Identification and Characterization of Bmi-1-responding Element within the Human p16 Promoter*§

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Bmi-1, the first functionally identified polycomb gene family member, plays critical roles in cell cycle regulation, cell immortalization, and cell senescence. Bmi-1 is involved in the development and progression of carcinomas and is a potent target for cancer therapy. One important pathway regulated by Bmi-1 is that involving two cyclin-dependent kinase inhibitors, p16\(^{ink4a}\) and p19\(^{arf}\), as Bmi-1 represses the INK4a locus on which they are encoded. A close correlation between the up-regulation of Bmi-1 and down-regulation of p16 has been demonstrated in various tumors; however, how Bmi-1 regulates p16 expression is not clear. In this study, we revealed that Bmi-1 regulates the expression of p16 by binding directly to the Bmi-1-responding element (BRE) within the p16 promoter. The BRE resided at bp −821 to −732 upstream of the p16 ATG codon. BRE alone was sufficient to allow Bmi-1-mediated regulation of the CMV promoter. Bmi-1 typically functions by forming a complex with Ring2; however, regulation of p16 was independent of Ring2. Chromatin immunoprecipitation sequencing of Bmi-1-precipitated chromatin DNA revealed that 1536 genes were targeted by Bmi-1, including genes involved in tissue-specific differentiation, cell cycle, and apoptosis. By analyzing the binding sequences of these genes, we found two highly conserved Bmi-1-binding motifs, which were required for Bmi-1-mediated p16 promoter regulation. Taken together, our results revealed the molecular mechanism of Bmi-1-mediated regulation of the p16 gene, thus providing further insights into the functions of Bmi-1 as well as a sensitive high-throughput platform with which to screen Bmi-1-targeted small molecules for cancer therapy.

Bmi-1, the first identified polycomb gene family member, plays important roles in cell cycle regulation, cell immortalization, and cell senescence (1, 2). Bmi-1 was first isolated as an oncogene that cooperated with c-Myc in the generation of lymphomas in mice (3). Previous reports have shown that Bmi-1 is associated with the initiation and progression of various types of tumor-initiating cells and plays important roles in the development and progression of carcinomas (4–8). Various types of human cancers display a similar pattern in the overexpression of Bmi-1, such as mantle cell lymphoma, non-small cell lung cancer, B-cell non-Hodgkin’s lymphoma, breast cancer, colorectal cancer, prostate cancer, and nasopharyngeal carcinoma (9–13). A high expression of Bmi-1 positively correlated with poor prognosis (11); and overexpression of Bmi-1 induced malignant transformation both in vitro and in vivo (14). Increasing evidence in the literature supports the clinical significance of Bmi-1 in the development of carcinomas; thus, Bmi-1 can be used as a valuable marker for the assessment and prognosis of different cancers (7, 11).

Consistent with its roles in the development of carcinoma, numerous studies have demonstrated that Bmi-1 plays vital roles in the self-renewal and differentiation of normal and cancer stem cells through multiple pathways. Bmi-1-deficient mice died by the time they reached early adulthood and displayed growth retardation, signs of hematopoietic failure, and neurological abnormalities (15). Accordingly, Bmi-1 was required for the postnatal maintenance of hematopoietic stem cells (HSCs) and neural stem cells (16–18). The proliferation of leukemic stem cells (LSCs) in a mouse model of AML was also promoted by Bmi-1 (16). Bmi-1-expressing LSCs were able to induce leukemia when transplanted into irradiated mice, whereas Bmi-1\(^{−/−}\) LSCs had only limited proliferative potential and were unable to induce disease (16). As an important regulator in tumorigenesis and proliferation of normal and cancer stem cells, Bmi-1 is a potent target candidate in cancer stem cells for cancer therapy. However, a sensitive high-throughput system with which to monitor Bmi-1 activity has not yet been established.

One important pathway in which Bmi-1 acts to promote the overall growth of mice and cellular proliferation includes Cdkn2a; Bmi-1 represses the expression of Cdkn2a, which encodes two cyclin-dependent kinase inhibitors, p16\(^{ink4a}\) (p16) and p19\(^{arf}\) (p19), also known as p14 in humans (9–11,19,20). p16 and p19 are transcribed from the same gene, INK4a, which
is tightly associated with the regulation of the cell cycle, senescence, and apoptosis (21, 22). p16 inactivates Cdk, leading to repression of the retinoblastoma (Rb) gene and subsequent cell cycle arrest in the G1/S phase (23, 24). p19 stabilizes p53 by antagonizing MDM2 and activating p53-dependent transcription and then arrests the cell cycle in G1 and G2/M phases, which in turn results in apoptosis (25, 26). Down-regulation of p16 and p19 by Bmi-1 facilitates cells to bypass senescence and become immortalized (19). Increasing evidence indicates that Bmi-1 might act on cancer cells in part by blocking p16-mediated pathways. Frequent mutations and deletions of the p16 protein were often seen in human cancer cell lines and malignant tumors (12). Tumors with moderate or strong Bmi-1 expression were more likely to have low levels of p16 (12). A close correlation between the up-regulation of Bmi-1 and down-regulation of p16 has been demonstrated in lung cancer and neuroblastoma tumors (9); however, the precise mechanism by which Bmi-1 regulates p16 expression remains unclear.

In this study, using a luciferase reporter system, we found that Bmi-1 regulated the expression of p16 by binding directly with the Bmi-1-responding element (BRE) within the p16 promoter. The BRE localized to base pairs (bp) −821 to −732 upstream of the p16 ATG codon, which was sufficient to enable Bmi-1-mediated regulation of the CMV promoter. Although Bmi-1 normally forms a complex with Ring2, in the case of p16 regulation, Bmi-1 is Ring2-independent. Chromatin immunoprecipitation sequencing of Bmi-1-precipitated chromatin DNA revealed that many genes were targeted by Bmi-1, including genes involved in tissue-specific differentiation, cell cycle and apoptosis. By analyzing the binding sequences of these genes, we found two highly conserved Bmi-1-binding motifs, which were required for Bmi-1-mediated regulation of the p16 promoter. Taken together, our results revealed the molecular mechanism by which Bmi-1 regulates the p16 gene, thus providing new insights into the function of Bmi-1 as well as a sensitive high-throughput platform with which to screen Bmi-1-targeted agents for cancer therapy.

**EXPERIMENTAL PROCEDURES**

**Modification of Vector and p16 Promoter Constructs**—The promoter-less Luciferase reporter vector, N3-MCS-Luc, was constructed on the basis of pEGFP-N3 (Clontech). The CMV promoter and the EGFP region of the pEGFP-N3 were mutated and removed, and the original MCS fragment was substituted by a newly synthesized fragment: GCTAGCGGATCCGAAT-

**P16 Promoter Assays**—P16 promoter luciferase assays were performed with the luciferase assay system (Promega). Thirty-six hours after transfection, HeLa cell and MCF7 cells were extracted with the use of a lysis buffer, a 30-μl aliquot was used for luminescence measurements with 50 μl of substrate. These experiments were carried out in triplicate, and all experiments were performed twice for confirmation.

**Western Blotting**—After being resolved on 6–15% SDS-PAGE gels, the protein samples were transferred onto nitrocellulose membrane in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) for 20 min at 30 V. The NC membrane was then blocked in TTBS (20 mM Tris-HCl pH 7.4, 500 mM NaCl, and 0.3% Tween-20) containing 5% nonfat milk for 1 h under room temperature and blotted for 1 h with antibody at 37 °C: Bmi-1 (Santa Cruz Biotechnology 1:5000), Ring2 (Upstate Signaling 1:2000), and tubulin (Upstate Signaling 1:2000).
The Activity of the p16 Promoter (p1214) Is Regulated by Bmi-1—To investigate how Bmi-1 regulates the expression of the p16 gene, we cloned the p16 promoter, a ~1.2-kb genomic sequence upstream of the p16 ATG initiation codon (31), designated p1214, into a promoterless luciferase reporter vector. Because p16 is expressed highly in HeLa cells, but is not expressed in MCF7 cells (Fig. 1, A and B), we use these two cell lines as the positive and negative controls with which to examine the activity of p1214. The results showed that p1214 displayed greater activity in HeLa rather than MCF7 cells (Fig. 1C), consistent with the expression patterns of the endogenous p16 gene in these cells. We then examined whether the activity of p1214 could be regulated by Bmi-1. For overexpression of Bmi-1, HeLa cells were transfected with pcDNA3.1-Bmi-1; for knockdown of Bmi-1, MCF7 cells were infected with pLL3.7-Bmi-1-Si-expressing virus (supplemental Fig. S1). The results showed that p1214 activity decreased about 30% upon Bmi-1 overexpression and increased about 30% when Bmi-1 was knocked down, and overexpression or knockdown of Bmi-1 had no such effect on CMV promoter activity (Fig. 1, D and E). The above results indicated that the activity of the p16 promoter, p1214, could be regulated by the Bmi-1 protein and that p1214 contains the BRE; thus, p1214 can be used to study the mechanism by which Bmi-1 regulates p16 expression.

Bmi-1-responding Element Is Localized to bp ~821 to ~732 of the p16 Promoter—To define the BRE sequence required for Bmi-1-mediated inhibition of p1214, a series of truncated p16 promoters was constructed (Fig. 2A) and then co-transfected with pcDNA3.1-Bmi-1 into HeLa cells or with pLL3.7-Bmi-1-Si into MCF7 cells. The results showed that overexpression and knockdown of Bmi-1 both greatly affected the activities of p1214, p1036, and p860, but had little effect on the activities of

**RESULTS**

- **ChIP and ChIP Sequencing (ChIP-seq) —** The ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) was used according to the supplier’s instructions. Approximately 1 × 10^6 HeLa or MCF7 cells were used per assay. Briefly, cells were cross-linked by adding formaldehyde (270 μl of 37% formaldehyde/10 ml) and incubate at 37 °C for 10 min. Then, chromatin was sonicated to an average size of about 300 bp and immunoprecipitated with antibody: Bmi-1 (Santa Cruz Biotechnology), Ring2 (Upstate Signaling), and actin (Upstate Signaling). Histone-DNA cross-links were reversed by heating at 65 °C. DNA was recovered by phenol/chloroform extraction and ethanol precipitation and then used for Q-PCR.

- **For ChIP sequencing, 1–10 ng of ChIP DNA were end-repaired and 5′-phosphorylated using ENDIt DNA End-Repair kit (Epicenter). A single adenine was added to the 3′-end by Klenow (3′-5′ exo), and double-stranded Illumina Adapters were ligated to the ends of the ChIP fragments. Adapter-ligated ChIP DNA fragments between 275 and 700 bp were gel-purified and subjected to 18 cycles of PCR. The prepared DNA library was used directly for cluster generation and sequencing analysis using the Illumina Genome Analyzer (Illumina, San Diego, CA) according to the manufacturer’s instructions. The image files generated by the sequencer were then processed to produce digital-quality data. After masking of adaptor sequences and removal of contaminated reads, clean reads were processed for computational analysis.

- **Quantitative Real-time PCR —** DNA was generated from Bmi-1 immunoprecipitation, and quantitative real-time PCR was performed using SYBR Premix Ex Taq (Takara) according to the provided manual (primer in supplemental Table S8). ABI PRISM 7300 system was used under normal amplification conditions for 40 cycles. Assays were performed in triplicate.

- **In Silico Prediction of Motif —** According to the results of ChIP-Seq, we collected those sequences both at the vicinity of peak summit position (100 bp) and in the 2000-bp upstream region of any gene transcription start site. 84 sequences were chosen and subjected to MEME software with the following parameters: motif width, 8, distribution of motifs, oops, minimum number of sites for each motif, 30. After MEME estimation, those potential motifs were manually curated, and some less conserved and simple tandem repeated motifs were discarded. Then the candidates were tried mapping to the promoter of p16.
Bmi-1-responding Element within the Human p16 Promoter

p650 and the shorter promoters (Fig. 2, B and C), indicating that the −860 to −650 bp region (BRE-containing region, designated BREr) of the p16 promoter contains a BRE that is required for Bmi-1-mediated regulation. For further confirmation, we inserted this region upstream of the CMV promoter, which is not regulated by Bmi-1, to examine whether the activ-
results showed that the p16 promoter BRER was sufficient for Bmi-1-mediated regulation.

To identify the Bmi-1-responding element more precisely, the BRER was divided into two fragments, F1 and F2, and each was inserted upstream of the CMV promoter (Fig. 2F). The results showed that the activity of F1-CMV, but not F2-CMV, was regulated by Bmi-1, indicating that the Bmi-1-responding element resides in the F1 fragment (Fig. 2, G and H). The F1 fragment was then subdivided into three smaller fragments, A, B, and C. Fragments A, B, or C or fragment combinations AB, AC, or BC was each inserted upstream of the CMV promoter (Fig. 2F), and the Bmi-1-responding activities of these six constructs were measured. The results showed that only fragment BC (bp −821 to −732) allowed CMV to be regulated by Bmi-1, and the regulation level was almost the same as that of the F1-CMV and the BRER-CMV (Fig. 2, G and H). The above results indicated that bps −821 to −732 of the p16 promoter contained the Bmi-1 responding element, which is necessary and sufficient for Bmi-1-mediated regulation of p16.

Bmi-1 Binds Specifically to the BRE of the p16 Promoter in Vivo through Its HT and RF Domains—

We next explored whether Bmi-1 regulated p16 expression by binding specifically to the BRE region of the p16 promoter. For ChIP analysis, five sets of primers were designed, and each pair amplified a ~200-bp amplicon covering −1214 bp to −250 bp of the genomic p16 promoter region. The amplicons were designated F1, F2, BRER, F3, and F4 (Fig. 3A). Bmi-1-overexpressing HeLa cells were used for ChIP assays with anti-Bmi-1 antibody, and
**Bmi-1-responding Element within the Human p16 Promoter**

FIGURE 4. **Ring2 is not dependent on Bmi-1 for regulation of p16.** A, HeLa cells were transfected separately with Bmi-1 wt, Bmi-1 ΔHT, or Bmi-1 ΔRF. ChIPs were then performed using anti-Bmi-1 antibody. The precipitated complex was analyzed by Western blot using anti-Bmi-1 or anti-Ring2 antibodies. B–D, MCF7 cells or Bmi-1 overexpressed HeLa cells were collected for ChIP using anti-Bmi-1 or anti-Ring2 antibody, and anti-actin antibody was used as a control. The precipitated chromatin was analyzed by PCR with p16 and Hox-B5 promoter-specific primers for gel visualization (B), and by real-time PCR using p16 promoter-specific primers (C and D). Results showed that Ring2 was not able to bind with the BRE of the p16 promoter. E and F, p1214-Luc or BRER-CMV-Luc were transfected into HeLa cells together with an overexpression vector containing Bmi-1, Ring2, or Bmi-1 plus Ring2 separately (E) or transfected into MCF7 cells that had been infected with Bmi-1ΔSi- or different Ring2ΔSi-containing viruses (F). Luciferase activities were then measured 48 h after transfection. Results showed that neither overexpression nor knockdown of Ring2 affected the activity of p1214 or BRER-CMV.

To examine the role of these two domains in the regulation of p16, we constructed two Bmi-1 deletion mutants lacking either the RF domain (ΔRF) or the HTHT domain (ΔHT). When over-expressed in HeLa cells, these two mutants had little effect on the activity of the p16 promoter, including p1214 and BREr-CMV (Fig. 3, C and D). The inability of these two mutants to inhibit p16 promoter activity might be due to a loss of their ability to bind the BRE region of the p16 promoter. To test this possibility, ChIP assays using anti-Bmi-1 antibody were performed in HeLa cells over-expressing the ΔRF or the ΔHT mutant. The results showed that little amplification of the p16 promoter was observed in ΔRF or ΔHT mutant-precipitated DNA (Fig. 3, E and F). These results indicate that the RF and HT domains are both necessary for Bmi-1 to bind the p16 promoter BRE and to regulate p16 expression.

Ring2 Is Not Required for Bmi-1-mediated Regulation of p16—Ring2 is another essential protein in the polycomb repression complex 1 (PRC1) and the main co-binding protein of Bmi-1. Bmi-1 represses the expression of numerous genes essential for genomic programming and development in the form of PRC1 (32, 33). We found that Ring2 co-precipitated with wild-type Bmi-1, but not with the ΔRF or ΔHT Bmi-1 mutants (Fig. 4A). Given that these two Bmi-1 mutants could not bind the p16 promoter BRE (Fig. 3), we reasoned that Ring2 may be necessary for Bmi-1 binding to the p16 promoter BRE.

To address this possibility, we first examined whether Ring2 protein is recruited to the p16 promoter region by ChIP. The chromatin of MCF7 cells or Bmi-1-overexpressed HeLa cells was precipitated with antibodies against Bmi-1, Ring2, and actin separately, and the precipitated DNA was analyzed by real-time PCR with p16 promoter region-specific primers. Hox-C5 was set as a positive control (30, 32, 34). The results showed that both Bmi-1 and Ring2 associated with the promoter region of Hox-C5, however, only Bmi-1 was recruited to the BRER of the p16 promoter (Fig. 4, B–D), indicating that Ring2 was not involved in the Bmi-1:p16 promoter complex. For further verification, we overexpressed Bmi-1 or Ring2 separately or in combination in HeLa cells to observe their effects on the activities of p1214 and BRE-CMV. The results showed that
Bmi-1-responding Element within the Human p16 Promoter

Conserved Bmi-1-binding Motif—Bmi-1 plays critical roles not only in tumorigenesis but also in the self-renewal of normal and cancer stem cells (16, 17, 19). To obtain a greater understanding of how Bmi-1 regulates these processes, we performed a ChIP experiment using anti-Bmi-1 antibody and then characterized, by sequencing, the total precipitated DNA population (ChIP-Seq). The reliability of the ChIP-seq data was confirmed by performing quantitative real-time PCR of p16. The results showed that there were 3559 Bmi-1 binding sites (peaks) in the whole genome from chr1 to chr22 as well as X and Y, and the peaks were abundant in chr1, chr2, chr8, and chr20; and chr 17 and chr20 showed the highest peak density (Fig. 5A and supplemental Table S1).

Bmi-1-targeted genes were identified as the gene’s related region, starting from 2 kb upstream of the transcriptional start site (TSS) (up 2k) and ending at the 3′-end of the last known exon, contained the Bmi-1-specific peaks. The global mapping analysis revealed that 1536 genes were targeted by Bmi-1 (Fig. 5B and supplemental Table S1), including a large number of tissue-specific differentiation genes, cell cycle and apoptosis-related genes (Fig. 5C and supplemental Tables S2–S4), which correlated with the proliferation promoting function of Bmi-1. Consistent with the previous report that Bmi-1 was required for HOX gene silencing during development (32), 12 homeobox-containing genes, including HOX-C4 and HOX-C5 that were reported to be regulated by Bmi-1 (32, 35) were found to be targeted by Bmi-1 (supplemental Table S5). These results suggested that Bmi-1 plays an important role in organism development and tumorigenesis and provides clues to guide further investigation into the function of Bmi-1 in these processes.

To investigate the conserved Bmi-1-binding motif, we compared the gene related peak sequences including that of p16, and obtained two motifs that were highly conserved in these peaks, and both motifs, especially motif-2, were GC-rich (Fig. 6A). In the whole Bmi-1-targeted sequences, motif-2 was more targeted genes were classified by the indicated functions: apoptosis, cell cycle, development, HOX-related, and other. There were 55 apoptosis-related genes, 56 cell cycle-related genes, 158 development-related genes, and 12 HOX-related genes, consistent with the roles of Bmi-1 in proliferation, development, and tumorigenesis. All the groups of genes are enriched significantly (p < 0.01).

Genome-wide Mapping of Bmi-1 Targets and Analysis of the Conserved Bmi-1-binding Motif—The results of ChIP-seq are derived from 2 parallel experiments. All antibodies were harvested for ChIP using anti-Bmi-1 antibody. The total population of the precipitated DNA was sequenced by HeLa cells overexpressing Bmi-1. The results of ChIP-seq are shown in Fig. 4E and supplemental Fig. S2. We also reduced the expression levels of Bmi-1 and Ring2 by knockdown (supplemental Fig. S1). Similar to the overexpression results, Ring2 knockdown had no effect; only Bmi-1 knockdown up-regulated the activities of p1214 and BRE-CMV, and the expression of endogenous p16 (Fig. 4F and supplemental Fig. S2). These results showed that although ΔRF or ΔHT Bmi-1 mutants could not bind with the BRE, this appeared to be unrelated to their inability to bind the Ring2 protein. These results also indicated that although Bmi-1 commonly forms a functional complex with Ring2, in the case of p16 repression by Bmi-1, Ring2 was not involved and was therefore not necessary for Bmi-1-mediated regulation of p16.

A

B

C

FIGURE 5. Genomic mapping of the Bmi-1 peaks from ChIP-seq dataset. HeLa cells overexpressing Bmi-1 were harvested for ChIP using anti-Bmi-1 antibody. The total population of the precipitated DNA was sequenced by ChIP-seq. The results of ChIP-seq are derived from 2 parallel experiments. All peaks were mapped to the genome. A, peaks were classified into four groups corresponding to: 1) the intergenic region, 2) the repeats only, 3) both gene-related regions and repeats, and 4) gene-related regions only. The distribution of these four types of peaks in each chromosome is shown in columns (the left y axis), and the average peak number in each chromosome is shown as curves (the right y axis). B, gene-related regions were subclassified into three types: up2k (indicates the region upstream to 2000 bp downstream of the transcription start site), intron or exon. The distribution of these three peak types was analyzed for each chromosome. Genes that contain a peak in the gene-related region were designated Bmi-1-targeted genes. C, all Bmi-1-targeted genes were classified by the indicated functions: apoptosis, cell cycle, development, HOX-related, and other. There were 55 apoptosis-related genes, 56 cell cycle-related genes, 158 development-related genes, and 12 HOX-related genes, consistent with the roles of Bmi-1 in proliferation, development, and tumorigenesis. All the groups of genes are enriched significantly (p < 0.01).
frequent than motif-1, and motif-2 often clustered by at least 2 repeats (Fig. 6B). To examine whether these two motifs are required for Bmi-1 binding, we searched for them in the BRE region of the p16 promoter and found that there was one motif-1 sequence and a motif-2 cluster consisting of three motif-2 sequences (Fig. 6C). Then we constructed a BRE mutant lacking either the motif-1 or the motif-2 cluster to examine their Bmi-1-binding and responding activities.

**Figure 6. Analysis of Bmi-1-binding consensus motif.** A, sequence of conserved consensus motifs-1 and -2. B, percentage of Bmi-1 peaks that contains motif-1, motif-2, motif 1+2, or motif-2 cluster was shown. Results showed that motif-2 was more frequent than motif-1 in the Bmi-1-targeted sequences, and motif-2 often clustered by at least 2 repeats. The numbers of peaks are indicated in parentheses. Motif-2 cluster refers to the cluster that contains at least two motif-2 sequences between which there is no more than 20 nucleotides. C, motif-1 (green) and motif-2 cluster (red) in the BRE of the p16 promoter. Motif-1 or motif-2 cluster was removed from the BRE and then designated M1-BRE or M2-BRE, respectively. D, stable cell lines of MCF7 and HeLa expressing CMV-Luc, BRE-CMV-Luc, M1-BRE-CMV-Luc, or M2-BRE-CMV-Luc were used for ChIP with anti-Bmi-1 antibody. For those HeLa cell lines, pcDNA3-1-Bmi-1 was transfected before ChIP experiment. The precipitated DNA was analyzed by real-time PCR using CMV-specific primer. Results showed that deletion of either the motif-1 or the motif-2 cluster from the BRE significantly reduced their binding activities with Bmi-1.

**H**. Sequences of human BRE and human RD were aligned. Results showed that both sequences were similar in the motif-2 cluster region of BRE. Human RD sequence was derived from Gonzalez et al. (37).
results showed that both mutants displayed lower binding activity with Bmi-1 protein (Fig. 6D), and were unable to respond to Bmi-1 (Fig. 6, E and F), thus indicating that both motifs are necessary for Bmi-1-mediated regulation of p16 expression.

Recently, Agherbi et al. (36) demonstrated that Bmi-1 was recruited to the RD (Regulatory Domain) region of Ink4a/Arf through CDC6 to regulate this locus. By comparing the sequences of RD and BRer, we found intriguingly that they are similar in the motif-2 cluster region of BRer (Fig. 6G). In addition, we searched motif-1 and -2 in the previously published database of anti-Ring2 ChIP-seq in human ES (34) to explore the relationship of these two motifs with the PRC1 complex. We found that as well as in the Bmi-1-targeted region, motif-2 appeared more frequently than motif-1 in the PRC1-binding region (data not shown). We randomly took out 18 genes, where the promoter region displays different binding activity with PRC1 (34), for comparison. Results showed that except in the promoter region of Hox-B6 and CHRD, the number of motif-2 and clustered motif-2 highly correlated with the binding activity to PRC1 (Fig. 6H), and most PRC1 tight-binding promoter regions contained at least two large motif-2 clusters, such as Pou3f3, Hox-B9, Hox-C9, Hox-C11, RFX4, and Bmi-1 itself (supplemental Table S7). Therefore, these results suggested that motif-2 cluster might be one characteristic regulating element for Bmi-1 and PRC1.

DISCUSSION

Bmi-1 promotes cell proliferation through suppression of the p16/Rb and/or p19/MDM2/p53 tumor suppressor pathways (9–11, 19, 20). Because of the close correlation between Bmi-1 and p16 in various tumors (9, 11, 12, 20), it is important to understand the molecular mechanism by which Bmi-1 regulates p16 expression. In this study, we found that Bmi-1 bound directly to the BRer of the p16 promoter to repress its expression. The BRer alone was sufficient to allow Bmi-1-mediated regulation of CMV. By comparing all of the Bmi-1-targeted sequences in the whole genome, we found two highly conserved motifs existed in most of the Bmi-1-binding gene regions including the BRer of the p16 promoter. Deletion of either motif from the BRer of the p16 promoter abrogated its ability to be regulated by Bmi-1.

Previous studies showed that the Ink4a/Arf locus could be regulated by CDC6 through binding with its RD region (37). Recently, Bmi-1 was demonstrated to be recruited to the RD region through CDC6 (36). The RD region resides in a ~35 kbp upstream translation start site of p16 in human genome (37), while the BRer locates at about 700-bp upstream that of p16. Intriguingly, the alignment of BRE and human RD regions exhibits an aligned G-rich area, where the human RD region is similar to the BRE in the motif-2 cluster. Given that Bmi-1 is recruited to the RD region by CDC6 (36), whether the motif-2 region of RD will strengthen the association of Bmi-1-CDC6 to RD region for Ink4a/Arf regulation needs further study. Further work is also needed to determine whether and how BRE and RD regions co-operate in Bmi-1-mediated regulation of the Ink4a/Arf locus.

Besides the BRE region of p16, motif-2 is often clustered in many other Bmi-1-targeted sequences. In total, about 20% Bmi-1-targeted sequences contain the motif-2 cluster, and the percentage of motif-2 cluster-containing peaks was more in the upstream 2k region, in which cis- or trans-elements often reside, rather than in other regions (Fig. 6B). Additionally, we found that the motif-2 cluster also resided in many PRC1-associated gene promoter regions. In our tested 18 gene promoters (although there were exceptions such as the promoter of Hox-B5 and CHRD) the numbers of motif-2 and clustered motif-2 highly correlated with the promoter binding activities to PRC1. These findings suggested that the motif-2 cluster might be one characteristic regulating element for Bmi-1 and PRC1, and the numbers of motif-2 could be used as a parameter to predict Bmi-1- and PRC1-regulated genes.

Bmi-1 is a core component of PRC1, which contains at least 10 subunits including Ring2, another core component of PRC1 (38). To our surprise, Ring2 was not necessary for Bmi-1 to regulate p16. Neither overexpression nor knockdown of Ring2 had an effect on Bmi-1-mediated regulation of p16. In addition, Ring2 was not involved in the Bmi-1-p16 promoter complex. Therefore, although deletion of the Ring or HT domain abrogated the ability of Bmi-1 to regulate p16, it did not seem as if this was due to the inability of these two mutants to bind Ring2. Interestingly, by comparing the previous anti-Ring2 ChIP-seq or ChIP-Chip data base, we found that Ring2 was recruited to the p16 promoter in human ES cells but not in mouse ES cells (34, 39). Together with our results, it implied that the recruitment of Ring2 to p16 promoter differs in different cell types. However, in our tested cells, Ring2 was not involved in Bmi-1-mediated p16 regulation, and Bmi-1 seems more likely to act in a Ring2-less PRC1 complex.

Bmi-1 plays important roles in cell cycle regulation, cell immortalization, and cell senescence (1, 2, 19). Using ChIP-seq and global mapping analysis, we revealed that many genes in apoptosis and cell cycle regulation were targeted by Bmi-1, consistent with the proliferation-promoting function of Bmi-1 and confirming that Bmi-1 acts partly by preventing apoptosis and cell cycle arrest (40). Bmi-1 is essential for organism development (15). We found that many tissue-specific differentiation genes were targeted by Bmi-1, especially genes involved in central nervous system development; this also correlates with evidence that Bmi-1 plays an important role in the proliferation and self-renewal of neural stem cells (17, 40–42). Previous reports indicated that the proliferation and differentiation of stem cells might be related to the regulation of HOX genes, which are crucial for cell fate determination and proliferation and for the developmental regulation of an organism (43, 44). Here, we found 12 HOX-related genes that were directly targeted by Bmi-1, which correlates with previous data showing that the transcriptional repression and activation of HOX genes could be regulated by the Bmi-1-polycomb group (32, 35). Among the 3559 Bmi-1-targeted peaks throughout the whole genome, 1536 were gene-related; the remainders were all intergenic sequences, thus raising questions of the role of these sequences and the effect incurred upon Bmi-1 binding. Future studies will be required to investigate these questions further. Importantly, our results pro-
vided insightful clues that will further our understanding of Bmi-1 functions.

Targeting cancer stem cells (CSCs) represents a new strategy for the cure of tumors (45–47). CSCs represent a small population of tumor cells that are capable of causing constant expansion of existing tumors or the formation of new tumors in the body, and have been characterized in both liquid tumors and solid tumors (45). Interestingly, CSCs display increased resistance to chemotherapy and apoptosis induction relative to other tumor cells (48–51), and often survive after traditional therapies and regenerate the tumor. Bmi-1 was demonstrated to be an important regulator in tumorigenesis and proliferation of normal and cancer stem cells (16–18).

We identified a highly conserved motif 

\[ \text{Bmi-1-responding Element} \]

within the human p16 promoter. Taken together, our results revealed the molecular mechanism of Bmi-1-mediated regulation of the p16 gene, thus providing important insights into the function of Bmi-1 as well as a sensitive high-throughput platform with which to screen Bmi-1-targeted agents for cancer therapy.

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