1x10^6 ES cells were injected into the flanks of nude mice in Matrigel Basement Membrane Matrix (BD Bioscience). I performed three injections each for wt, *Eed*$^{-/-}$, *Ring1B*$^{-/-}$ and two independent dKO ES cell lines. After three weeks teratomas were excised, embedded in O.C.T. compound (Tissue Tek) for cryosections and subsequently analysed using immunohistochemistry. The genotype was confirmed by PCR. Further characterization included H3K27me3 immunofluorescence analysis. dKO ES cells did not give rise to teratomas within six months after injection.

Immunofluorescence, Immunohistochemistry and image acquisition

For immunofluorescence (IF), ES cells were grown on Roboz slides (CellPoint Scientific, USA). Cells were fixed for 10 min in 4% PFA in PBS, permeabilized for 5 min in 0.1% Na Citrate/0.5% Triton-X100, blocked for 30 min in PBS containing 5% BSA, 0.1% Tween-20. Incubations with the primary antibodies were performed for three hours at room temperature in a humid chamber. Following a washing step in PBS/0,1% Tween, slides were incubated with secondary antibody for one hour. DAPI (4',6'-diamidino-2-phenylindole) was used to couterstain the DNA. Vectashield (Vector Laboratories) was used as imaging medium. Immunohistochemistry was performed on frozen sections using the Ventana Discovery System. IF images were obtained 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, USA) at 20x magnification. Images of ES cell colonies were taken with a Zeiss Axiovert 200 microscope. A Zeiss Axioplan 2 microscope was used to analyse IHC stainings.

Northern analysis

Northern analysis was performed using 5-15 µg of RNA purified with Trizol (Invitrogen) as described previously (Leeb and Wutz, 2007). Primers used to generate Northern probes are listed in Table 5. Images were obtained using a STORM 860 Scanner (Molecular Dynamics) and the Image Quant TL software.

Protein Analysis

Proteins were extracted in RIPA buffer. Histones were acid extracted in 0.2N HCl. α-Lamin B1, α-H3 and Ponceau S staining was 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 Biotechnology), α-Suz12 (Upstate Biotechnology), α-Ezh2, α-H3K27me3 (6), α-H2AK119ub1 (Upstate Biotechnology), α-H3 (Abcam), α-Nestin (Developmental Studies Hybridoma Bank, Iowa City), α-Lamin B1 (Abcam), Smooth muscle actin (Thermo Scientific), Glial Fibrillary Acidic Protein (GFAP; Dako), Alpha-1-Fetoprotein

(Dako) and Troma-1 (Developmental Studies Hybridoma Bank, Iowa City). Secondary antibodies: Alexa Fluor 488 goat anti rabbit IgG, Alexa Fluor 488 goat anti mouse IgG (Molecular Probes), HRP conjugated Affinipure goat α-rabbit IgG, HRP conjugated, Affinipure goat α-mouse IgG (Jackson Immuno Research Laboratories).

Quantitative Gene expression analysis

cDNA was generated from 4 µg DNase treated total RNA from wild type, *Eed*$^{-/-}$, *Ring1B*$^{-/-}$, two independent dKO, and dKO$^{Eed\text{GFP}}$ ES cells using the Superscript II Reverse transcription kit (Invitrogen) and random hexamere primers. Quantitative real time PCR analysis was performed in triplicates using the Biorad iQ Taq SYBR green master mix on a Biorad 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. Primer sequences are listed Table 5.

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 (Berlin, Germany) using Affymetrix GeneChip 430 2.0 arrays. Normalization was performed using the MAS 5 algorithm. Pairwise comparisons between all genotypes were performed. Genes regulated at least two-fold with a p-value of less than 0.05 were analysed further. PcG target gene sets, CpG promoter classes and pluripotency transcription factor bound genes were annotated from published datasets (Boyer et al., 2006; Kim et al., 2008; Ku et al., 2008). PcG target genes were defined as genes with a bivalent promoter status (Ku et al., 2008). 168 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 heatmap. Bioinformatic analysis of Affymetric microarray datas was performed with the help of Dr. Maria Novatchkova and Dr. Markus Jaritz (IMP, Vienna)

Chromatin Immunoprecipatation (ChIP)

ChIP was performed as described previously with minor modifications (Sado et al., 2005). $2x10^6$ ES or RA differentiated cells were used for each experiment. Rabbit IgG was used as mock control. Immunoprecipitation was performed using Dynabeads Protein G (Invitrogen). The following antibodies were used: α-H3K27me3 (Upstate Biotechnology) rabbit monoclonal α-H3K27me3 (Cell signalling) and α-H3 (Abcam). Quantitative PCR was performed in duplicates. Error bars represent standard deviation. ChIP results were confirmed in two independent experiments.

Quantifiation of MLV copy number

To quantitate the MLV copy number I performed qPCR with primers specific or the MLV sequence. The copy numbers were normalized to an intergenic sequence on chromosome 8. Error bars represent the standard deviation.

DNA methylation analysis

DNA samples of wt, *Eed*$^{-/-}$, *Ring1B*$^{-/-}$, *Eed*$^{-/-}$:*Ring1B*$^{-/fl}$, dKO, *Eed*$^{-/-Eed\text{GFP}}$ and dKO$^{\text{EedGFP}}$ ES cells were digested with MspI and the CpG methylation sensitive isoschizomere HapII. Primers used to generate the IAP and MLV specific Southern probe can be found in Table 5.

Tables

Table 1 – Top 100 upregulated PcG target genes in PcG KO ES cells

The 100 most upregulated PcG target genes with a p-value smaller than 0.05 in PcG mutant ES cells are listed with fold change (fc) compared to wild type ES cells.

Table 1

PcG target genes

Ring1B-/- vs wt			Eed-/- vs wt			dKO vs wt		
Entrez GeneID	Annotation	fc	Entrez GeneID	Annotation	fc	Entrez GeneID	Annotation	fc
22771	Zic1	79,57	83555	Tex13	50,52	83555	Tex13	72,93
15423	Hoxc4	41,81	15423	Hoxc4	39,17	20671	Sox17	50,07
15405	Hoxa9	34,54	16869	Lhx1	33,21	22771	Zic1	43,82
13593	Ebf3	22,12	18505	Pax3	31,49	15423	Hoxc4	43,06
18505	Pax3	21,87	20671	Sox17	25,58	15220	Foxq1	40,37
19332	Rab20	20,51	231440	9130213B05Rik	23,25	18505	Pax3	31,59
15430	Hoxd10	16,49	22771	Zic1	22,98	15371	Hmx1	28,19
83555	Tex13	15,06	15405	Hoxa9	21,28	16869	Lhx1	28,03
13132	Dab2	14,35	27206	Nrk	20,45	17153	Mal	26,28
20671	Sox17	14,19	26410	Map3k8	17,21	13593	Ebf3	26,07
15371	Hmx1	13,89	13082	Cyp26a1	16,12	14120	Fbp2	24,98
11819	Nr2f2	13,80	15220	Foxq1	15,58	13380	Dkk1	24,64
21384	Tbx15	13,65	12590	Cdx1	14,60	71908	Cldn23	23,58
15220	Foxq1	12,21	15371	Hmx1	13,63	27206	Nrk	22,53
15395	Hoxa10	12,03	21384	Tbx15	13,26	231440	9130213B05Rik	22,01
13036	Ctsh	11,52	13132	Dab2	13,06	16772	Lama1	21,85
71712	1200002N14Rik	11,30	16772	Lama1	12,93	13082	Cyp26a1	21,56
14120	Fbp2	9,53	15430	Hoxd10	12,71	15405	Hoxa9	20,89
15433	Hoxd13	9,45	12705	Cited1	12,19	26410	Map3k8	19,54
15427	Hoxc9	8,91	20312	Cx3cl1	12,16	12827	Col4a2	19,44
24113	Vax2	8,70	12826	Col4a1	12,15	15430	Hoxd10	18,74
18423	Otx1	8,55	19332	Rab20	12,03	12826	Col4a1	17,81
71592	Pogk	8,40	24113	Vax2	11,90	19332	Rab20	17,27
21410	Tcf2	7,79	12156	Bmp2	11,76	21384	Tbx15	17,03
21386	Tbx3	7,64	12827	Col4a2	11,56	57246	Tbx20	16,79
16772	Lama1	7,62	57246	Tbx20	11,46	71712	1200002N14Rik	15,37
18595	Pdgfra	7,47	18423	Otx1	11,43	13036	Ctsh	15,33
20927	Abcc8	7,43	17300	Foxc1	11,25	16002	Igf2	15,07
13380	Dkk1	7,40	13380	Dkk1	10,89	12705	Cited1	14,64
15438	Hoxd9	7,36	15427	Hoxc9	10,86	11881	Arsb	14,45
15229	Foxd1	7,31	16002	Igf2	10,83	16939	Lor	14,41
12826	Col4a1	7,12	22750	Zfp9	9,71	15427	Hoxc9	14,36
83558	Tex11	7,10	209195	Clic6	9,57	12394	Runx1	14,32
224796	Clic5	6,69	15395	Hoxa10	9,20	20312	Cx3cl1	13,76
22066	Trpc4	6,57	27205	Podxl	8,98	12831	Col5a1	13,73
12827	Col4a2	6,52	24059	Slco2a1	8,97	15395	Hoxa10	13,47
14180	Fgf9	6,46	66859	Slc16a9	8,96	18186	Nrp1	13,46
50916	Irx4	6,30	15433	Hoxd13	8,67	24059	Slco2a1	13,16
20680	Sox7	6,21	20927	Abcc8	8,60	15229	Foxd1	12,93
15404	Hoxa7	6,08	71712	1200002N14Rik	8,50	18595	Pdgfra	12,90
18227	Nr4a2	6,03	15426	Hoxc8	8,34	20680	Sox7	12,66
17536	Mrg1	5,97	24056	Sh3bp5	7,81	18423	Otx1	12,65
14465	Gata6	5,94	15376	Foxa2	7,76	20745	Spock1	12,61
214763	E330016A19Rik	5,92	12475	Cd14	7,49	56753	Tacstd2	12,55
15424	Hoxc5	5,92	227545	5430407P10Rik	7,47	14254	Flt1	12,43
15431	Hoxd11	5,88	12394	Runx1	7,32	60527	Fads3	11,93
240590	Dmrt3	5,84	16668	Krt18	7,23	54670	Atp8b1	11,75
223433	BC052328	5,77	18595	Pdgfra	7,14	12156	Bmp2	11,74
12705	Cited1	5,72	15438	Hoxd9	7,04	14465	Gata6	11,49
11554	Adrb1	5,70	20378	Frzb	7,01	213696	Duoxa1	11,19
235135	Tmem45b	5,36	20361	Sema7a	6,98	21859	Timp3	10,88
170765	Ripply3	5,34	60527	Fads3	6,80	209195	Clic6	10,87
12590	Cdx1	5,33	15229	Foxd1	6,77	13132	Dab2	10,45
73333	Slc25a31	5,33	20680	Sox7	6,76	224796	Clic5	10,34
57246	Tbx20	5,29	14120	Fbp2	6,70	108116	Slco3a1	10,26
74318	Hod	5,21	108116	Slco3a1	6,64	12153	Bmp1	10,12
13082	Cyp26a1	5,17	14465	Gata6	6,59	171180	Syt12	10,00
17762	Mapt	5,16	54670	Atp8b1	6,56	53623	Gria3	9,95
15376	Foxa2	5,09	21814	Tgfbr3	6,51	15433	Hoxd13	9,92
15939	Ier5	5,06	19227	Pthlh	6,44	15376	Foxa2	9,74
66654	Tex12	4,99	15424	Hoxc5	6,41	50766	Crim1	9,73
13813	Eomes	4,89	13731	Emp2	6,30	14118	Fbn1	9,68
14463	Gata4	4,83	18511	Pax9	6,25	21410	Tcf2	9,34
22348	Slc32a1	4,81	227753	Gsn	6,16	20429	Shox2	9,32
12156	Bmp2	4,81	15228	Foxg1	6,11	12475	Cd14	9,28
66859	Slc16a9	4,80	17702	Msx2	6,09	14421	B4galnt1	9,21
69219	Ddah1	4,77	18027	Nfia	6,06	230316	Megf9	9,16
12418	Cbx4	4,76	21386	Tbx3	5,99	16668	Krt18	9,08
15412	Hoxb4	4,69	13655	Egr3	5,97	227753	Gsn	8,75
21375	Tbr1	4,63	19263	Ptprb	5,96	20378	Frzb	8,62
15228	Foxg1	4,59	57342	Parva	5,95	15438	Hoxd9	8,42
237339	L3mbtl3	4,58	12153	Bmp1	5,84	15424	Hoxc5	8,41
16870	Lhx2	4,56	75746	Morc4	5,78	15426	Hoxc8	8,29
215798	Gpr126	4,45	18205	Ntf3	5,78	71592	Pogk	8,26
14586	Gfra2	4,42	231510	A230097K15Rik	5,73	13731	Emp2	8,19
27206	Nrk	4,39	21410	Tcf2	5,70	14066	F3	8,15
27205	Podxl	4,37	11819	Nr2f2	5,67	218820	Zfp503	7,85
209448	Hoxc10	4,32	108058	Camk2d	5,58	99887	Tmem56	7,83
17268	Meis1	4,32	70564	5730469M10Rik	5,57	225642	Grp	7,66
15364	Hmga2	4,24	11856	Arhgap6	5,48	11856	Arhgap6	7,65
12159	Bmp4	4,22	20745	Spock1	5,39	13655	Egr3	7,60
15426	Hoxc8	4,16	22634	Plagl1	5,37	14461	Gata2	7,59
77485	Stk31	4,05	14254	Flt1	5,35	17702	Msx2	7,55
105785	Kdelr3	4,03	14701	Gng12	5,28	226922	Kcnq5	7,51
17919	Myo5b	4,01	107587	Osr2	5,24	12505	Cd44	7,47
16508	Kcnd2	4,00	14066	F3	5,22	16777	Lamb1-1	7,41
75388	Boll	3,99	50766	Crim1	5,09	77097	Tanc2	7,39
16392	Isl1	3,98	12444	Ccnd2	5,08	231510	A230097K15Rik	7,32
72828	2810457I06Rik	3,96	16870	Lhx2	5,07	21814	Tgfbr3	7,25
14234	Foxc2	3,95	110796	Tshz1	5,06	22634	Plagl1	7,23
30785	Cttnbp2	3,88	69219	Ddah1	5,04	72828	2810457I06Rik	7,19
81907	Tmem108	3,88	17919	Myo5b	4,91	231503	BC062109	7,14
13392	Dlx2	3,85	12418	Cbx4	4,91	22750	Zfp9	7,12
13482	Dpp4	3,82	14461	Gata2	4,89	22793	Zyx	7,01
107587	Osr2	3,80	22417	Wnt4	4,87	23984	Pde10a	6,96
74190	1200009I06Rik	3,79	16007	Cyr61	4,85	170441	Slc2a10	6,95
93835	Amn	3,79	218454	Lhfpl2	4,85	69219	Ddah1	6,95
15227	Foxf1a	3,75	72828	2810457I06Rik	4,83	56811	Dkk2	6,93
67155	Smarca2	3,73	171180	Syt12	4,82	27205	Podxl	6,92
232146	Tmem166	3,70	16370	Irs4	4,81	15228	Foxg1	6,87
70564	5730469M10Rik	3,62	223433	BC052328	4,80	78923	4833446K15Rik	6,84

Table 2 - Top 100 upregulated genes in PcG KO ES cells

The 100 most upregulated genes with a p-value smaller than 0.05 in PcG mutant ES cells are listed with fold change (fc) compared to wild type ES cells.

Table 2

all genes

Ring1B-/- vs wt			Eed-/- vs wt			dKO vs wt		
Entrez GeneID	Annotation	fc	Entrez GeneID	Annotation	fc	Entrez GeneID	Annotation	fc
21426	Tcfec	129,33	12309	S100g	150,58	22139	Ttr	330,83
12309	S100g	80,04	21426	Tcfec	81,71	12309	S100g	259,16
22771	Zic1	79,57	83555	Tex13	50,52	21426	Tcfec	94,47
15423	Hoxc4	41,81	15423	Hoxc4	39,17	83555	Tex13	72,93
70678	3021401C12Rik	39,30	16869	Lhx1	33,21	20755	Sprr2a	50,56
15405	Hoxa9	34,54	18505	Pax3	31,49	20671	Sox17	50,07
65969	Cubn	29,90	70678	3021401C12Rik	26,78	70125	2210016H18Rik	47,08
15397	Hoxa11os	24,18	20671	Sox17	25,58	22771	Zic1	43,82
19073	Srgn	24,09	231440	9130213B05Rik	23,25	15423	Hoxc4	43,06
70125	2210016H18Rik	23,20	22771	Zic1	22,98	15220	Foxq1	40,37
13593	Ebf3	22,12	15405	Hoxa9	21,28	18828	Plscr2	38,66
18505	Pax3	21,87	27206	Nrk	20,45	70678	3021401C12Rik	36,69
83561	Tdrd1	21,54	65969	Cubn	19,78	70574	Cpm	36,09
19332	Rab20	20,51	320047	4833444G19Rik	19,35	18505	Pax3	31,59
329416	Nostrin	20,31	100727	Ugt2b34	19,06	15371	Hmx1	28,19
17276	Mela	19,73	19202	Rhox6	17,82	16869	Lhx1	28,03
15430	Hoxd10	16,49	19073	Srgn	17,52	15476	Hs3st1	26,55
83555	Tex13	15,06	26410	Map3k8	17,21	15397	Hoxa11os	26,44
107753	Lgals2	14,49	15476	Hs3st1	17,14	17153	Mal	26,28
13032	Ctsc	14,43	15397	Hoxa11os	16,80	13593	Ebf3	26,07
13132	Dab2	14,35	13082	Cyp26a1	16,12	109222	Rarres1	25,78
622402	Akr1c12	14,34	11576	Afp	15,83	14120	Fbp2	24,98
20671	Sox17	14,19	15220	Foxq1	15,58	13380	Dkk1	24,64
15371	Hmx1	13,89	12590	Cdx1	14,60	217169	Tns4	23,93
11819	Nr2f2	13,80	217169	Tns4	14,14	71908	Cldn23	23,58
21384	Tbx15	13,65	243659	Styk1	14,05	27206	Nrk	22,53
21946	Pglyrp1	12,42	622402	Akr1c12	14,02	11576	Afp	22,52
243659	Styk1	12,34	21957	Tnnt3	13,75	231440	9130213B05Rik	22,01
15436	Hoxd4	12,28	15371	Hmx1	13,63	16772	Lama1	21,85
74129	Dmgdh	12,24	21384	Tbx15	13,26	13082	Cyp26a1	21,56
15220	Foxq1	12,21	13132	Dab2	13,06	65969	Cubn	20,94
71089	Sep.12	12,14	16772	Lama1	12,93	15405	Hoxa9	20,89
15395	Hoxa10	12,03	268780	Egflam	12,89	67896	Ccdc80	20,35
15476	Hs3st1	11,97	15430	Hoxd10	12,71	73690	Glipr1	19,99
72906	2900042A17Rik	11,62	78365	1500016L03Rik	12,35	100727	Ugt2b34	19,70
13036	Ctsh	11,52	20724	Serpinb5	12,19	26410	Map3k8	19,54
73429	1700109F18Rik	11,39	12705	Cited1	12,19	12827	Col4a2	19,44
71712	1200002N14Rik	11,30	20312	Cx3cl1	12,16	15430	Hoxd10	18,74
56746	Tex101	10,88	12826	Col4a1	12,15	212326	BC035537	18,16
15437	Hoxd8	10,69	19332	Rab20	12,03	12826	Col4a1	17,81
68614	Letmd1	10,30	109246	Tspan9	11,97	19332	Rab20	17,27
171284	Timd2	9,97	24113	Vax2	11,90	21384	Tbx15	17,03
100042000	LOC100042000	9,92	12156	Bmp2	11,76	57246	Tbx20	16,79
72512	Tmem173	9,82	12827	Col4a2	11,56	74129	Dmgdh	16,31
14120	Fbp2	9,53	57246	Tbx20	11,46	110454	Ly6a	16,06
15433	Hoxd13	9,45	18423	Otx1	11,43	22361	Vnn1	15,57
15427	Hoxc9	8,91	73690	Glipr1	11,27	71712	1200002N14Rik	15,37
78365	1500016L03Rik	8,73	17300	Foxc1	11,25	17178	Fxyd3	15,35
24113	Vax2	8,70	94242	Tinagl	10,96	13036	Ctsh	15,33
110454	Ly6a	8,68	13380	Dkk1	10,89	16002	Igf2	15,07
70846	4921506M07Rik	8,64	15427	Hoxc9	10,86	19073	Srgn	14,82
18423	Otx1	8,55	16002	Igf2	10,83	22296	V1ra1	14,77
11576	Afp	8,42	17276	Mela	10,51	12705	Cited1	14,64
98558	Mael	8,41	14766	Gpr56	10,28	27384	Akr1c13	14,46
71592	Pogk	8,40	18826	Lcp1	10,28	11881	Arsb	14,45
68338	Golt1a	8,18	319876	Cobll1	10,26	16939	Lor	14,41
69852	Tcf23	8,14	100223	9630041G16Rik	10,18	15427	Hoxc9	14,36
64918	Bhmt2	8,12	104384	Rhox9	9,75	12394	Runx1	14,32
73690	Glipr1	7,96	22750	Zfp9	9,71	67405	Nts	14,09
268780	Egflam	7,91	209195	Clic6	9,57	94242	Tinagl	13,84
21410	Tcf2	7,79	228576	Mall	9,51	20312	Cx3cl1	13,76
21386	Tbx3	7,64	72512	Tmem173	9,39	12831	Col5a1	13,73
16772	Lama1	7,62	72297	B3gnt3	9,34	72297	B3gnt3	13,73
100552	AA675344	7,62	216019	Hkdc1	9,28	15395	Hoxa10	13,47
18595	Pdgfra	7,47	15395	Hoxa10	9,20	18186	Nrp1	13,46
15408	Hoxb13	7,47	15437	Hoxd8	9,02	83379	Klb	13,44
105855	Nckap1l	7,46	27205	Podxl	8,98	24059	Slco2a1	13,16
20927	Abcc8	7,43	24059	Slco2a1	8,97	14013	Evi1	13,00
13380	Dkk1	7,40	66859	Slc16a9	8,96	19202	Rhox6	12,97
15438	Hoxd9	7,36	73429	1700109F18Rik	8,91	15229	Foxd1	12,93
15229	Foxd1	7,31	330577	1700129I04Rik	8,82	18595	Pdgfra	12,90
83379	Klb	7,23	15433	Hoxd13	8,67	20680	Sox7	12,66
20503	Slc16a7	7,14	20927	Abcc8	8,60	18423	Otx1	12,65
12826	Col4a1	7,12	20459	Ptk6	8,54	20745	Spock1	12,61
83558	Tex11	7,10	71712	1200002N14Rik	8,50	16177	Il1r1	12,58
13070	Cyp11a1	7,08	16691	Krt8	8,44	56753	Tacstd2	12,55
320898	A430107P09Rik	7,07	27494	Amot	8,38	12955	Cryab	12,53
53814	Oaz3	7,06	15426	Hoxc8	8,34	20708	Serpinb6b	12,47
27384	Akr1c13	7,03	71217	4933431I19Rik	8,31	14254	Flt1	12,43
171168	Asah3	6,92	30937	Lmcd1	8,28	60527	Fads3	11,93
14776	Gpx2	6,89	21946	Pglyrp1	8,13	16691	Krt8	11,89
231147	Sh3tc1	6,82	381677	Vgf	8,03	54670	Atp8b1	11,75
69454	Clic3	6,77	100213	Rusc2	7,94	12156	Bmp2	11,74
224796	Clic5	6,69	19124	Procr	7,83	69454	Clic3	11,50
13884	Es1	6,69	24056	Sh3bp5	7,81	107753	Lgals2	11,49
27494	Amot	6,69	15376	Foxa2	7,76	14465	Gata6	11,49
22066	Trpc4	6,57	207792	BC034090	7,72	68850	1110062M06Rik	11,43
12827	Col4a2	6,52	14405	Gabrg1	7,66	64918	Bhmt2	11,34
66805	Tspan1	6,46	320860	B130021B11Rik	7,60	213696	Duoxa1	11,19
14180	Fgf9	6,46	76509	1600029D21Rik	7,54	21957	Tnnt3	11,13
237310	Il22ra2	6,39	74129	Dmgdh	7,52	102097	AI465270	10,88
216019	Hkdc1	6,38	12475	Cd14	7,49	78365	1500016L03Rik	10,88
69836	Pla2g12b	6,37	227545	5430407P10Rik	7,47	21859	Timp3	10,88
330450	Mlstd1	6,33	12394	Runx1	7,32	209195	Clic6	10,87
50916	Irx4	6,30	71237	4933435G04Rik	7,30	73241	3110080O07Rik	10,71
20680	Sox7	6,21	17178	Fxyd3	7,29	14960	H2-Aa	10,67
268859	A2bp1	6,12	68338	Golt1a	7,26	15437	Hoxd8	10,62
15404	Hoxa7	6,08	16668	Krt18	7,23	13132	Dab2	10,45
18227	Nr4a2	6,03	18595	Pdgfra	7,14	73429	1700109F18Rik	10,43
20724	Serpinb5	6,03	11833	Aqp8	7,06	17110	Lyz	10,41
74333	4122401K19Rik	6,00	110454	Ly6a	7,06	53603	Tslp	10,35

Table 3 - Top 100 down regulated PcG target genes in PcG KO ES cells

The 100 most down regulated PcG target genes with a p-value smaller than 0.05 in PcG mutant ES cells are listed with fold change (fc) compared to wild type ES cells.

Table 3

PcG target genes

Ring1B-/- vs wt			Eed-/- vs wt			dKO vs wt		
Entrez GeneID	**Annotation**	**fc**	**Entrez GeneID**	**Annotation**	**fc**	**Entrez GeneID**	**Annotation**	**fc**
14169	Fgf14	10,17	18508	Pax6	9,98	14675	Gna14	42,84
20564	Slit3	9,01	14675	Gna14	6,85	237868	A830091I15Rik	10,93
16552	Kif12	7,10	320127	Dgki	6,75	16994	Ltb	5,96
66864	Clec14a	6,32	21417	Zeb1	6,08	216881	Wscd1	5,94
260408	BC107230	5,81	20672	Sox18	6,00	20358	Sema6a	5,74
217371	Rab40b	5,73	14169	Fgf14	5,13	17863	Myb	4,95
14765	Gpr50	5,35	16994	Ltb	5,03	211232	Cpne9	4,90
216881	Wscd1	5,09	12829	Col4a4	4,76	257882	Olfr1344	4,72
320840	Negr1	5,09	15208	Hes5	4,61	381813	Prmt8	4,72
57765	Tbx21	4,85	13590	Lefty1	4,59	14025	Bcl11a	4,46
14675	Gna14	4,81	239857	Cadm2	4,30	329154	Ankrd44	4,16
19157	Pscd1	4,71	19277	Ptpro	3,87	15162	Hck	4,13
22141	Tub	4,59	64705	Dpys	3,77	64705	Dpys	4,11
237868	A830091I15Rik	4,53	68428	Steap3	3,59	18508	Pax6	4,10
239857	Cadm2	4,36	269784	Cntn4	3,46	22239	Ugt8a	4,08
76459	Car12	4,21	72147	Zbtb46	3,34	14115	Fbln2	3,96
12307	Calb1	4,18	14427	Galr1	3,26	243923	Rgs9bp	3,94
13590	Lefty1	4,13	72978	Cnih3	3,26	171209	Accn3	3,92
497097	Xkr4	4,13	12942	Pcdha11	3,17	19157	Pscd1	3,70
22342	Lin7b	4,11	14403	Gabrd	3,02	50523	Lats2	3,69
239827	Pigz	4,03	14395	Gabra2	3,02	12142	Prdm1	3,28
20672	Sox18	4,01	20451	St8sia3	2,95	64242	Ngb	3,00
213469	Lgi3	3,98	103967	Dnm3	2,92	14633	Gli2	2,99
13003	Vcan	3,90	13003	Vcan	2,88	242022	Frem2	2,99
16994	Ltb	3,89	22141	Tub	2,83	226518	Nmnat2	2,98
76850	Eif2c4	3,85	16913	Psmb8	2,83	14388	Gab1	2,91
18508	Pax6	3,76	14585	Gfra1	2,78	57915	Tbc1d1	2,91
16560	Kif1a	3,54	14417	Gad2	2,69	15485	Hsd17b1	2,90
20358	Sema6a	3,29	57265	Fzd2	2,61	238323	Rps6kl1	2,89
269642	Nat8l	3,27	19157	Pscd1	2,59	14580	Gfap	2,86
18751	Prkcb1	3,22	13591	Ebf1	2,58	216991	Centa2	2,84
103967	Dnm3	3,22	80883	Ntng1	2,55	232975	Atp1a3	2,84
21417	Zeb1	3,17	94089	Trim7	2,54	107227	Macrod1	2,81
71310	Tbc1d9	3,16	74596	Cds1	2,53	214048	Larp2	2,77
20349	Sema3e	3,12	17391	Mmp24	2,53	16764	Aff3	2,67
20348	Sema3c	3,05	101744	C330005M16Rik	2,45	18162	Npr3	2,62
15162	Hck	2,95	12824	Col2a1	2,41	213469	Lgi3	2,49
224024	Scarf2	2,93	15162	Hck	2,35	100129	Gpr153	2,47
23923	Aadat	2,80	226610	C030014K22Rik	2,33	29857	Mapk12	2,46
329154	Ankrd44	2,79	329154	Ankrd44	2,32	14585	Gfra1	2,43
13446	Doc2a	2,78	20349	Sema3e	2,32	19395	Rasgrp2	2,41
14585	Gfra1	2,76	17863	Myb	2,29	211147	AK162044	2,39
18549	Pcsk2	2,76	16180	Il1rap	2,28	14924	Magi1	2,37
242022	Frem2	2,74	11622	Ahr	2,26	140709	Emid2	2,37
232975	Atp1a3	2,72	66873	1200009O22Rik	2,25	277973	Slc9a5	2,35
14465	Gata6	2,69	230576	Ttc22	2,23	272031	E130309F12Rik	2,34
216991	Centa2	2,65	140577	Ankrd6	2,19	14451	Gas1	2,31
18671	Abcb1a	2,61	17172	Ascl1	2,18	320560	D030011O10Rik	2,28
108100	Baiap2	2,55	67874	Rprm	2,16	140577	Ankrd6	2,20
208869	Dock3	2,52	216049	Zfp365	2,14	94219	Cnnm2	2,19
52882	Rgs7bp	2,52	242022	Frem2	2,13	78283	Mtap7d2	2,19
269784	Cntn4	2,50	17967	Ncam1	2,12	432628	Gm1964	2,16
54712	Plxnc1	2,48	23920	Insrr	2,09	58208	Bcl11b	2,15
12569	Cdk5r1	2,48	93961	B3galt5	2,08	19337	Rab33a	2,14
12942	Pcdha11	2,43	237465	Ccdc38	2,08	13841	Epha7	2,14
19277	Ptpro	2,41				11496	Adam22	2,11
17967	Ncam1	2,40				20349	Sema3e	2,09
58887	Repin1	2,39				18212	Ntrk2	2,07
13508	Dscam	2,38				382034	Gse1	2,04
103149	Upb1	2,38				12286	Cacna1a	2,04
320405	Cadps2	2,37				57340	Jph3	2,03
227325	Dner	2,36				116837	Rims1	2,01
116837	Rims1	2,35				56741	Nope	2,01
15485	Hsd17b1	2,33				216049	Zfp365	2,00
24084	Tekt2	2,33				56508	Rapgef4	2,00
18760	Prkcm	2,32						
50931	Il27ra	2,30						
13841	Epha7	2,30						
381813	Prmt8	2,29						
56741	Nope	2,27						
14417	Gad2	2,26						
319387	Lphn3	2,26						
57265	Fzd2	2,24						
72147	Zbtb46	2,17						
67557	Larp6	2,15						
16979	Lrrn1	2,12						
19876	Robo1	2,12						
235505	Cd109	2,11						
19242	Ptn	2,11						
65079	Rtn4r	2,10						
13649	Egfr	2,08						
19088	Prkar2b	2,07						
17863	Myb	2,06						
13388	Dll1	2,04						
19337	Rab33a	2,04						
14119	Fbn2	2,01						
241263	Gpr158	2,00						

Table 4 - Top 100 down regulated genes in PcG KO ES cells

The 100 most down regulated genes with a p-value smaller than 0.05 in PcG mutant ES cells are listed with fold change (fc) compared to wild type ES cells.

Table 4

all genes

Ring1B-/- vs wt			Eed-/- vs wt			dKO vs wt		
Entrez GeneID	Annotation	fc	Entrez GeneID	Annotation	fc	Entrez GeneID	Annotation	fc
17263	Gtl2	72,10	13626	Eed	37,98	14675	Gna14	42,84
373070	Mirg	33,77	78887	Sfi1	17,46	78887	Sfi1	27,05
320085	B830012L14Rik	27,53	17263	Gtl2	16,51	105398	9330199F22Rik	26,73
231098	Dnajc5g	18,62	75745	Rian	15,57	13626	Eed	20,96
17294	Mest	17,12	236219	Tcstv3	15,19	14164	Fgf1	19,98
75745	Rian	17,05	231098	Dnajc5g	14,87	234395	Ushbp1	19,56
268482	A830036E02Rik	14,86	234395	Ushbp1	14,58	77836	Mlana	17,66
13526	Adam24	11,21	373070	Mirg	13,60	17294	Mest	16,56
76217	Jakmip2	10,83	18545	Pcp2	11,84	14171	Fgf17	15,68
14169	Fgf14	10,17	18802	Plcd4	11,49	14936	Gys1	13,61
53901	Rcan2	9,07	72484	2610300M13Rik	11,29	69707	Iqcg	13,46
20564	Slit3	9,01	14164	Fgf1	11,02	108101	BC032204	13,34
17246	Mdm2	8,59	108655	Foxp1	10,74	14910	Gt(ROSA)26Sor	12,66
245610	Nxf3	8,17	70879	4921511E18Rik	10,64	18682	Phkg1	12,50
108655	Foxp1	8,04	18508	Pax6	9,98	68507	Ppfia4	12,42
209294	Csta	7,93	319530	Zfp750	9,54	17916	Myo1f	10,93
22290	Uty	7,85	76218	6430710C18Rik	9,25	237868	A830091I15Rik	10,93
14559	Gdf1	7,77	99055	AI662476	8,75	407790	Ndufa4l2	10,43
26900	Ddx3y	7,75	18111	Nnat	8,12	74407	Ttc25	10,28
68678	Smtnl1	7,68	17339	Mip	8,09	19712	Rest	9,89
242891	Gm443	7,67	207565	Camkk2	8,08	79455	Pdcl2	9,71
16534	Kcnn4	7,27	76059	5830442K09Rik	7,36	71137	Rfx4	9,53
216164	Dos	7,25	71140	4933413I22Rik	7,23	56615	Mgst1	9,51
14164	Fgf1	7,21	71872	Aox4	7,16	99055	AI662476	9,48
16552	Kif12	7,10	14675	Gna14	6,85	55993	Msh4	9,39
230793	Ahdc1	6,92	75357	4930557J02Rik	6,75	17263	Gtl2	9,36
73094	Sgip1	6,80	320127	Dgki	6,75	373070	Mirg	9,12
79455	Pdcl2	6,77	110876	Scn2a1	6,68	18111	Nnat	9,10
66864	Clec14a	6,32	16988	Lst1	6,64	75692	2310073E15Rik	8,91
18816	Serpinf2	6,21	78822	5830411K21Rik	6,63	73239	3110054G05Rik	8,90
74405	Efhc2	6,21	18012	Neurod1	6,57	212516	BC060267	8,74
269846	Tcrb-V13	6,15	407797	BC030308	6,46	73094	Sgip1	8,74
20274	Scn9a	6,09	73239	3110054G05Rik	6,37	78286	5330421F07Rik	8,72
626123	9130004C02Rik	5,94	103814	AI662270	6,34	18041	Nfs1	8,55
68867	Rnf122	5,87	12991	Csn2	6,26	97775	D930048N14Rik	8,53
260408	BC107230	5,81	23859	Dlg2	6,16	20667	Sox12	8,32
171543	Bmf	5,78	329906	C030014L02	6,16	66776	4933439C20Rik	8,26
208079	A530053G22Rik	5,76	69397	1700019A02Rik	6,10	26970	Pla2g2e	8,18
217371	Rab40b	5,73	69861	2010003K11Rik	6,08	19206	Ptch1	7,92
18012	Neurod1	5,73	21417	Zeb1	6,08	330890	Piwil4	7,80
226251	Ablim1	5,63	67340	1700052I22Rik	6,02	80720	Pbx4	7,75
78887	Sfi1	5,46	20672	Sox18	6,00	56069	Il17b	7,71
97243	Ard1b	5,37	71338	5430420C16Rik	5,97	672498	LOC672498	7,53
21687	Tek	5,37	16412	Itgb1	5,95	75745	Rian	7,40
14765	Gpr50	5,35	330355	Dnahc6	5,95	67392	4833420G17Rik	7,39
277468	Slc39a12	5,33	17246	Mdm2	5,74	66793	Efcab1	7,17
74898	4930456K20Rik	5,25	69690	2310057B04Rik	5,71	100045988	LOC100045988	7,10
70084	2300004M11Rik	5,16	58203	Zbp1	5,65	21366	Slc6a6	7,08
94179	Krt23	5,14	18012	Neurod1	5,64	26896	Med14	7,06
11474	Actn3	5,14	103358	AU022077	5,63	109225	Ms4a7	6,98
216881	Wscd1	5,09	104936	AU017263	5,59	399595	6430514M23Rik	6,91
320840	Negr1	5,09	21334	Tac2	5,58	108655	Foxp1	6,85
72014	1500005I02Rik	5,07	52662	D18Ertd653e	5,52	14555	Gpd1	6,75
52662	D18Ertd653e	5,06	19266	Ptprd	5,51	105425	B930053N05Rik	6,74
16663	Krt13	5,03	70785	Dennd1c	5,46	14469	Gbp2	6,69
20345	Selplg	5,03	67058	2810428J06Rik	5,37	320000	C630016I17Rik	6,68
105989	AU045094	4,99	116852	Akr1c20	5,36	16656	Hivep3	6,59
74269	1700063H04Rik	4,96	233649	Cnga4	5,31	50789	Fbxl3	6,54
70831	4733401H21Rik	4,96	68527	1110017I16Rik	5,28	72991	2900075N08Rik	6,54
97136	C77438	4,94	223666	D15Wsu169e	5,20	21423	Tcfe2a	6,52
57765	Tbx21	4,85	433719	OTTMUSG00000001246	5,18	75600	Calml4	6,49
103503	BB001228	4,84	14169	Fgf14	5,13	97928	C85363	6,40
14675	Gna14	4,81	378466	ENSMUSG00000057924	5,06	17878	Myf6	6,39
117160	Ttyh2	4,72	16994	Ltb	5,03	72215	1700001P01Rik	6,37
20607	Sstr3	4,71	16663	Krt13	4,96	72897	2900006B11Rik	6,36
19157	Pscd1	4,71	270035	Letm2	4,94	74076	4933406C10Rik	6,36
68152	5830415L20Rik	4,70	68991	Ssu72	4,93	110876	Scn2a1	6,34
22141	Tub	4,59	208666	Diras1	4,93	433766	Trim63	6,28
13114	Cyp3a16	4,54	26365	Ceacam1	4,87	208666	Diras1	6,27
71682	Wdr27	4,54	20274	Scn9a	4,84	20904	Strm	6,21
77836	Mlana	4,53	103012	6720401G13Rik	4,84	58198	Sall1	6,17
237868	A830091I15Rik	4,53	18125	Nos1	4,83	75083	Usp50	6,14
11770	Fabp4	4,46	17227	Mcpt4	4,83	223262	Timm8a2	6,13
328795	Ubash3a	4,40	74269	1700063H04Rik	4,77	11634	Aire	6,09
12994	Csn3	4,37	12829	Col4a4	4,76	102085	AI451606	6,06
239857	Cadm2	4,36	105398	9330199F22Rik	4,75	338535	E030013I19Rik	6,01
399595	6430514M23Rik	4,35	97928	C85363	4,75	18012	Neurod1	5,97
93874	Pcdhb3	4,33	78288	5330421F21Rik	4,72	16994	Ltb	5,96
76459	Car12	4,21	52711	D11Ertd729e	4,71	216881	Wscd1	5,94
12307	Calb1	4,18	333307	Trim75	4,71	52480	D7Ertd715e	5,94
55963	Slc1a4	4,18	672498	LOC672498	4,71	68867	Rnf122	5,90
22619	Siae	4,18	12530	Cdc25a	4,69	66211	Rpl3l	5,88
13590	Lefty1	4,13	109225	Ms4a7	4,61	320352	E230002P03Rik	5,86
497097	Xkr4	4,13	15208	Hes5	4,61	54158	Copg2as2	5,85
22342	Lin7b	4,11	14171	Fgf17	4,60	228858	Gdap1l1	5,80
67578	4930424G05Rik	4,08	13590	Lefty1	4,59	103268	2410017P07Rik	5,79
66441	2010012C16Rik	4,08	399595	6430514M23Rik	4,57	320635	Cyb5r2	5,76
16656	Hivep3	4,07	107526	Gimap4	4,57	16534	Kcnn4	5,75
235472	Prtg	4,07	71860	Wdr16	4,56	20358	Sema6a	5,74
67410	4930449I24Rik	4,05	76413	1700016D06Rik	4,55	381411	Gm1967	5,73
239827	Pigz	4,03	72986	2900078E11Rik	4,54	52711	D11Ertd729e	5,72
13666	Eif2ak3	4,02	20607	Sstr3	4,48	53868	Rab25	5,70
20672	Sox18	4,01	17916	Myo1f	4,44	103784	Wdr92	5,66
52711	D11Ertd729e	3,99	77481	C030048H21Rik	4,44	228421	Kif18a	5,65
213469	Lgi3	3,98	16866	Lhb	4,40	17425	Foxk1	5,63
55993	Msh4	3,93	66399	Tsfm	4,39	108655	Foxp1	5,61
58243	Nap1l5	3,92	68553	1110001D15Rik	4,36	18778	Pla2g1b	5,57
76024	5830431A10Rik	3,91	68606	Ppm1f	4,36	380713	Scarf1	5,48
71321	4933440J02Rik	3,91	170938	Zfp617	4,30	78751	Zc3h6	5,45
13003	Vcan	3,90	239857	Cadm2	4,30	66720	Klhl10	5,35
16994	Ltb	3,89	50789	Fbxl3	4,27	234311	BC013672	5,34

Table 5

List of PCR primer sequences used in this study

Table 5

primer	5'-Sequence-3'
Arg2 FW	AGAAGCTGGCTTGCTGAAGA
Arg2 RV	AGGGATCATCTTGTGGGACA
Chr6:72,811,719-72,811,833 FW	CCCCTTTCTGAAGCACTCTG
Chr6:72,811,719-72,811,833 RV	TAAGGCGTCATTTCCCAAAG
Dkk1 FW	CTCATCAATTCCAACGCGATCA
Dkk1 RV	GCCCTCATAGAGAACTCCCG
Dlk1 FW	CGGGAAATTCTGCGAAATAG
Dlk1 RV	TGTGCAGGAGCATTCGTACT
Foxa2 FW	CCCTACGCCAACATGAACTCG
Foxa2 RV	GTTCTGCCGGTAGAAAGGGA
Hoxd11 FW	ACACCAAGTACCAGATCCGC
Hoxd11 RV	AGTGAGGTTGAGCATCCGAG
Hoxd13 FW	CCCATTTTTGGAAATCATCC
Hoxd13 RV	TGGTGTAAGGCACCCTTTTC
Gapdh promoter FW	AGCATCCCTAGACCCGTACAGT
Gapdh promoter RV	GGGTTCCTATAAATACGGACTGC
IAP probe FW	CCCAGTTGGGTTGTGAAAGAAAACT
IAP probe RV	TTTACTAGTTAGGCACAGCGGAGGC
Klf4 FW	GTGCCCCGACTAACCGTTG
Klf4 RV	GTCGTTGAACTCCTCGGTCT
Lef1 promoter FW	ATCAGTCATCCCGAAGAGGA
Lef1 promoter RV	AGCTGCCCACTCACCTCAT
MLV probe FW	CCCCTGCAAGTGCTGACCCTAA
MLV probe RV	CCATCACAGTCTCTTTTCTGGCCTC
Nanog FW	TCTTCCTGGTCCCCACAGTTT
Nanog RV	GCAAGAATAGTTCTCGGGATGAA
Nrp1 FW	CCGGAACCCTACCAGAGAA
Nrp1 RV	CCCCATCAATTACTTCCACG
Oct4 FW	GGCTTCAGACTTCGCCTCC
Oct4 RV	AACCTGAGGTCCACAGTATGC
Oct4 promoter FW	CAAGTTGGCGTGGAGACTTT
Oct4 promoter RV	TTGGTTCCACCTTCTCCAAC
Rex1 FW	CCCTCGACAGACTGACCCTAA
Rex1 RV	TCGGGGCTAATCTCACTTTCAT
ribosomal protein L32 FW	GGCTTTTCGGTTCTTAGAGGA
ribosomal protein L32 RV	TTCCTGGTCCACAATGTCAA
Ring1B deletion check FW	TGCTCCTTTTTGATGGAAC
Ring1B deletion check RV	CAACCCACTCACCATCACAC
Sox7 FW	GGGTCTCTTCTGGGACAGTG
Sox7 RV	GGATGAGAGGAAACGTCTGG
Zic1 FW	CTTTTCCCTGCCCGTTTC
Zic1 RV	CTCGAACTCGCACTTGAAGG

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