Bmi1 cooperates with Dnmt1 associated protein 1 in gene silencing

Masamitsu Negishi^{1,3}, Atsunori Saraya¹, Satoru Miyagi¹, Kenji Nagao⁴, Yoshimasa Inagaki⁵, Mitsuo Nishikawa⁵, Shoji Tajima⁶, Haruhiko Koseki⁷, Hiroshi Tsuda³, Yoshinari Takasaki³, Hiromitsu Nakauchi², Atsushi Iwama¹

¹Department of Cellular and Molecular Medicine, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan. ²Laboratory of Stem Cell Therapy, Center for Experimental Medicine, Institute of Medical Sciences, University of Tokyo, Tokyo 108-8679, Japan. ³Department of Internal Medicine and Rheumatology, Juntendo University, Tokyo, 113-8421, Japan. ⁴Pharmaceutical Frontier Laboratory and ⁵Pharmaceutical Research Laboratories, Kirin Brewery Co. Ltd., Takasaki, Gunma 370-1295, Japan. ⁶Institute for Protein Research, Osaka University, Suita, Osaka 565-0871, Japan. ⁷RIKEN Research Center for Allergy and Immunology, Laboratory for Developmental Genetics, Yokohama 230-0045, Japan.

Correspondence to: Atsushi Iwama, Department of Cellular and Molecular Medicine, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba, 260-8670 Japan. Tel. 81-43-226-2187; Fax. 81-43-226-2191

E-Mail: aiwama@faculty.chiba-u.jp

Abstract

Polycomb group (PcG) proteins are involved in gene silencing through chromatin modifications. Among polycomb repressive complexes (PRC), PRC1 exhibits H2A-K119 ubiquitin E3 ligase activity. However, the molecular mechanisms underlying PRC1-mediated gene silencing remain largely obscure. In this study, we found that Bmi1 directly interacts with Dnmt1 associated protein 1 (Dmap1), which has been characterized to associate with the maintenance DNA methyltransferase, Dnmt1. Bmi1 was demonstrated to form a ternary complex with Dmap1 and Dnmt1 with Dmap1 in the central position. Chromatin immunoprecipitations confirmed the ternary complex formation within the context of the PRC1 at the Bmi1 target loci. Loss of Dmap1 binding to the Bmi1 target loci was tightly associated with derepressed gene expression in *Bmi1*^{-/-} cells. *Dmap1* knockdown exhibited the same impact as *Bmi1* knockout did on the expression of Bmi1 targets, including *Hox* genes. Collectively, our findings suggest that Bmi1 incorporates Dmap1 in polycomb gene silencing.

Key words: Bmi1 / Dmap1 / Dnmt1 / polycomb gene / gene silencing

Introduction

Cell-type specific gene expression profiles are stabilized by changes in chromatin structure and DNA methylation patterns. Cellular memory of chromatin modifications can be faithfully maintained through subsequent cell divisions by the counteractions of transcriptional activators of the trithorax group (TrxG) and repressors of the polycomb group (PcG) (1). PcG proteins form multiprotein complexes that play an important role in the maintenance of transcriptional repression of target genes. So far, four major PcG complexes have been identified and well characterized. PRC1 includes Bmi1/Pgcf4, Rnf110/Mel18, Phc1/Mph1/Rae28, Cbx2/M33, Scmh1, Ring1a and Rind1b, and PRC2/3 includes Eed, Enx1/Ezh2 and Suz12 (1, 2). A transformation-specific PRC4 complex includes Ezh2, Eed isoform 2, Suz12 and the NAD⁺-dependent histone deacetylase SirT1 (3). The PRC1 and PRC2/3/4 complexes possess H2A-K119 ubiquitin E3 ligase activity (4, 5) and H3-K27 methyltransferase activity (1), respectively. Both histone modifications contribute to PcG gene silencing. Although no physical associations have been demonstrated between the two complexes, H3-K27 methylation serves as a binding site for the recruitment of the PRC1 complex. Thus, the two PcG complexes could function in a cooperative manner to maintain gene silencing.

PRC1 has been implicated in stem cell self-renewal (6, 7), a process by which epigenetic cellular memory is precisely inherited to daughter cells through cell division. Among PRC1 components, we have demonstrated that Bmi1 plays a central role in the inheritance of stemness of hematopoietic stem cells in both loss-of-function and gain-of-function analyses (8, 9). Bmi1 in the PRC1 complex contributes to the H2A-K119 ubiquitin E3 ligase activity (5). However, its interacting molecules other than core PRC1 components remain to be determined.

In this study, we screened novel interacting molecules with Bmi1 and found that Bmi1 forms a complex with Dmap1 and the maintenance DNA methyltransferase Dnmt1 at its target loci. We further confirmed the involvement of Dmap1 in gene silencing at the Bmi1 target loci using *Dmap1* knockdown cells. Our finding unveils a novel aspect of gene silencing mediated by Bmi1.

Materials and Methods

Yeast two-hybrid screening

Mouse hematopoietic stem cell (CD34⁻c-Kit⁺Sca-1⁺lineage⁻) library (10) was screened using a MATCHMAKER GAL4 Two-Hybrid System 2 (Invitrogen). The transformants were selected on plates lacking histidine, leucine and tryptophan, and then assayed for β -galactosidase activity.

Immunoprecipitation and GST pull-down

Transfected COS7 cells and NIH3T3 cells were lysed in hypertonic buffer (20mM Na-PO₄ at pH7.0, 250mM NaCl, 0.1% NP-40, 30mM Na₄P₂O₇, 10mM NaF, 5mM EDTA, 1mM DTT and protease inhibitor cocktails (Roche), and then the immunoprecipitation was performed. For the GST pull-down assay, HA-tagged Dmap1 and myc-tagged Dnmt1 were translated *in vitro* using a TNT reticulocyte lysate transcription/translation system (Promega) and mixed with GST-Bmi1 fusion protein. The reaction was carried out at 4 °C for 2 hours and the beads were washed four times with NETN buffer (2mM Tris-HCl, pH8.0, 1% NP-40, 0.1% glycerol, 150 mM NaCl, 1mM EDTA and a proteinase inhibitor cocktail). The antibodies used were a monoclonal anti-Bmi1 (229F6; Upstate Biotechnology), a polyclonal anti-Dmap1 (ab2848; abcam), a polyclonal anti-Dnmt1 (kindly provided by Dr. S. Tajima), a monoclonal anti-Flag (Sigma), a monoclonal anti-Myc (9E10; Santa Cruz) and a polyclonal anti-HA (Y-11; Santa Cruz) antibody.

Glycerol gradient sedimentation analysis

2.5×10^7 NIH3T3 cells were treated on ice for 10 min in lysis buffer [50mM Hepes (pH7.6), 150mM NaCl, 0.1% Triton, 1mM EDTA] and were sonicated sequentially for 10min in 15s burst with 30s cooling in between. The whole cell lysate (0.25 ml) was loaded onto a 10.4 ml of 10-50% glycerol gradients in 50 mM HEPES (pH 7.6), 150 mM NaCl, 1 mM EDTA

and 0.1% Triton X, and spun at 38,000 rpm for 13 hr. The supernatant were separated into 36 fractions (250 μ l each), which were analyzed by Western blot using anti-Bmi1, Dmap1 and Dnmt1 antibodies. To confirm fractionation efficacy, thyroglobulin and aldolase (SIGMA) were fractioned as size markers on a separate gradient that was run in parallel, then subjected to SDS-PAGE followed by coomassie brilliant blue staining analysis.

MS-RDA, RT-PCR analysis and 5-aza-dC treatment

MS-RDA screening was performed as described (11). Genomic DNA isolated from Lineage⁻ immature hematopoietic cells from E14.5 wild-type and *Bmi1*^{-/-} littermate fetal livers were subjected to MS-RDA. Differentially methylated DNA fragments were cloned into a TA vector, sequenced, and their genomic localization determined with a BLAST search. RT-PCR was carried out using normalized cDNA by quantitative PCR using TaqMan rodent GAPDH control reagent (Perkin-Elmer Applied Biosystem). Primer sequences are available on request. MEL cells and MEFs were treated with 5-aza-dC (1 μ M; Sigma) for 4 days. Lentivirus vectors (CS-H1-shRNA-EF-1 α -EGFP) expressing shRNA against murine *Dmap1* (target sequence: GGCACAGATCTCA AGATAC) and Luciferase were prepared and the viruses were produced as previously described (9).

Chromatin immunoprecipitation

Cross-linked chromatin was sonicated to generate fragments with an average length of 300-400bp, and was subjected to immunoprecipitation using the following antibodies; anti-Bmi1 and anti-Dmap1; anti-Dnmt1 (H-300), anti-Mel-18 (H-115), anti-H3-acethyl-K9/K14 (sc-8655-R), and anti-rabbit IgG (Santa Cruz); anti-Ring1b and anti-Scmh1 (kindly provided by Dr. H. Koseki); anti-H3-trimethyl K27 and anti-ubiquityl H2A (E6C5)(Upstate Biotechnology).

Results

Bmi1 forms a ternary complex with Dmap1 and Dnmt1.

To identify the proteins that cooperate with Bmi1 in gene silencing, we performed a yeast two-hybrid screening of a cDNA library prepared from mouse bone marrow hematopoietic stem cells using Bmi1 lacking the C-terminal Proline/Serine-rich domain as a bait. One positive clone encoded full-length Dmap1, a co-repressor that interacts and co-localizes to DNA replication foci with Dnmt1 throughout the S phase (12) (Fig. 1A). Dmap1 interacts with Dnmt1 through its middle portion, which includes the SANT domain. Detailed mapping of interaction regions of the two proteins was carried out using a yeast two-hybrid system. The regions of Bmi1, which include a ring finger domain and helix-turn repeats, respectively, bound to the Dmap1 N-terminus, the region outside the Dnmt1 interaction site of Dmap1 (Fig. 1A). These findings evoked the possibility that Bmi1 forms a ternary complex with Dmap1 and Dnmt1. To address this issue, we performed a co-immunoprecipitation assay using transfected cells with Flag-Bmi1, HA-Dmap1 and Myc-Dnmt1. Both HA-Dmap1 and Myc-Dnmt1 co-immunoprecipitated with Flag-Bmi1 with an anti-Flag antibody, and both Flag-Bmi1 and Myc-Dnmt1 did so with HA-Dmap1 with an anti-HA antibody (Fig. 1B). The direct interaction of Bmi1 with Dmap1 was confirmed in GST pull-down assay; whereas, Bmi1 did not bind to Dnmt1 directly (Fig. 1C). These results indicate that Bmi1 forms a ternary complex with Dmap1 and Dnmt1 by directly interacting with Dmap1.

The ternary complex formation was further determined in Bmi1-transfected NIH3T3 fibroblasts, where endogenous Dmap1 and Dnmt1 co-immunoprecipitated with Bmi1 using an anti-Bmi1 antibody (Fig. 1D). We next fractionated whole cell lysates derived from untransfected NIH3T3 cells on a glycerol gradient. Importantly, Bmi1, Ring1b, Dmap1

and Dnmt1 were co-fractionated in high-molecular-weight fractions (>1 MDa; Fractions 30-33)(Fig. 1E). These findings indicate that the ternary complex is formed within the context of the PRC1.

Bmi1 cooperates with Dmap1 and Dnmt1 in gene silencing.

DNA methylation is an important component of the epigenetic control of gene silencing. DNA methyltransferases, namely Dnmt1, Dnmt3a and Dnmt3b, are required for the establishment and maintenance of genomic DNA methylation patterns. It is widely accepted that Dnmt3a and Dnmt3b function primarily as *de novo* methyltransferases that establish DNA methylation patterns; whereas, Dnmt1 functions primarily as a maintenance methyltransferase that copies the parental-strand methylation pattern onto the daughter strand after each round of DNA replication. A recent study has implicated Ezh2, a component of PRC2/3/4, in the control of DNA methylation (13). Ezh2 serves as a recruitment platform for DNA methyltransferases, including Dnmt1, Dnmt3a and Dnmt3b, thereby connecting chromatin modifications and DNA methylation. The finding that Bmi1 forms a complex with Dmap1 and Dnmt1 evoked the possibility that the ternary complex has a role in the control of local DNA methylation. We therefore took a genome-wide screening of *Bmi1*^{-/-} genomic DNA for differentially methylated genes using methylation-sensitive-representational difference analysis (MS-RDA)(11). This method enables us to identify DNA fragments differentially methylated between the two DNA sources. MS-RDA screening was performed using genomic DNA of fetal liver hematopoietic cells obtained from wild-type and *Bmi1*^{-/-} littermates (E14.5). We isolated a large number of genomic DNA fragments, and 30 of them were randomly sequenced. Among them, we selected 12 fragments located in the putative promoter/enhancer regions for further analysis (Supplementary Table S1). The expression of the 5 genes was depressed in *Bmi1*^{-/-} fetal

liver hematopoietic cells (Fig. 2A). Furthermore, 3 out of 5 genes – C1q and TNF related protein 6 (*C1qTr6*), Ring finger protein 103 (*RNF103*) and WD repeat domain 77 (*WDr77*), were clearly reactivated in an immature hematopoietic cell line, MEL, by treatment with the DNA methylation inhibitor 5-aza-deoxycytidine (5-aza-dC) (Fig. 2B), indicating that these genes are sensitive to DNA methylation in its transcriptional repression. Given that the PcG proteins are involved in gene silencing, the derepressed genes might represent direct Bmi1 targets. We therefore performed detailed analysis on these loci.

We first asked whether Bmi1 is targeted to the Bmi1 target loci and forms a ternary complex with Dmap1 and Dnmt1. Chromatin immunoprecipitations (ChIP) using MEL cells confirmed the ternary complex formation within the context of the PRC1 at these loci (Fig. 2C). Of note was that loss of Bmi1 binding in *Bmi1* knockout fetal liver cells profoundly affected the Dmap1 binding to the same loci and Dnmt1 binding to a lesser extent (Fig. 3). Consistent with an important role for Bmi1 in H2A ubiquitination, the *Bmi1* knockout resulted in a significant decrease in H2A ubiquitination, and in a significant increase in acetylation of H3K9, a hallmark of active chromatin (Fig. 3). In contrast, the tri-methylation status of H3K27 in the absence of Bmi1 varied among the analyzed loci.

To evaluate the effect of the ternary complex on the local CpG methylation status, we next carried out bisulfite genomic sequencing of the MS-RDA fragments at the putative enhancer and promoter regions of *C1qTr6* and *WDr77* genes, respectively (Supplementary Fig. S1). Genomic DNA was prepared from mouse fetal liver Lineage⁻ immature hematopoietic cells from wild-type and *Bmi1*^{-/-} littermates (E14.5). Interestingly, several CpGs located within analyzed region showed differential methylation patterns between wild-type and *Bmi1*^{-/-} fetal liver hematopoietic cells. The *C1qTr6* locus included several hypomethylated CpGs, while the *WDr77* locus (7414543-7414663) included both local hypomethylation and hypermethylation. These differential methylation patterns were

confirmed by repeated experiments using genomic DNA from three different littermates. In contrast, CpGs situated outside the *WDr77* MS-RDA fragment region (7414469-7414543) did not show significant difference in methylation status. These data indicate the involvement of DNA methylation control in gene silencing at the *Bmi1* target loci. However, *Bmi1* deficiency did not substantially alter the quantitative methylation status of both major and minor satellite sequences (Supplementary Fig. S2A).

***Hox* loci are derepressed in *Bmi1*^{-/-} and *Dmap1* knockdown MEFs.**

Finally, we examined the role of the *Bmi1*-*Dmap1* complex in the transcriptional control of *homeobox* (*Hox*) genes. Previous studies in mice indicated that loss of *Bmi1* results in a shift in *Hox* gene expression boundaries and shows posterior transformation (14). We analyzed the expression of *Hox* genes that are reportedly derepressed in expression in *Bmi1*^{-/-} mouse embryonic fibroblasts (MEF)(5). In addition to *Bmi1* knockout, we evaluated the role of *Dmap1* by using the *Dmap1* knockdown technique. MEFs were infected with a lentivirus expressing shRNA against *Dmap1*, which efficiently inhibited *Dmap1* expression (Fig. 4A). Although *Dmap1* has been reported to co-localize to DNA replication foci with *Dnmt1* throughout the S phase (12), *Dmap1* knockdown as well as *Bmi1* knockout did not significantly alter the quantitative methylation status of both major and minor satellite sequences (Supplementary Fig. S2B). The expression of *HoxA7*, *HoxA9* and *HoxA10* appeared to be regulated at least in part by DNA methylation, as their expression was derepressed by 5-aza-dC-treatment. Notably, their expression was derepressed not only by *Bmi1* knockout, but also by *Dmap1* knockdown (Fig. 4B), indicating that *Bmi1* and *Dmap1* cooperatively repress these *Hox* genes. Although *HoxD10* was downregulated by both *Bmi1* knockout and *Dmap1* knockdown (Fig. 4B), this might represent an indirect effect of the *Bmi1*-*Dmap1* complex. On the other hand, *HoxC13* expression was derepressed only

by *Bmi1* knockout, and not by 5-aza-dC-treatment or *Dmap1* knockdown (Fig. 4B). In this locus, H2A ubiquitination could be most critical in gene silencing as reported (5). Importantly, *Dmap1* knockdown in MEFs also resulted in the derepressed expression of *Bmi1* targets we identified in hematopoietic cells (Fig. 4C). In contrast to *Dmap1* knockdown, however, *Bmi1* knockout did not affect the expression of *Rnf103* and *ClqTr6* in MEFs. The different cell type-specificities of *Bmi1* and *Dmap1* in terms of gene silencing could be ascribed to a major role of Rnf110/Mel18, another mammalian homologue of *Drosophila* Psc, in this process in MEFs or are indicative of more general contribution of *Dmap1* to gene silencing.

Discussion

Bmi1 has been characterized as an essential molecule in the maintenance of cellular memory. It contributes to the stable gene silencing at least in part by cooperating with Ring1A and Ring1B in H2A-K119 ubiquitination (5). In the present study, we identified a novel Bmi1 complex operating in gene silencing. Bmi1 recruits Dmap1 and Dnmt1 to its target loci and forms a ternary complex within the context of the PRC1. As demonstrated at the Bmi1 target loci we identified in this study, the loss of Bmi1-Dmap1 complex was tightly associated with gene reactivation in *Bmi1*^{-/-} cells and *Dmap1* knockdown cells, indicating that Bmi1 cooperates with Dmap1 in the maintenance of gene silencing.

The function of Dmap1 has not yet been fully clarified. It reportedly interacts and co-localizes to DNA replication foci with Dnmt1 throughout the S phase, and also interacts with the transcriptional co-repressor TSG101 (12). The Dmap1-Dnmt1 complex interacts with the p33ING1-Sin3-HDAC complex physically through direct interaction between Dmap1 and p33ING1 at pericentric heterochromatin in the late S phase, and both complexes contribute to the maintenance of pericentric heterochromatin structure (15). In this study, we unveiled a novel aspect of Dmap1 function in gene silencing in collaboration with Bmi1. It has recently been shown that the localization of Bmi1 to PcG bodies is disrupted when Dnmt1 is depleted by RNAi (16). This raises the possibility that at certain gene loci, Bmi1 and Dmap1-Dnmt1 mutually reinforce each other's recruitment to specific promoters and cooperate to repress transcription. On the other hand, Dmap1 has also been characterized as one of the component molecules of the NuA4b HAT multisubunit complex (17). The functional impact of Dmap1 in this HAT complex remains to be determined.

At the Bmi1 target loci we analyzed, the knockout of Bmi1 resulted in a substantial decrease in H2A ubiquitination and in a considerable increase in acetylation of H3K9 (Fig.

3A). The former finding is consistent with an important role for Bmi1 in H2A ubiquitination (5). Regarding the latter finding, it is possible that Bmi1 affects HAT-containing complex. Or, given that Dnmt1 directly binds HDACs, and that Dnmt1 is tightly associated with histone deacetylase activity *in vivo* (12, 18), the Bmi1-Dmap1-Dnmt1 complex might also be connected with histone deacetylation activity.

Long-term gene silencing involves maintenance of proper CpG methylation patterns and tethering repressive regulators to methylated CpG dinucleotides, including methyl-CpG binding proteins (MBPs), which contribute to establish and maintain transcriptional repression. It should be noted that most of the Bmi1 targets we analyzed in this study are DNA methylation-sensitive. In addition, we observed altered CpG methylation patterns in *Bmi1*^{-/-} cells, which could lead to disruption in gene silencing. Altered DNA methylation patterns in *Bmi1*^{-/-} cells included both local hypomethylation and hypermethylation. The similar dysregulation of the CpG methylation patterns has been observed at the imprinting gene loci on distal chromosome 7 in embryo deficient for Eed (19), a PRC2 component that makes direct contact with Dnmt1, Dnmt3a and Dnmt3b, like Ezh2 (13). Given that Bmi1 forms a complex with Dnmt1 through Dmap1, it is possible that Bmi1 as well as Eed/Ezh2 controls DNA methyltransferases to precisely propagate DNA methylation patterns of their target loci. Detailed analyses are required to address whether Bmi1 is really involved in the control of local DNA methylation.

CpG gene silencing is a diversified process that involves both DNA methylation and various chromatin modifications. The way to establish gene silencing should be unique to each gene loci. Our findings provide the Bmi1-Dmap1-Dnmt1 complex as one of the core complexes that govern this process and might be relevant to various gene-silencing processes, such as imprinting, X-chromosome inactivation and heterochromatinization. The biological significance of this complex should be tested.

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References

1. A.H. Lund, M. van Lohuizen, M. Polycomb complexes and silencing mechanisms, *Curr. Opin. Cell Biol.* 16 (2004) 239-246.
2. A. Kuzmichev, T. Jenuwein, P. Tempst, D. Reinberg D, Different Ezh2-containing complexes target methylation of histone H1 or nucleosomal histone H3, *Mol. Cell* 14 (2004) 183-193.
3. A. Kuzmichev, R. Margueron, A. Vaquero, T.S. Preissner, M. Scher, A. Kirmizis, X. Ouyang, N. Brockdorff, C. Abate-Shen, P. Farnham, D. Reinberg, Composition and histone substrates of polycomb repressive group complexes change during cellular differentiation, *Proc. Natl. Acad. Sci. U.S.A.* 102 (2005) 1859-1864.
4. H. Wang, L. Wang, H. Erdjument-Bromage, M. Vidal, P. Tempst, R.S. Jones, Y. Zhang, Role of histone H2A ubiquitination in Polycomb silencing, *Nature* 431 (2004) 873-878.
5. R. Cao, Y.I. Tsukada Y. Zhang, Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing, *Mol. Cell* 20 (2004) 845-854.
6. M.E. Valk-Lingbeek, S.W.M. Bruggeman, M. van Lohuizen, Stem cells and cancer: the polycomb connection, *Cell* 118 (2004) 409-418.
7. A. Iwama, H. Oguro, M. Negishi, Y. Kato, H. Nakauchi, Epigenetic regulation of hematopoietic stem cell self-renewal by polycomb group genes, *Int. J. Hematol.* 81 (2005) 294-300.
8. A. Iwama, H. Oguro, M. Negishi, Y. Kato, Y. Morita, H. Tsukui, H. Ema, T. Kamijo, Y. Katoh-Fukui, H. Koseki, M. van Lohuizen, H. Nakauchi, Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product, Bmi-1. *Immunity* 21 (2004) 843-851.

9. H. Oguro, A. Iwama, Y. Morita, T. Kamijo, M. van Lohuizen, H. Nakauchi, Differential impact of *Ink4a* and *Arf* on hematopoietic stem cells and their bone marrow microenvironment in *Bmi1*-deficient mice, *J. Exp. Med.* 203 (2006) 2247-2253.
10. M. Masuhara, K. Nagao, M. Nishikawa, M. Sasaki, A. Yoshimura, M. Osawa, Molecular cloning of murine STAP-1, the stem-cell-specific adaptor protein containing PH and SH2 domains. *Biochem. Biophys. Res. Commun.* 268 (2000) 697-703.
11. T. Ushijima, K. Morimura, Y. Hosoya, H. Okonogi, M. Tatematsu, T. Sugimura, M. Nagao, Establishment of methylation-sensitive-representational difference analysis and isolation of hypo- and hypermethylated genomic fragments in mouse liver tumors, *Proc. Natl. Acad. Sci. U.S.A.* 94 (1997) 2284-2289.
12. M.R. Rountree, K.E. Bachman, S.B. Baylin, DNMT1 binds HDAC2 and a new co-repressor, DMAP1, to form a complex at replication foci, *Nat. Genet.* 25 (2000) 269-277.
13. E. Vire, C. Brenner, R. Deplus, L. Blanchon, M. Fraga, C. Didelot, L. Morey, A. Van Eynde, D. Bernard, J.M. Vanderwinden, M. Bollen, M. Esteller, L. Di Croce, Y. de Launoit, F. Fuks, The polycomb group protein EZH2 directly controls DNA methylation, *Nature* 439 (2006) 871-874.
14. N.M. van der Lugt, M. Alkema, A. Berns, J. Deschamps, The Polycomb-group homolog Bmi-1 is a regulator of murine Hox gene expression, *Mech. Dev.* 58 (1996) 153-164.
15. H. Xin, H. -G. Yoon, P.B. Singh, J. Wong, J. Qin, Components of a pathway maintaining histone modification and heterochromatin protein 1 binding at the pericentric heterochromatin in mammalian cells, *J. Biol. Chem.* 279 (2004) 9539-

9546.

16. I. Hernandez-Munoz, P. Taghavi, C. Kuijl, J. Neefjes, M. van Lohuizen, Association of BMI1 with polycomb bodies is dynamic and requires PRC2/EZH2 and the maintenance DNA methyltransferase DNMT1, *Mol. Cell. Biol.* 25 (2005) 11047-11058.
17. Y. Doyon, W. Selleck, W.S. Lane, S. Tan, J. Cote, Structural and functional conservation of the NuA4 histone acetyltransferase complex from yeast to humans, *Mol. Cell. Biol.* 24 (2004) 1884-1896.
18. F. Fuks, W.A. Burgers, A. Brehm, L. Hughes-Davies, T. Kouzarides, T, DNA methyltransferase Dnmt1 associates with histone deacetylase activity, *Nat. Genet.* 24 (2000) 88-91.
19. J. Mager, N.D. Montgomery, F.P. de Villena, T. Magnuson, Genome imprinting regulated by the mouse Polycomb group protein Eed, *Nat. Genet.* 33 (2003) 502-507.

Figure legends

Figure 1. Bmi1 forms a ternary complex with Dmap1 and Dnmt1.

(A) Mapping of the interaction domains between Bmi1 and Dmap1. Interaction of Bmi1 and Dmap1 was evaluated by the growth on selection plates lacking histidine (HIS-) and by β -galactosidase activity in a yeast two-hybrid system. Rnf, ring finger domain; HT, helix-turn-helix-turn-helix-turn domain; P/S, Proline/Serine-rich domain; SANT, SANT domain. The region of Dmap1 (amino acids 212-422) that interacts with Dnmt1 is indicated by an asterisk. **(B)** The whole-cell extracts from COS-7 cells transfected with Flag-Bmi1, HA-Dmap1 and Myc-Dnmt1 were immunoprecipitated by anti-Flag antibody (left panel) or anti-HA antibody (right panel). Western blot analysis was performed using the indicated antibodies. **(C)** *In vitro* translated HA-Dmap1 or Myc-Dnmt1 was incubated with GST or GST-Bmi1 fusion proteins. **(D)** Endogenous Dmap1 and Dnmt1 were co-immunoprecipitated with Bmi1. The whole-cell extracts from Bmi1-transfected NIH3T3 cells were immunoprecipitated with either control IgG or anti-Bmi1 antibody. Western blot analysis was performed using the indicated antibodies. **(E)** Co-fractionation of Bmi1, Dmap1 and Dnmt1 on a glycerol gradient. Whole cell lysate of NIH3T3 cells was separated on a 10-50% glycerol gradient. Fractions were probed with antibodies against Bmi1, Dmap1, Dnmt1 and Ring1b. Fraction number is indicated on the top of the panel. 'In' represents input. An asterisk indicates non-specific band. Thyroglobulin (660kDa) and aldorase (160kDa) were used as protein markers and were fractionated on a separate gradient that was run in parallel, and fractions containing these proteins are indicated on the top of the panel.

Figure 2. Identification of Bmi1 target loci.

(A) RT-PCR demonstrated the de-repressed expression of genes related to MS-RDA loci in *Bmi1*^{-/-} lineage-marker⁻ immature fetal liver hematopoietic cells (E14.5). *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase. (B) Reactivation of *Bmi1* target genes by DNA demethylation. MEL cells were treated with 5-aza-dC for 96 hours. RT-PCR analysis was performed on MS-RDA-related genes in 'A'. *Hprt*, hypoxanthine-Guanine phosphoribosyl transferase. (C) ChIP assays of the *Bmi1* target loci show the association of *Bmi1*, *Dmap1* and *Dnmt1* at the *ClqTr6* gene locus and the *Rnf103* locus, and (D) the *Bmi1*-*Dmap1*-*Dnmt1* ternary complex formation within the context of the PRC1 at the *WDr77* locus in *Bmi1*-overexpressing MEL cells. The top panels are the schematic representations of each gene locus. Asterisks indicate the genomic fragments identified by MS-RDA screening. The genomic regions analyzed cover about 200 bp and are indicated in numbers.

Figure 3. *Bmi1* recruits *Dmap1* and *Dnmt1* to its target loci.

ChIP assays of the *Bmi1* target loci in *Bmi1*^{-/-} cells demonstrate a significant reduction in *Dmap1* and *Dnmt1* binding concomitant with the decreased H2A ubiquitination and increased H3K9 acetylation. The promoter regions of the indicated gene loci and a distal region of the *WDr77* gene locus were analyzed by ChIP using wild-type and *Bmi1*^{-/-} fetal liver cells (E14.5). The promoter regions detected encompass *WDr77* regions 4 and 5, *ClqTr6* region 3 and *Rnf103* regions 3 and 4 depicted in Fig. 4, and the distal region was designed at 4 kb upstream of the *WDr77* ATG.

Figure 4 *Hox* loci are derepressed in *Bmi1*^{-/-} MEFs and *Dmap1* knockdown MEFs.

(A) Knockdown of the endogenous *Dmap1* in infected MEFs was confirmed by western blotting. Tubulin served as a loading control. The antibodies used are indicated on the

left. **(B, C)** RT-PCR analysis of the *Hox* genes **(B)** and the *Bmi1* target genes **(C)** in *Bmi1*^{-/-} MEFs, MEFs treated with 5-aza-dC for 96 hours, *Dmap1* knockdown MEFs and control MEFs infected with shRNA virus against the *Luciferase* gene.

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Figure 2 Negishi et al

Figure 3 Negishi et al

Figure 4 Negishi et al