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

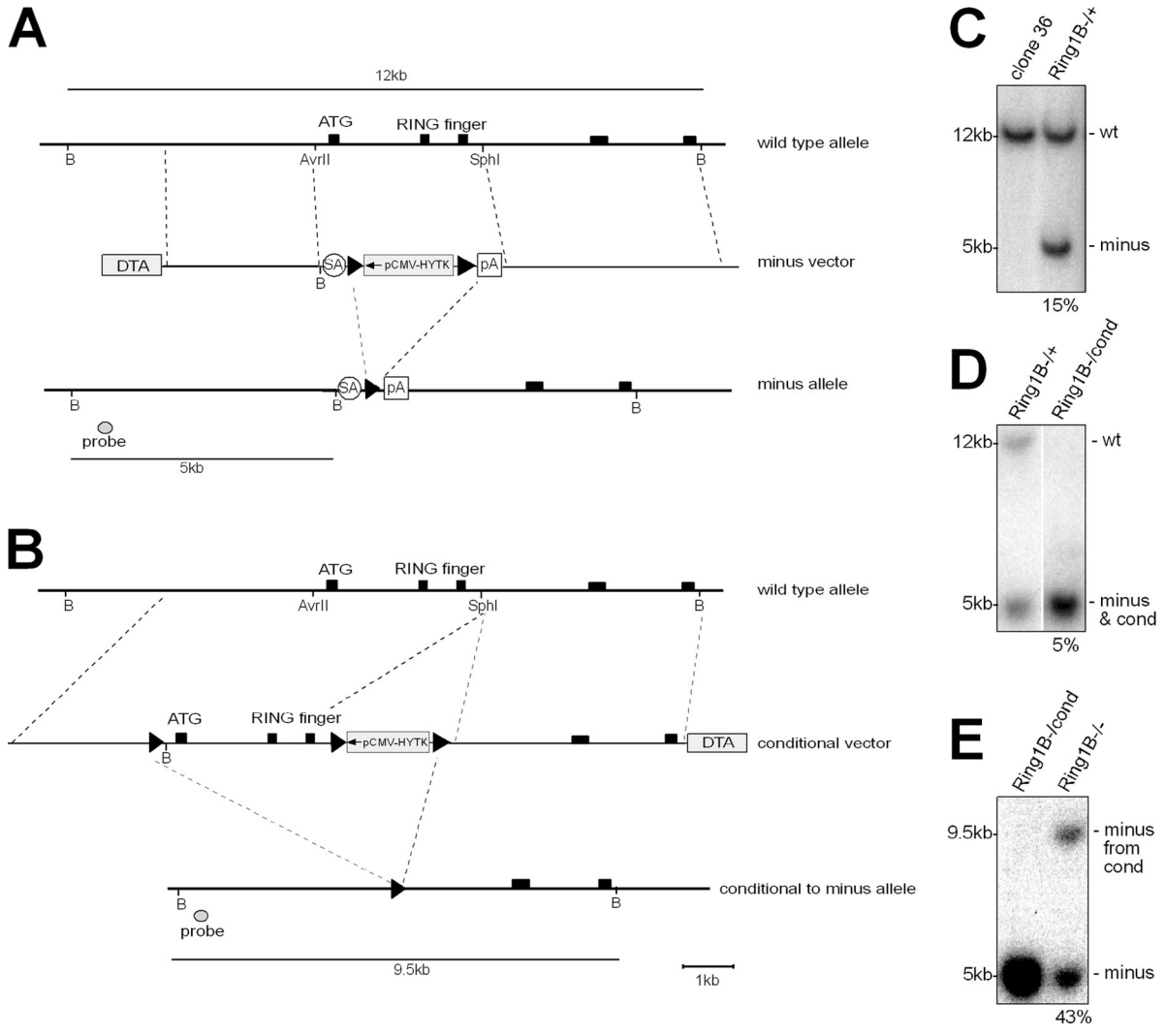

Figure 1. **Generation of *Ring1B*-deficient ES cells.** (A) Schematic representation of the *Ring1B* locus and the minus targeting vector replacing the start codon and the RING finger domain with a stop cassette. (B) *Ring1B* conditional targeting vector allowing for deletion of the *Ring1B* locus after 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.

(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

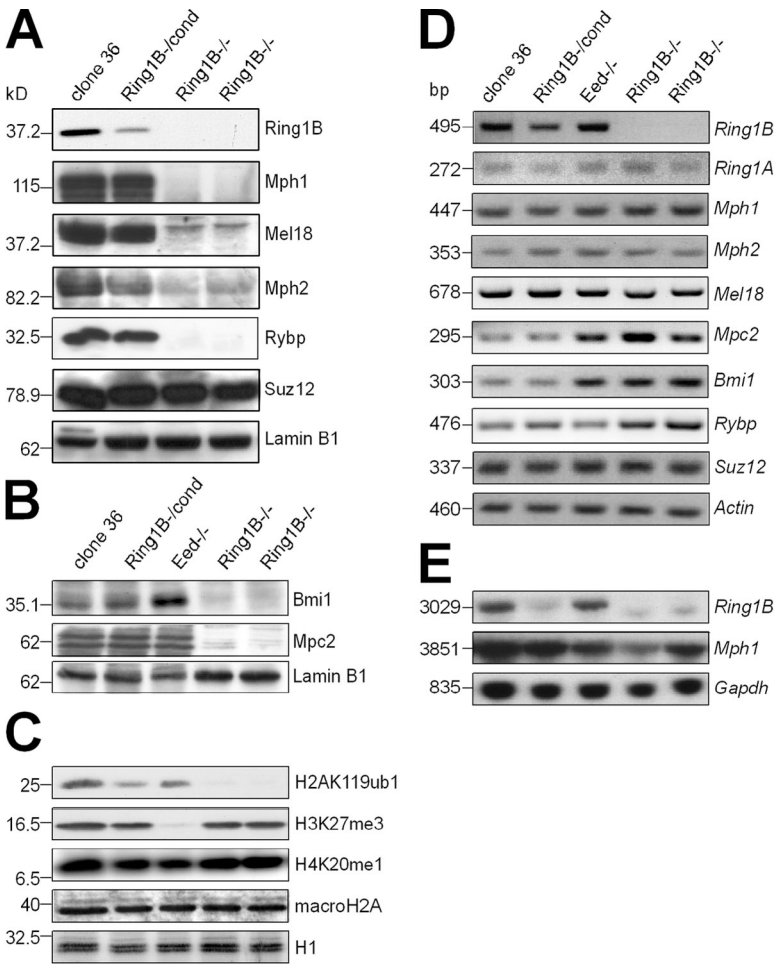

Figure 2. **Analysis of PcG expression in clone $36^{Ring1B-/-}$ cells.** (A) Western analysis of PcG proteins in nuclear extracts from control clone 36, $36^{Ring1B-/cond}$, and $36^{Ring1B-/-}$ ES cells. A representative lamin B1 loading control is shown. (B) Western analysis of Bmi1 and Mpc2 in nuclear 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.

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.

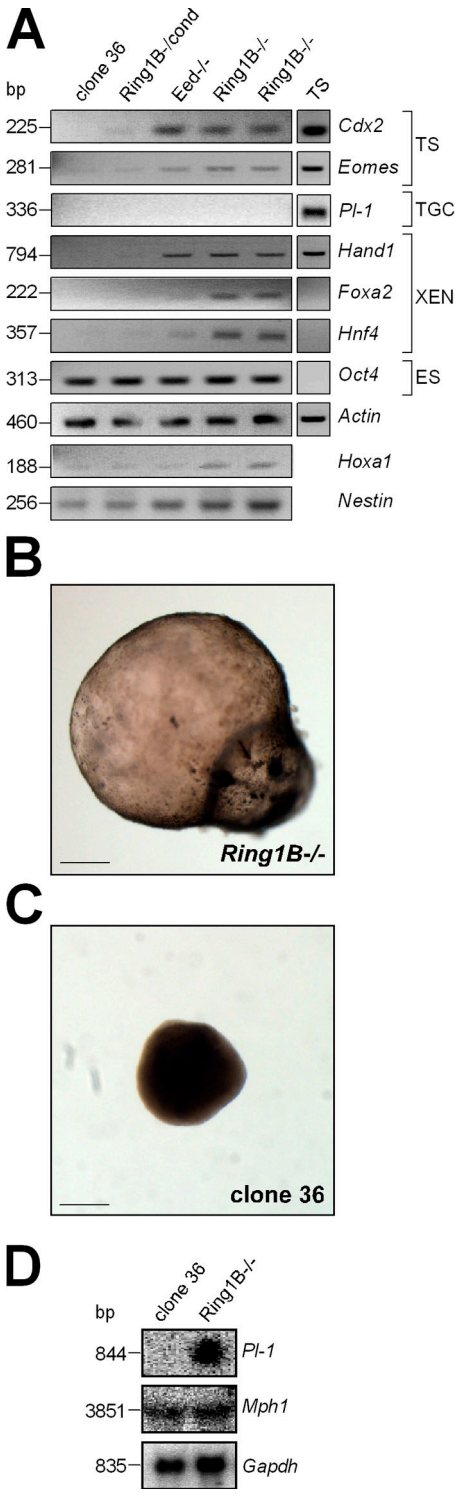

Figure 3. **Deregulation of developmental control genes upon the loss of *Ring1B*.** (A) Expression analysis of *Cdx2*, *Eomes*, *Pl-1*, *Hand1*, *Foxa2*, *Hnf4*, *Oct4*, *Hoxa1*, *Nestin*, and the loading control *β-actin* using RNA from ES cells as indicated by RT-PCR. (B and C) EBs derived from clone 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

$36^{Ring1B-/-}$ 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).

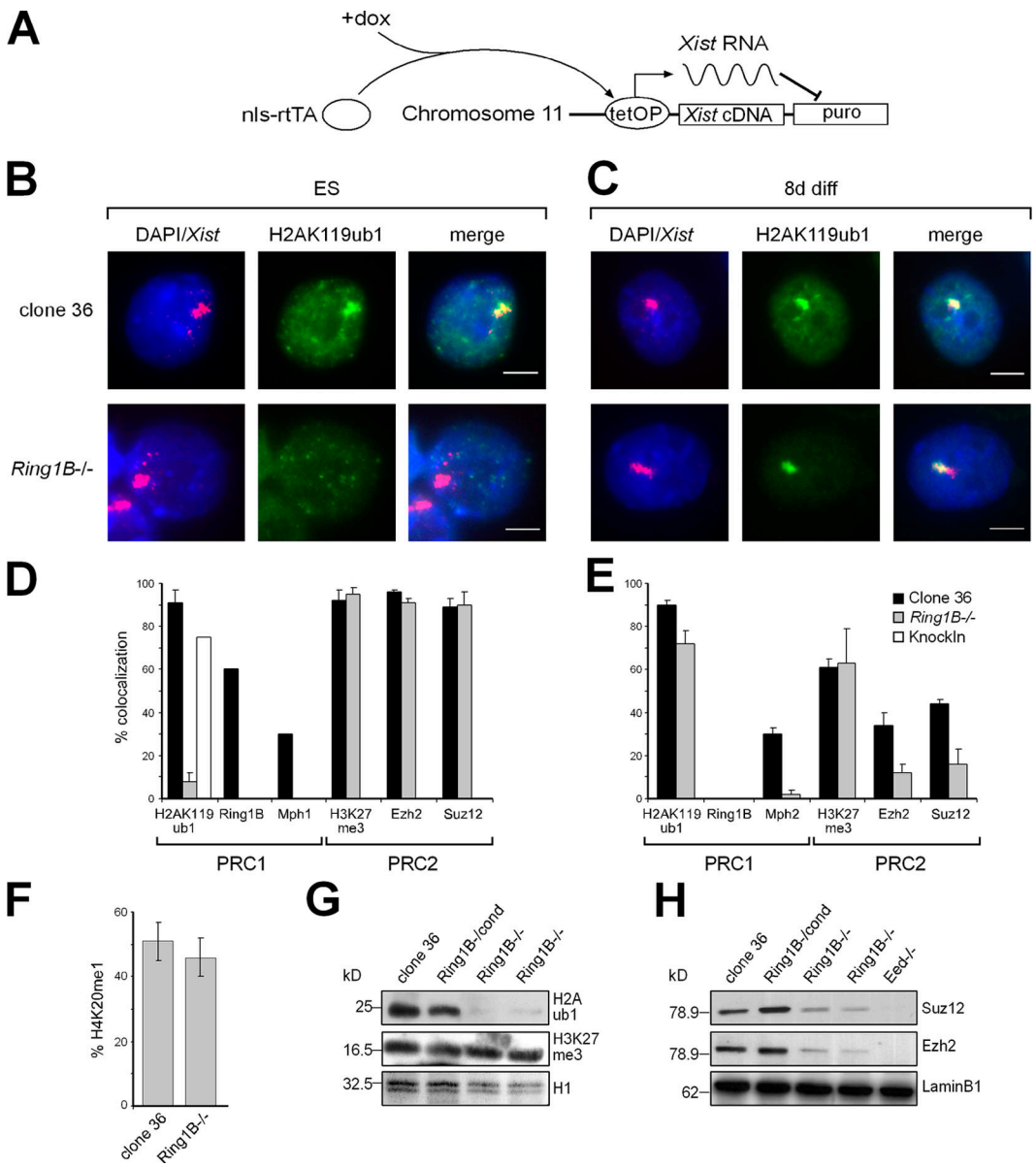

Figure 4. **Recruitment of histone modifications and PcG proteins by *Xist* in *Ring1B*-deficient cells.** (A) Scheme showing the inducible *Xist* expression system in clone 36 ES cells. In the presence of the inducer doxycycline, the tetracycline-regulated transactivator (nls-rtTA) binds to the inducible 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

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

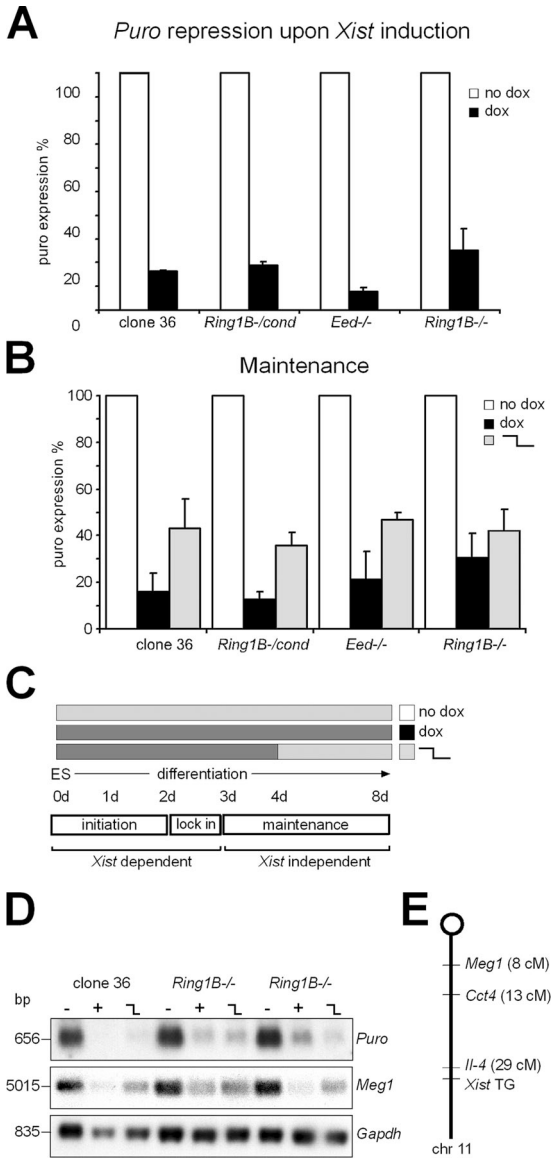

Figure 5. **Initiation and maintenance of *Xist*-mediated silencing in *Ring1B*-deficient cells.** (A) Quantification of puro repression upon *Xist* induction with doxycycline (dox) in clone 36, $36^{Ring1B-/cond}$, $36^{Eed-/-}$, and $36^{Ring1B-/-}$ ES cells by Northern analysis. (B) Maintenance of 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.

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

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

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 cell 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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