Role of Bmi-1 and Ring1A in H2A Ubiquitylation and Hox Gene Silencing

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Summary

Polycomb group (PcG) proteins exist in at least two biochemically distinct protein complexes, the EED-EZH2 complex and the PRC1 complex, that respectively possess H3-K27 methyltransferase and H2A-K119 ubiquitin E3 ligase activities. How the enzymatic activities are regulated and what their role is in Hox gene silencing are not clear. Here, we demonstrate that Bmi-1 and Ring1A, two components of the PRC1 complex, play important roles in H2A ubiquitylation and Hox gene silencing. We show that both proteins positively regulate H2A ubiquitylation. Chromatin immunoprecipitation (ChIP) assays demonstrate that Bmi-1 and other components of the two PcG complexes bind to the promoter of HoxC13. Knockout Bmi-1 results in significant loss of H2A ubiquitylation and upregulation of Hoxc13 expression, whereas EZH2-mediated H3-K27 methylation is not affected. Our results suggest that EZH2-mediated H3-K27 methylation functions upstream of PRC1 and establishes a critical role for Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing.

Introduction

Maintenance of spatially restricted homeobox (Hox) gene expression patterns in both flies and vertebrates is controlled by PcG and trithorax group (trxG) proteins (Ringrose and Paro, 2004). Biochemical and genetic studies indicate that PcG proteins exist in at least two different protein complexes: the Polycomb repressive complex 1 (PRC1) and the ESC-E(Z) complex (Francis and Kingston, 2001; Simon and Tamkun, 2002). Consistent with the notion that PcG proteins function at the level of chromatin, studies in the past several years have revealed that both protein complexes possess intrinsic enzymatic activities that catalyze the covalent modification of histones. Whereas the ESC-E(Z) complex in fly and its mammalian counterpart EED-EZH2 complex possess H3-K27 methyltransferase activity (Beisel et al., 2002; Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Milne et al., 2002; Muller et al., 2002; Nakamura et al., 2002), the PRC1 complex has been recently demonstrated to possess H2A-K119 ubiquitin E3 ligase activity (Wang et al., 2004a). Interestingly, PRC1-mediated H2A ubiquitylation has also been linked to X inactivation (de Napoles et al., 2004; Fang et al., 2004). Genetics and biochemical studies suggest that the two PcG complexes function in a cooperative manner to maintain long-term gene silencing (Francis

and Kingston, 2001; Simon and Tamkun, 2002). Accumulating evidence supports a sequential model (Wang et al., 2004b) in which ESC-E(Z)-mediated H3-K27 methylation serves as a binding site for the recruitment of the PRC1 complex through the specific recognition of the H3-K27 methyl mark by the chromo domain of the Polycomb (Pc) protein (Fischle et al., 2003; Min et al., 2003). How recruitment of the PRC1 complex leads to gene silencing is not understood, although the PRC1 complex has been shown to be capable of inhibiting chromatin remodeling by the SWI/SNF complex in vitro (Shao et al., 1999).

Recent studies demonstrating an intrinsic H2A-K119 ubiquitin E3 ligase activity have shed some light on how PRC1 participates in the silencing process (Wang et al., 2004a). The mammalian PRC1 ubiquitin E3 ligase complex consists of several PcG proteins, including three ring domain-containing proteins termed RING1/Ring1A, RING2/Ring1B, and BMI-1/Bmi-1. Analysis of the three ring domain-containing proteins indicated that RING2/Ring1B is the catalytic subunit (Wang et al., 2004a), although both Ring1A and Ring1B contribute to H2A ubiquitylation in vivo (de Napoles et al., 2004). Genetic studies in mice revealed that both Ring1A and Bmi-1 have an important role in anterior-posterior axis specification and in maintaining the correct spatial Hox gene expression pattern (del Mar Lorente et al., 2000; van der Lugt et al., 1996). In addition, Bmi-1 has also been shown to play an important role in cell proliferation (Jacobs et al., 1999a), stem cell self-renewal (Lessard and Sauvageau, 2003; Park et al., 2003), and cancer (Jacobs et al., 1999b). Therefore, understanding the biochemical function of Ring1A and Bmi-1 in the context of the PRC1 ubiquitin E3 ligase complex may shed some light on understanding how they participate in these diverse biological processes. Studies in yeast have revealed that methylation on both H3-K4 and H3-K79 is dependent on Rad6-mediated H2B ubiquitylation (Briggs et al., 2002; Dover et al., 2002; Ng et al., 2002; Sun and Allis, 2002). Given that both H3-K27 methylation and H2A-K119 ubiquitylation participate in PcG silencing (Cao et al., 2002; Wang et al., 2004a), it will be important to determine if and how the two histone modifications cooperate to mediate PcG silencing.

With the above questions in mind, here we report on the functional characterization of the individual components of the E3 ligase complex. By comparing the enzymatic activity of different subcomplexes, we demonstrated that both Ring1A and Bmi-1 contribute to the E3 ligase activity. Analysis of Hox gene expression followed by ChIP revealed a role for Bmi-1 in promoting H2A ubiquitylation and HoxC13 silencing. Manipulation of the H3-K27 methylation and H2A-K119 ubiquitylation by knockdown SUZ12 or knockout Bmi-1, respectively, allowed us to address the relationship between the two enzymatic activities. Thus, our work not only provided evidence that supports an important role for Ring1A and Bmi-1 in H2A ubiquitylation and Hox gene silencing but also revealed the sequential events of H3-K27 methylation and H2A-K119 ubiquitylation. The

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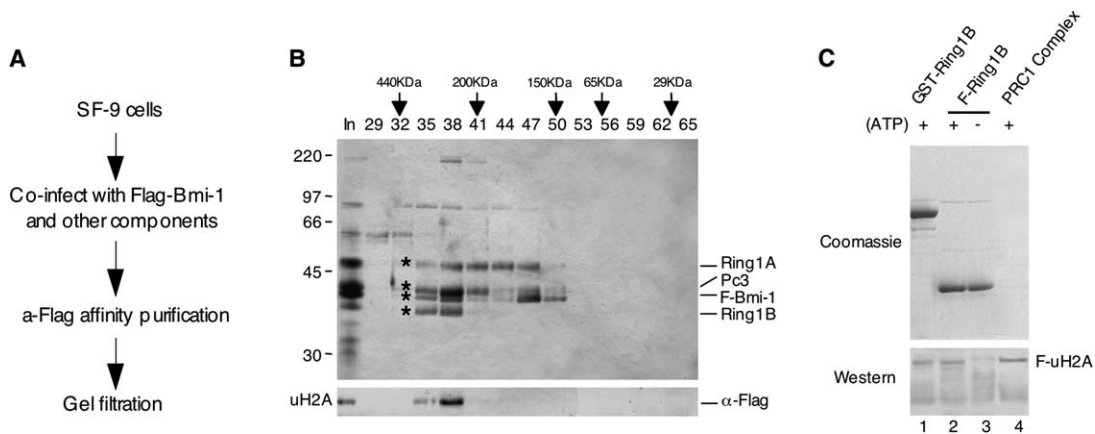
Figure 1. Reconstituted PRC1 Complex Is Significantly More Active Than Ring1B Alone

(A) Schematic representation of the steps used in PRC1 reconstitution.

(B) Silver staining of a polyacrylamide-SDS gel (top) and an E3 ligase activity assay (bottom) of the fractions derived from the Sephadex 200 gel-filtration column. The four components of the recombinant PRC1 subcomplex are indicated by asterisks (*). The elution profile of the protein markers is indicated at the top of the panel. The positions of the protein size markers are indicated to the left of the panel.

(C) Comparison of the E3 ligase activity (bottom) of the recombinant Ring1B made in *E. coli* (lane 1) and in Sf9 cells (lanes 2 and 3) either alone or in the context of the four-component complex (lane 4). The amount of enzymes required for equal activity is dramatically different as revealed by Coomassie staining (top).

demonstration that Bmi-1 regulates gene expression through modulating H2A ubiquitylation has important implications in understanding the molecular mechanisms by which Bmi-1 participates in cancer and stem cell self-renewal.

Results

The PRC1 Complex Is Significantly More Active Than Ring1B Alone in H2A Ubiquitylation

Previously, we purified a multisubunit PRC1-like complex from HeLa cells that possesses H2A-K119 ubiquitin E3 ligase activity (Wang et al., 2004a). Components of the complex include three ring domain-containing proteins, RING2/Ring1B, RING1/Ring1A, and BMI-1/Bmi-1, in addition to hPH2 and PC3 (present in some batches of purification). Analysis of the three ring domain-containing proteins indicated that RING2/Ring1B is the catalytic subunit (Wang et al., 2004a). To dissect the function of individual subunits and to obtain large amounts of purified enzyme complex for detailed functional analysis, we attempted to reconstitute the enzymatic activity with recombinant proteins encoded by mouse cDNAs. To this end, Sf9 cells were coinfected with baculoviruses expressing Flag-Bmi-1, Ring1A, Ring1B, and Pc3. Flag-Bmi-1 and associated proteins were purified by affinity chromatography followed by gel filtration to separate the four-component complex from free Flag-Bmi-1 and partial complex (Figure 1A). Silver staining and ubiquitin E3 ligase assays of the gel filtration column fractions indicate that the four-component complex copurified with the enzymatic activity as a protein complex of about 250 kDa (Figure 1B). We note that, under our purification conditions, Ph2 appears to be easily dissociated from the four-component complex in a gel-filtration column when it is coexpressed with other components (data not shown). Therefore, a stable five-component protein complex was not formed under our purification conditions.

To determine whether other components of the complex contribute to the enzymatic activity of Ring1B, we purified Flag-Ring1B from infected Sf9 cells and compared its E3 ligase activity with GST-Ring1B expressed in *E. coli* and the four-component complex described above. Results shown in Figure 1C indicate that Flag-Ring1B is an active ubiquitin E3 ligase for H2A (compare lanes 2 and 3) and its activity is comparable with that of GST-Ring1B (compare lanes 1 and 2). Surprisingly, the four-component complex is significantly more active, as much less enzyme is required to achieve a similar level of H2A ubiquitylation (compare lanes 2 and 4). We estimate that an equal molar amount of Ring1B in the complex is about 200-fold more active than Ring1B alone (data not shown). These results suggest that one or more than one of the other three subunits in the complex can stimulate the E3 ligase activity of Ring1B.

Physical Relationship among Components of the PRC1 Complex

Of the five components present in the PRC1 complex, Ring1B is the only subunit demonstrated to have E3 ligase activity by itself (Wang et al., 2004a). To address the effect of other subunits on Ring1B enzymatic activity, we attempted to reconstitute Ring1B-containing subcomplexes. Toward this end, we first defined the precise spatial relationship among components of the complex by GST pull-down assays. Individual components were expressed as GST fusion proteins, and equal amounts of the fusion proteins, as well as GST alone (Figure 2A, fifth panel), were immobilized onto beads and incubated with ^{35}S labeled proteins. After washing and electrophoresis, the pair-wise protein-protein interactions were revealed by fluorography. Results shown in Figure 2A indicate that Ring1B can interact with Bmi-1, Pc3, and Ph2 (first panel). Similar interaction patterns were also observed for Ring1A (second panel), consistent with the high similarity of the two proteins. Interestingly, Bmi-1 appears to be capable of interacting with each of the
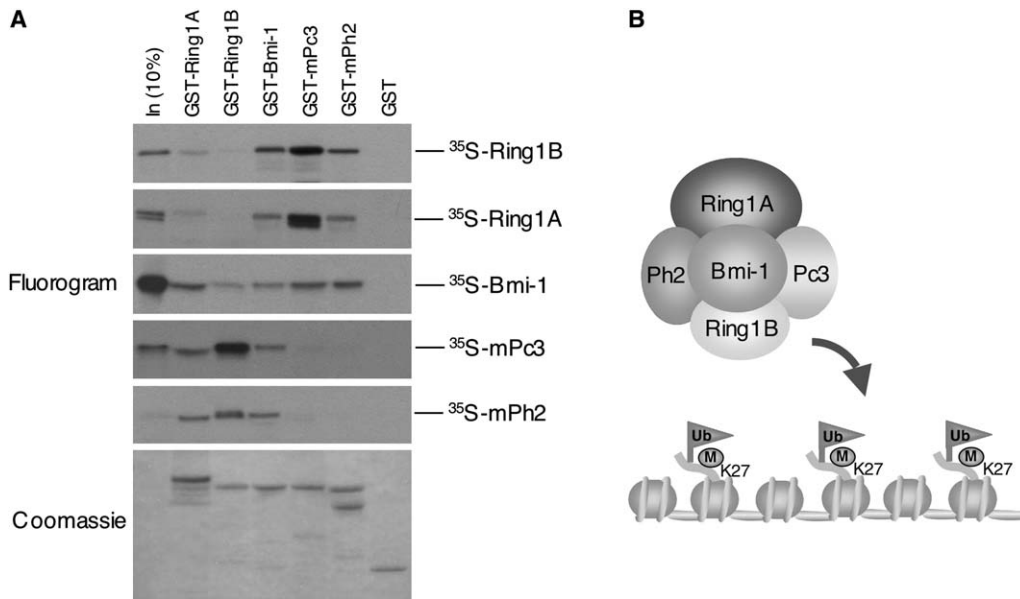
Figure 2. Protein-Protein Interactions among Components of the PRC1 Complex

(A) GST pull-down assays using equal amounts of GST fusion proteins (bottom) and in vitro-translated ^{35}S labeled proteins indicated on right (top five panels). Abbreviation: In, 10% of the total input.

(B) Schematic representation of the interactions detected in (A).

components (third panel), suggesting that Bmi-1 might be an essential component for maintaining the complex integrity. Both Pc3 and Ph2 appear to be capable of interacting with all three ring domain-containing proteins, but they do not seem to interact with each other (fourth and fifth panels). These pair-wise interaction data are best summarized in Figure 2B, which shows Bmi-1 making multiple contacts with other components and occupying a central position in this complex.

### Ring1B-Associated Proteins Stimulate Its E3 Ligase Activity

After defining the spatial relationship among the five components, we used a similar two-step purification procedure and reconstituted Ring1B-containing subcomplexes with two (Flag-Ring1B and Bmi-1), three (Ring1B, Flag-Bmi-1, and Ring1A), and four (Ring1B, Flag-Bmi-1, Ring1A, and Pc3) subunits. Silver staining revealed that these reconstituted subcomplexes are near homogeneity (Figure 3A). To evaluate the relative activities of these purified complexes, various amounts of the different subcomplexes, as indicated by Western blot analysis of Ring1B (Figure 3B, top), were used to ubiquitylate equal amounts of nucleosomal substrates. Results shown in Figure 3B (bottom) indicate that association of Bmi-1 with Ring1B stimulated its E3 ligase activity (compare lanes 1 and 2). However, the enzymatic activity of the two-component complex is apparently not comparable to that of the four-component complex (Figure 3B, compare lanes 4 and 6). Incorporation of Ring1A into the two-component subcomplex (F-Ring1B + Bmi-1) also stimulated the ubiquitin ligase activity (Figure 3C, compare lanes 3 and 6). These data suggest that Bmi-1, Ring1A, and Pc3 all contribute to optimal activity of Ring1B in vitro.

To substantiate the above observations in vivo, we took advantage of the availability of the Bmi-1 and Ring1A null

mouse embryonic fibroblast (MEF) cells and analyzed the effects of knockout of these two genes on the level of H2A ubiquitylation. Equal amounts of histone extracts prepared from wild-type (wt) and Bmi-1 or Ring1A knockout MEFs were subjected to Western blot analysis with antibodies specific for ubiquitinated H2A. Results shown in Figures 3D and 3E indicate that knockout of either of the two genes greatly reduced the H2A ubiquitylation level. Together, these data suggest that Bmi-1 and Ring1A contribute to H2A ubiquitylation in vivo.

Knockout of Ring1A and Bmi-1 Results in Alterations in Hox Gene Expression

Having demonstrated that knockout of Ring1A and Bmi-1 results in a significant decrease in the H2A ubiquitylation level, we investigated their role in Hox gene expression. Previous studies in mice indicate that loss of Ring1A and Bmi-1 function resulted in anterior and posterior transformations, respectively (del Mar Lorente et al., 2000; van der Lugt et al., 1996). Analysis of the expression patterns of selected Hox genes by RNA in situ hybridization revealed a shift in their expression boundaries. The shift is subtle in Ring1A$^{-/-}$ mice, whereas the shift is more significant in Bmi-1$^{-/-}$ mice (del Mar Lorente et al., 2000; van der Lugt et al., 1996). These studies, however, did not analyze changes in levels of Hox gene expression. To analyze the potential effects of Ring1A and Bmi-1 knockout on Hox gene expression levels, we analyzed expression of 33 out of the 39 Hox genes by RT-PCR using RNAs isolated from MEFs of wt and mutant littermates as templates. It is not clear whether failure of amplifying the other six Hox genes is due to a low expression level of these Hox genes or to primer problems. Results shown in Figure 4A indicate that Ring1A knockout only modestly affected HoxC13, HoxD3, and HoxD10 expression. In contrast to the subtle changes in the Ring1A knockout, knockout of Bmi-1
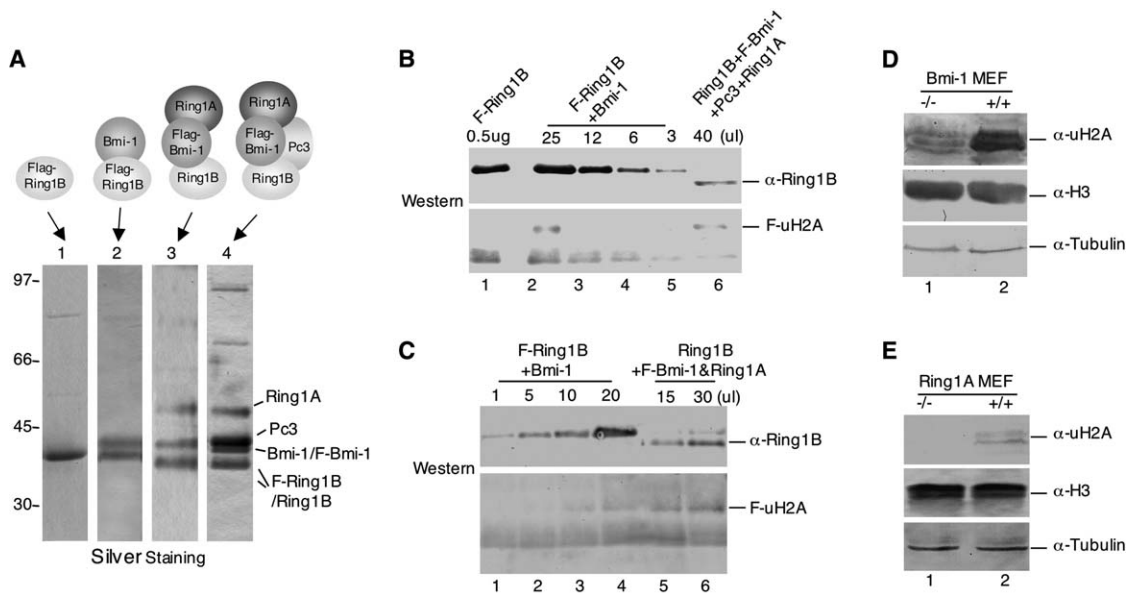
Figure 3. Bmi-1 and Ring1A Stimulate the E3 Ligase Activity of Ring1B In Vitro and In Vivo

(A) Silver-stained gels reveal the purity of the different Ring1B-containing subcomplexes.

(B) Bmi1 stimulates the ubiquitin E3 ligase activity of Ring1B in vitro. The amounts of Ring1B in different lanes are quantified by Western blotting using an anti-Ring1B antibody (top). Although lanes 1 and 2 contain similar amounts of Ring1B, lane 2 has higher enzymatic activity. Lanes 4 and 6 have similar amounts of Ring1B, but lane 6 exhibited much higher E3 ligase activity.

(C) Incorporation of Ring1A into the Ring1B/Bmi-1 subcomplex further stimulates the ubiquitin E3 ligase activity in vitro. The E3 ligase activity of the two-component F-Ring1B/Bmi1 complex (lanes 1–4) was compared to that of the three-component complex of Ring1B/F-Bmi1/Ring1A (lanes 5 and 6). The amounts of Ring1B and ubiquitylated H2A in the different reactions were detected by Western blotting using Ring1B and Flag antibodies, respectively.

(D) Western blot analysis of H2A ubiquitylation levels of histones extracted from wild-type (wt) and Bmi-1 null MEFs with an antibody specific for ubiquitylated H2A. Equal loading was confirmed by Western blot using anti-H3 and anti-tubulin antibodies.

(E) Western blot analysis of H2A ubiquitylation levels of histones extracted from wt and Ring1A null MEFs with an antibody specific for ubiquitylated H2A. The controls for loading are similar to those in (D).

resulted in significant changes in the expression of most of the Hox genes (Figure 4B). Of the 33 Hox genes analyzed, 12 (underlined) are significantly upregulated, and 13 (indicated by an asterisk [*]) are significantly downregulated. It is interesting to note that most late Hox genes are upregulated, whereas most early Hox genes are downregulated. Given that the PcG proteins are believed to be involved in gene silencing, downregulation in response to loss of Bmi-1 function might not represent a direct effect. Therefore, our further analysis has focused on upregulated genes, particularly HoxC13 because upregulation of this gene was observed in MEFs of both Bmi-1 and Ring1A knockouts.

Derepression of HoxC13 Correlates with Decreased H2A Ubiquitylation at Promoter

To understand the relationship between Bmi-1, H2A ubiquitylation, and HoxC13 derepression, we first examined Bmi-1 binding across the entire HoxC13 gene locus by ChIP assays. Results shown in Figure 5A indicate that Bmi-1 is highly enriched in a 500 bp region that encompasses the transcription start site. The observed binding is specific, as a similar signal is not observed in a parallel ChIP experiment with the Bmi-1$^{-/-}$ cells. Because Bmi-1 is part of the PRC1 H2A ubiquitin E3 ligase complex (Wang et al., 2004a), and PRC1 functions together with the EZH2 H3-K27 methyltransferase complex in Hox gene silencing (Ringrose and Paro, 2004), we analyzed

the presence in this region of other PcG proteins belonging to the two protein complexes as well as H3-K27 methylation and H2A ubiquitylation. We found that all the components of the two complexes analyzed are present (Figure 5B, region A). Consistent with the two protein complexes respectively mediating H3-K27 methylation and H2A ubiquitylation, both histone modifications are observed in the region. Protein binding and histone modifications are restricted to the promoter region, as a parallel analysis of a region in the intron between exon1 and exon2 did not detect their presence (region B). Consistent with an important role for Bmi-1 in H2A ubiquitylation, knockout of Bmi-1 resulted in significant decrease of H2A ubiquitylation in the promoter region (Figure 5B, lane 7). Interestingly, loss of Bmi-1 binding or H2A ubiquitylation does not affect the binding of Suz12, a component of the Ezh2 complex, or H3-K27 methylation (lanes 6 and 8). This is consistent with the notion that PRC1 recruitment and H2A ubiquitylation are downstream events of Ezh2-mediated H3-K27 methylation (Wang et al., 2004a). Collectively, the above results support a critical role for Bmi-1 in H2A ubiquitylation and Hox gene silencing.

Mel-18 Can Substitute for Bmi-1 to Stabilize PRC1 but Is Not Functionally Redundant

It is interesting to note that both Ring1A and Ring1B are present in the promoter region in the absence of Bmi-1
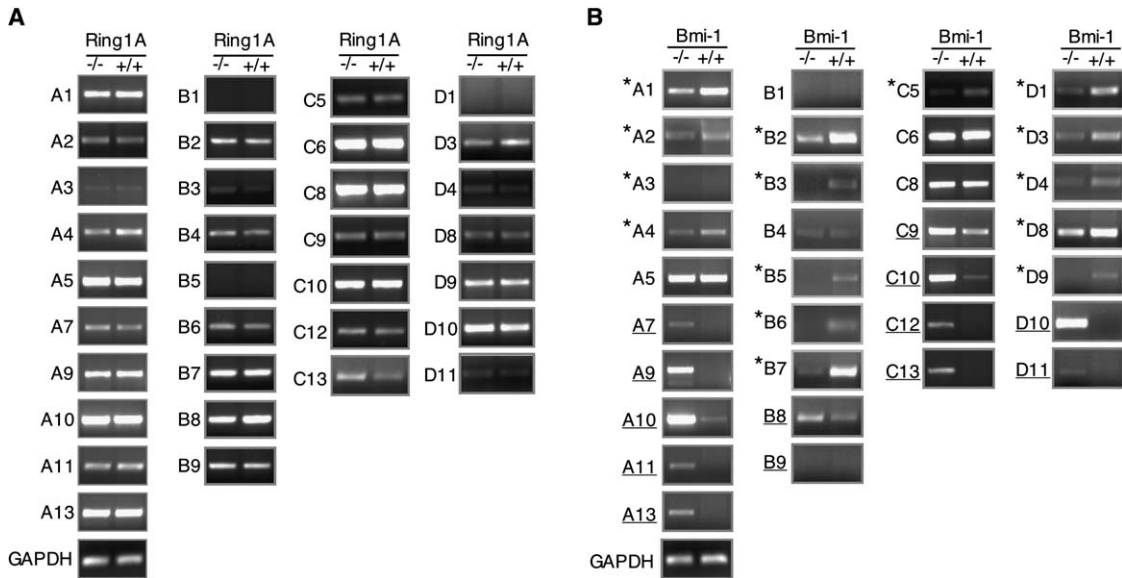
Figure 4. Effects of Ring1A and Bmi-1 Knockout on Hox Gene Expression

Hox gene expression was analyzed by RT-PCR using total RNA isolated from Ring1A null (A) or Bmi-1 null (B) MEFs and compared with that isolated from wt MEFs. GAPDH was a control for equal RNA input in RT-PCR. In the absence of reverse transcriptase (RT), no PCR product was detected in any of the reactions (data not shown), showing that the signals observed were not from amplification of genomic DNA. Upregulated genes in Bmi-1 null cells are underlined, downregulated genes in Bmi-1 null cells are indicated by asterisks.

(Figure 5B, lanes 3 and 5, left panels). This appears to be inconsistent with the finding that Bmi-1 mediates Ring1A and Ring1B interactions (Figure 2B). To determine whether knockout of Bmi-1 affects the integrity of the PRC1 complex, we compared the elution profile of the PRC1 components over a gel-filtration column by using protein extracts derived from wt and Bmi-1$^{-/-}$ MEFs. Results shown in Figure 6A indicate that the Ring1A and Ring1B elution profile is not altered by Bmi-1 knockout, suggesting that the integrity of the PRC1 complex is not affected by loss of Bmi-1. One possible explanation is that Mel-18, a protein that is 70% identical to Bmi-1, may replace Bmi-1 to maintain complex integrity. To address this possibility directly, Sf9 cells were coinfected with baculoviruses that express Ring1A, Ring1B, Pc3, and Flag-Mel-18. After affinity purification using Flag-antibody-coated beads, the bound proteins were eluted and separated on a gel-filtration column. Results shown in Figure 6B indicate that Mel-18 copurified with the other components as a protein complex.

Having demonstrated that Mel-18 can replace Bmi-1 in forming the PRC1 complex, we asked whether the Mel-18-containing complex is functionally similar to the Bmi-1 containing complex with regard to H2A ubiquitylation. After quantifying the two protein complexes through Western analysis of the Ring1B component, their E3 ligase activities were directly compared. Results shown in Figure 6C indicate that the Bmi-1 containing complex is significantly more active than the Mel-18 containing complex (compare lanes 4 and 5). To further evaluate the capability of Bmi-1 and Mel-18 to stimulate Ring1B E3 ligase activity, we purified Ring1B alone and Ring1B in complex with Bmi-1 or Mel-18 from infected insect cells and compared their E3 ligase activity directly. Results shown in Figure 6D demonstrate that

Bmi-1 greatly stimulated the Ring1B E3 ligase activity, whereas Mel-18 does not have a detectable effect (compare lanes 2 and 6). These results collectively support that Mel-18 can be incorporated into the PRC1 complex like Bmi-1, but it has little effect on the E3 ligase activity of Ring1B. Our results support a nonredundant function for Bmi-1 and Mel-18 and are consistent with the fact that Bmi-1 and Mel-18 knockout mice exhibited different phenotypes (Akasaka et al., 1996; van der Lugt et al., 1994).

SUZ12 Knockdown Affects Chromatin Binding by the PRC1 and H2A Ubiquitylation

Our previous studies in *Drosophila* have suggested a hierarchical PcG protein recruitment pathway in Ubx gene silencing (Wang et al., 2004b). Data presented in Figure 5 are consistent with the notion that EZH2-mediated H3-K27 methylation precedes H2A ubiquitylation. To analyze the relationship between H3-K27 methylation and H2A-K119 ubiquitylation further, we asked whether impaired H3-K27 methylation affects PRC1 recruitment and H2A ubiquitylation. We previously demonstrated that SUZ12 knockdown resulted in reduced H3-K27 methylation and HoxA9 upregulation (Cao and Zhang, 2004). Analysis of the same SUZ12 knockdown cell line indicated that HOXC13 is also upregulated when compared with the mock knockdown (Figure 7A). Thus, we analyzed binding of SUZ12 and two different components of the PRC1 complex: Bmi-1 and Ring1A. In addition, H3-K27 methylation and H2A ubiquitylation in the promoter and intron of the corresponding regions analyzed in Figure 5 were also analyzed. Results shown in Figure 7B indicated enrichment of SUZ12, BMI-1, RING1, and the corresponding histone modifications in the promoter region (region A), but not in the intron (region B). However, knockdown of SUZ12 not only
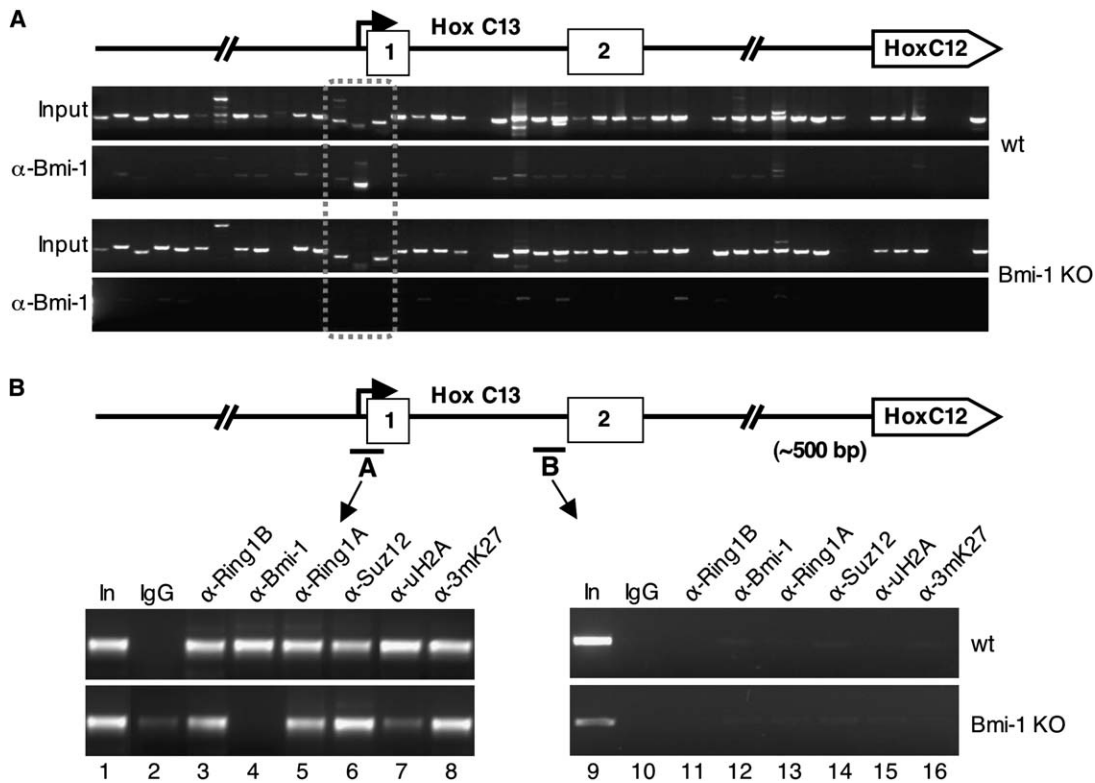
Figure 5. Derepression of HoxC13 in Bmi-1 Knockout MEFs Correlates with a Decreased Level of Promoter H2A Ubiquitylation

(A) ChIP analysis across the HoxC13 gene with an antibody against Bmi-1 in wt (top two panels) and Bmi-1 null (bottom two panels) MEFs. The diagram on top of the panel represents the entire HoxC13 gene where the two exons are indicated by two boxes.

(B) ChIP analysis of the HoxC13 promoter (region A) and a downstream region (region B) of the HoxC13 gene as indicated in the diagram. Each region covers about 500 bp. Antibodies against Ring1B, Ring1A, Bmi-1, SUZ12, ubiquitylated H2A, trimethyl-H3-K27, and an IgG control were used in the ChIP assays with wt (top panels) and Bmi-1 null (bottom panels) MEF cells. ChIP results were revealed by EtBr staining of agarose gels containing PCR amplified ChIP DNA.

affected H3-K27 methylation (lane 6) but also affected BMI-1 and RING1 binding and, consequently, H2A ubiquitylation (lanes 4, 5, and 7). These data are consistent with the notion that H3-K27 methylation provides a binding site for Pc (Fischle et al., 2003; Min et al., 2003) and thus contributes to PRC1 recruitment and H2A ubiquitylation. Collectively, the above results indicate that the sequential PcG protein recruitment model, previously demonstrated in *Drosophila* (Wang et al., 2004b), is also conserved in mammals.

Discussion

Bmi-1 and Ring1A Contribute to H2A Ubiquitylation and Hox Gene Silencing

By reconstitution of the PRC1 complex and subcomplexes, we demonstrate that the H2A ubiquitin E3 ligase activity of Ring1B can be stimulated by incorporation of Bmi-1 and Ring1A (Figure 3). Importantly, knockout of Bmi-1 and Ring1A greatly reduced the H2A ubiquitylation level (Figure 3), supporting an in vivo function for Bmi-1 and Ring1A in H2A ubiquitylation. Given that our previous studies have already demonstrated that recombinant Bmi-1 or Ring1A is not an active ubiquitin E3 ligase for H2A (Wang et al., 2004a), the contribution of Bmi-1 and Ring1A to H2A ubiquitylation is likely achieved through modulating the catalytic activity of

Ring1B. This possibility is supported by a cocrystal structure study revealing a specific interaction of Bmi-1 with the active site of Ring1B (Z. Li, R.C., M. Wang, M.P. Myers, Y.Z., and R.-M. Xu, unpublished data).

One important question regarding the role of H2A ubiquitylation is whether it contributes to PcG silencing. Consistent with a role for H2A ubiquitylation in Ubx gene silencing, we previously demonstrated that a loss-of-function dRing mutant, R65C, identified in a genetic screen (Fritsch et al., 2003), also lacks H2A E3 ligase activity (Wang et al., 2004a). The role of H2A ubiquitylation in Hox gene silencing is conserved in mammals, as knockout of Ring1A, particularly Bmi-1, resulted in derepression of many Hox genes, including HoxC13 (Figure 4). Importantly, we provide evidence that HoxC13 is a direct target of the PRC1 ubiquitin E3 ligase (Figure 5), as Ring1B, Bmi-1, Suz12, and other PcG proteins directly bind to the promoter of HoxC13 to mediate H3-K27 methylation and H2A-K119 ubiquitylation (Figure 5). Knockout of Bmi-1 resulted in a decrease in H2A ubiquitylation concomitant with derepression of HoxC13, supporting a role of H2A ubiquitylation in Hox gene silencing. Our data thus demonstrate a role of H2A ubiquitylation in Hox gene silencing in mammalian cells.

Bmi-1 has been shown to have diverse biological functions and has been linked to cancer (Jacobs et al., 1999b) and stem cell self-renewal (Lessard and
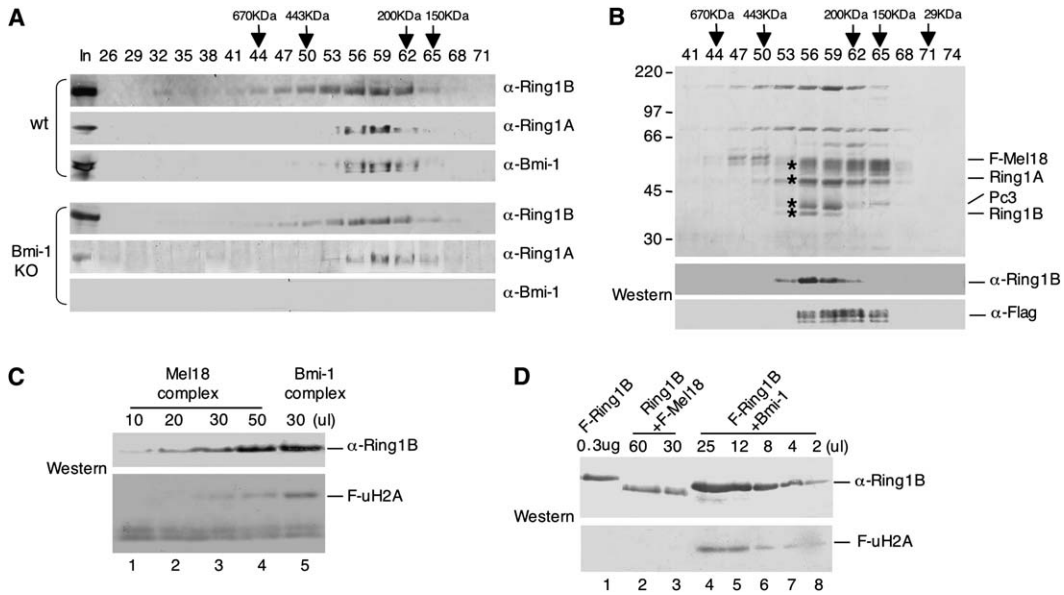
Figure 6. Replacement of Bmi-1 with Mel-18 Maintains the Complex Integrity, but Not Its E3 Ligase Activity

(A) Ring1A and Ring1B have similar elution profiles on a Superose-6 column with protein extracts prepared from wt or Bmi-1 null MEFs. The elution profile of the protein markers is indicated at the top of the panel.

(B) Silver staining of an SDS-gel containing the reconstituted Mel-18 containing complex. Mel-18 copurifies with the other components as a stable protein complex.

(C) Comparison of the E3 ligase activity of the four-component complexes reconstituted with Ring1A, Ring1B, Pc3 plus Bmi-1 or Mel-18. Western blot (top) serves to quantify the complex amounts.

(D) Comparison of the E3 ligase activity of Ring1B/Bmi1-1, and Ring1B/Mel-18 with Ring1B alone. Western blot (top) serves to quantify the protein amounts.

Sauvageau, 2003; Molofsky et al., 2003; Park et al., 2003). In addition to regulating Hox gene expression, Bmi-1 is known to regulate Ink4a and ARF (Jacobs et al., 1999b; Molofsky et al., 2005), two important cell cycle regulators. It is tempting to speculate that the role of Bmi-1 in cancer and stem cell self-renewal is linked to its function in H2A ubiquitylation. Further characterization of this property of Bmi-1 and identification of a mutant that is defective in stimulating the Ring1B E3 ligase activity will allow this issue to be investigated.

Interplay between H3-K27 Methylation and H2A-K119 Ubiquitylation

Studies in budding yeast have revealed that H3-K4 and -K79 methylation depends on H2B ubiquitylation (Briggs et al., 2002; Dover et al., 2002; Ng et al., 2002; Sun and Allis, 2002). The fact that the EZH2 and the PRC1 PcG protein complexes, which respectively possess H3-K27 methyltransferase activity and H2A-K119 ubiquitin E3 ligase activity (Ringrose and Paro, 2004; Zhang et al., 2004), are both required for Hox gene silencing
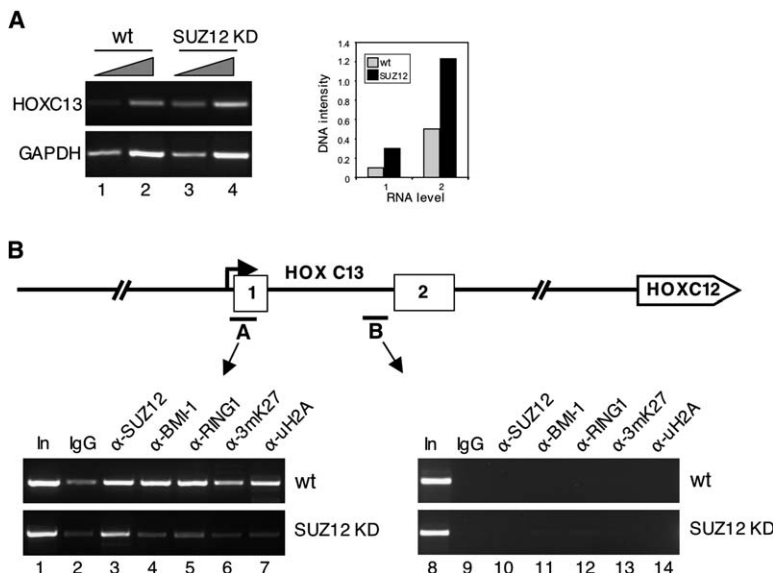
Figure 7. SUZ12 Knockdown Affects PRC1 Recruitment and H2A Ubiquitylation Concomitant with HoxC13 Derepression

(A) Semiquantitative RT-PCR analysis of HOXC13 expression in mock and SUZ12 knockdown cells. GAPDH was used as a control for equal RNA amounts. A quantification of the results is shown in the right panel.

(B) ChIP analysis of promoter (region A) and a downstream region (region B) analogous to the regions analyzed in Figure 5. ChIP was performed with mock knockdown (top panels) and SUZ12 knockdown cells (bottom panels) with the antibodies indicated.

prompted us to ask whether the two histone modifying enzymatic activities are interdependent. Unlike the case of H2B ubiquitylation, which is upstream of the histone H3 methylation, our data are most consistent with H2A ubiquitylation being downstream of H3-K27 methylation. First, knockout of Bmi-1, although clearly affecting H2A ubiquitylation, does not seem to affect recruitment of the Ezh2 complex or H3-K27 methylation at the HoxC13 promoter (Figure 5). However, knockdown of Suz12 not only affected H3-K27 methylation but also affected recruitment of the PRC1 complex and H2A ubiquitylation (Figure 7). These data support a hierarchical recruitment model previously proposed for silencing of the homeotic genes by the *Drosophila* PcG proteins (Wang et al., 2004b).

Based on the hierarchical recruitment model and the fact that H2A ubiquitylation plays an important role in PcG silencing, it will be important to understand how H2A ubiquitylation might lead to gene silencing. Analogous to the role of methyl-K27 serving as a binding site for the recruitment of PRC1, it is possible that H2A-K119 ubiquitylation might serve as a binding site for a protein factor or protein complex that in turn blocks recruitment of the basal transcriptional machinery, including the RNA polymerase, to promoters. Alternatively, H2A ubiquitylation could directly block recruitment of the basal transcriptional machinery. It is worth noting that although the emphasis of our work has been on covalent modifications of histones, in vitro studies have indicated that PcG proteins, particularly PSC and dRing, the *Drosophila* Bmi-1 and Ring1B homologs, can represses chromatin remodeling and transcription in the absence of histone modifications (Lavigne et al., 2004), raising the possibility that multiple mechanisms might be used in PcG silencing.

Experimental Procedures

Purification of Recombinant PRC1 Complex and Subcomplexes

Ring1B cDNA was PCR amplified from I.M.A.G.E cDNA clone (4021046) and inserted into BamHI and HindIII sites of a pFASTBAC (GIBCO) vector with or without an N-terminal Flag tag. Pc3 cDNA was amplified from I.M.A.G.E cDNA clone (4456896) and inserted into HindIII and NcoI sites of pFASTBAC (GIBCO) without Flag. Ph2 cDNA was amplified from I.M.A.G.E cDNA clone (6410302) and inserted into BamHI and HindIII sites of pFASTBAC (GIBCO) without Flag. All the sequences have been verified by DNA sequencing. Baculovirus expression vectors for Bmi-1, Mel-18, and Ring1A were provided by Dr. Kingston and were previously described (Lavigne et al., 2004). All the baculoviruses were generated and amplified per the manufacturer's protocol. To purify the recombinant PRC1 complex and subcomplexes, different baculoviruses were used to coinfect SF9 cells. After 2 days of infection, cells were collected and resuspended in F lysis buffer (20 mM Tris [pH 7.9], 500 mM NaCl, 4 mM MgCl$_2$, 0.4 mM EDTA, 2 mM DTT, 20% glycerol, and 0.1% NP40) with proteinase inhibitors. Then, cells were homogenized with pestle A three times (ten strokes each) in a period of 30 min. The supernatant was recovered by centrifuging at 11,000 rpm for 10 min. The supernatant was adjusted to 300 mM NaCl by adding dilution buffer (20 mM Tris [pH 7.9] and 10% glycerol), then incubated with the M2 α-Flag agarose (Sigma) equilibrated with F lysis buffer for 4 hr at 4°C. After washing with F washing buffer (20 mM Tris [pH 7.9], 150 mM NaCl, 2 mM MgCl$_2$, 0.2 mM EDTA, 1 mM DTT, 15% glycerol, and 0.01% NP40) until no protein came out, bound proteins were eluted with 0.2 mg/ml Flag peptide (DYKDDDDK) in F washing buffer for 20 min each time at room temperature. The eluted complexes were further purified through a gel-filtration Sephadex 200 or Superose 6 column.

Ubiquitin E3 Ligase Assay, Endogenous ubH2A Analysis, and Antibodies

Oligonucleosomes (5 μg) were incubated with 30 μl protein fractions or recombinant proteins in a 40 μl reaction containing 50 mM Tris-HCl (pH 7.9), 5 mM MgCl$_2$, 2 mM NaF, 0.6 mM DTT, 2 mM ATP, 10 μM Okada acid, 0.1 μg ubiquitin activating enzyme E1 (Calbiochem), 0.6 μg ubiquitin conjugating enzyme Ubc5c, and 1 μg Flag-ubiquitin (Sigma). After incubation at 37°C for 1 hr, reaction was terminated by the addition of SDS-PAGE loading buffer. The proteins were resolved in 8%−15% SDS-PAGE and blotted with anti-Flag antibody. To evaluate H2A ubiquitylation in vivo, cells were collected from a 100 mm plate, washed with cold PBS twice, and then suspended in 200 μl PBS with 0.2 N HCl. The suspension was kept on ice for 30 min and centrifuged for 10 min at 13,000 rpm. The extracted supernatant was mixed with 200 μl 25% TCA and kept on ice for another 10 min. The precipitated proteins were collected by centrifugation and washed by acetone. After air dry, proteins were dissolved in 1 × SDS loading buffer and analyzed by Western blotting. Antibodies against uH2A, tubulin, and Bmi-1 were purchased from Upstate and Sigma. Antibodies against Ring1A, Ring1B, SUZ12, and tri-methyl-H3 K27 have been previously described (Cao and Zhang, 2004; Wang et al., 2004a). Antibodies against H3 were kindly provided by Dr. Verrault.

Plasmids and GST Pull-Down Assay

Full-length cDNAs for Ring1B, Ring1A, Bmi-1, Pc3, and Ph2 were inserted into pCITE vector for in vitro translation by using the rabbit reticulocyte lysate kit according to the manufacturer's instructions (Promega). Full-length cDNAs for Ring1B, Ring1A, Bmi-1, Pc3, and Ph2 were also cloned into pGEX-KG vector for the production of GST fusion proteins. About 3 μg of GST or GST fusion proteins were bound to 10 μl of glutathione-immobilized agarose beads (Sigma) and incubated with in vitro-translated products in 500 μl buffer A (50 mM Tris-HCl [pH 7.9], 0.5 mM EDTA, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, and 10% glycerol) containing 150 mM KCl and 0.05% NP-40. After incubation at 4°C for 2 hr, the beads were washed three times with buffer A containing 300 mM KCl and 0.05% NP-40 and then washed once in buffer A containing 50 mM KCl before being subjected to SDS-PAGE and autoradiogram.

Cell Lines and RT-PCR Analysis of Hox Gene Expression

Ring1A$^{-/-}$ and Ring1A$^{+/+}$ MEFs were provided by Dr. Vidal and were previously described (del Mar Lorente et al., 2000). Immortalized Bmi-1$^{-/-}$ and Bmi-1$^{+/+}$ MEFs were provided by Dr. Lohuizen. Total RNAs were isolated from both −/− and +/+ MEFs by using RNeasy (Qiagen) and treated with RNase-free DNase I (Promega). For each sample, 1 μg RNA was used for a 20 μl reverse transcription reaction using ImProm-II (Promega). For Hox gene expression analysis, 1 μl cDNA was used as template for PCR. For control, 1/20 μl of cDNA was used for GAPDH amplification. The primer sequences for all the Hox genes and GAPDH are available in Table S1 available in the Supplemental Data with this article online.

ChIP Assay

For ChIP assays, ∼5 × 10^6 HeLa cells or 2 × 10^6 MEF cells in 150 mm dishes were treated with DMEM containing 1% formaldehyde for 10 min at RT. The crosslinking was stopped by the addition of 125 mM glycine and incubation for 10 min. After washing twice with PBS, the cells were resuspended in 300 μl of cell lysis buffer (10 mM HEPES [pH 7.9], 0.5% NP-40, 1.5 mM MgCl$_2$, 10 mM KCl, and 0.5 mM DTT) by pipetting and kept on ice for 10 min. After centrifugation at 4000 rpm for 5 min, the cell pellets were resuspended in nuclear lysis buffer (20 mM HEPES [pH 7.9], 25% glycerol, 0.5% NP-40, 0.42 M NaCl, 1.5 mM MgCl$_2$, and 0.2 mM EDTA) containing protease inhibitors to extract nuclear proteins at 4°C for 20 min. Then, the chromatin was sonicated into fragments with an average length of 0.5–3 kb. After centrifugation at 13,000 rpm for 10 min, the supernatants were diluted in equal volume of dilution buffer containing 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 7.9), 50 mM NaCl, and protease inhibitors. The extracts were precleaned by incubating with 60 μl protein A-Sepharose beads for at least 1 hr. ChIP assays were then performed with indicated antibodies as previously described (Cao and Zhang, 2004). ChIP DNA was detected by standard

PCR using Platinum Taq polymerase (Invitrogen). The primer pairs covering the mouse HoxC13 genome locus are designed by Array Designer from Premierbiosoft and available in Table S2. The following primer pairs are for the two regions of the human HOXC13: region A, 5′-TGCAGCGGAGCGAGCCCC-3′ and 5′-TCAACAGGGATGAGC GCGTCGTG-3′; region B, 5′-GGATTCCGGTCTAGGGAGTC-3′ and 5′-CAGCCAATACAGGGTAGGTGA-3′.

Supplemental Data

Supplemental Data include two tables and can be found with this article online at http://www.molecule.org/cgi/content/full/20/6/845/DC1/.

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