

# pRB family proteins are required for H3K27 trimethylation and Polycomb repression complexes binding to and silencing p16<sup>INK4a</sup> tumor suppressor gene

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**Genetic studies have demonstrated that Bmi1 promotes cell proliferation and stem cell self-renewal with a correlative decrease of p16<sup>INK4a</sup> expression. Here, we demonstrate that Polycomb genes *EZH2* and *BMI1* repress *p16* expression in human and mouse primary cells, but not in cells deficient for pRB protein function. The *p16* locus is H3K27-methylated and bound by BMI1, RING2, and SUZ12. Inactivation of pRB family proteins abolishes H3K27 methylation and disrupts BMI1, RING2, and SUZ12 binding to the *p16* locus. These results suggest a model in which pRB proteins recruit PRC2 to trimethylate *p16*, priming the BMI1-containing PRC1 ubiquitin ligase complex to silence *p16*.**

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The mammalian pRB family proteins, pRB, p107, and p130 (also known as pocket proteins), play a key role in controlling the G1-to-S transition of the cell cycle and maintaining differentiated cells in a reversible quiescent or permanent senescent arrest state (Weinberg 1995; Corbinik 2005). The pocket proteins are hypophosphorylated in cells exiting mitosis as well as in quiescent cells, where they bind to and negatively regulate the function of the E2F family transcription factors (Trimarchi and Lees 2002). In cells entering the cell cycle, extracellular mitogens first induce the expression of D-type cyclins, which bind to and activate CDK4 and CDK6, leading to the phosphorylation of pRB family proteins, causing functional inactivation by E2F dissociation, thereby promoting a G1-to-S transition. Inhibition of CDK4 and CDK6 by the INK4 family of CDK inhibitors (p16<sup>INK4a</sup>,

p15<sup>INK4b</sup>, p18<sup>INK4c</sup>, and p19<sup>INK4d</sup>) retains pRB family proteins in their hypophosphorylated, growth-suppressive states and prevents G1-to-S progression. Disruption of the INK4–RB pathway, consisting of INK4–cyclinDs–CDK4/6–RB–E2Fs, deregulates G1-to-S control and represents a common event in the development of most, if not all, types of cancer (Sherr 1996).

Among the major challenges toward a better understanding of G1 control by the INK4–pRB pathway is how different *INK4* genes are regulated, thereby linking G1 control to different cellular pathways. INK4 proteins are relatively stable, and the primary regulation of INK4 is through transcriptional control. The expression of each of the *INK4* genes is distinctly different during development, in different adult tissues, and in response to different cellular conditions (Roussel 1999). There have been only a few reports wherein a transcriptional regulator has been demonstrated to bind to an *INK4* promoter by either gel shift or chromatin immunoprecipitation (ChIP) assay. Identification of factors that directly bind to *INK4* promoters holds the key to linking different cellular pathways to G1 control by the INK4–pRB pathway, but these links remain disproportionately poorly understood in comparison with our knowledge of the function of the INK4–pRB pathway (Pei and Xiong 2005).

To elucidate the molecular mechanisms regulating *p16* expression, we tested whether *p16* gene expression is directly regulated by *BMI1*, an oncogene that encodes a transcriptional repressor of the Polycomb group (PcG) of proteins (van Lohuizen et al. 1991; Lund and van Lohuizen 2004). Deletion of *Bmi1* retards cell proliferation, causes premature senescence in mouse embryonic fibroblasts (MEFs), and reduces the number of hematopoietic stem cells, with an associated up-regulation of *p16* (and to a lesser extent of p19<sup>Arf</sup>). Codeletion of *p16* (or *p16-Arf*) partially rescued the proliferative defects of *Bmi1*-null cells (Jacobs et al. 1999a; Bruggeman et al. 2005; Molofsky et al. 2005), providing genetic evidence supporting a functional interaction between the *Bmi1* and *p16* genes. However, whether BMI1 directly binds to and regulates the transcription of the *p16* gene has not been demonstrated. A notable feature of *p16* is its high level of expression in virally transformed cells (Xiong et al. 1993) and its inverse correlation with pRB function (Tam et al. 1994; Hara et al. 1996), suggesting a negative regulation of *p16* gene expression by pRB (Li et al. 1994). We therefore also examined whether pRB and BMI1 collaboratively repress *p16* expression.

## Results and Discussion

### *pRB* family proteins negatively regulate *p16* gene expression

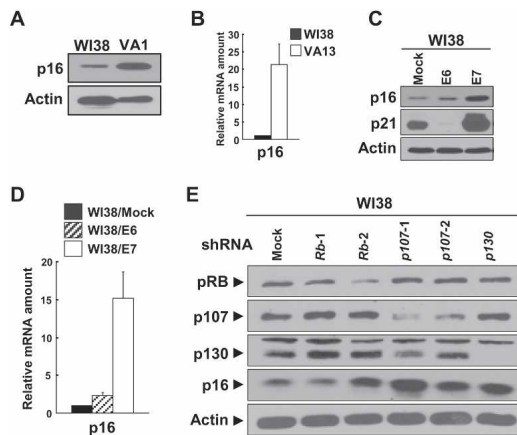
The *p16* protein is expressed at a high level in DNA tumor virus-transformed cells, including VA13 cells, that were transformed from normal human fibroblasts (WI38 cells) by SV40 (Fig. 1A; Xiong et al. 1993). The high level of *p16* protein in VA13 cells was correlated with a high level of *p16* mRNA (Fig. 1B), indicating transcriptional activation of the *p16* gene. We established WI38 stable cell lines expressing either type 16 papilloma viral oncoprotein E6 (inactivating p53) or E7 (inactivating pRB family proteins). To avoid the possible accumulation of

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**Figure 1.** pRB family proteins negatively regulate *p16* gene expression. The levels of p16, p18, and p21 proteins (A,C) and mRNA (B,D) were determined in WI38 and VA13 cells by direct immunoblotting or Q-RT-PCR. Data are expressed relative to the corresponding values for WI38 (B) or WI38/Mock (D) cells, and mean values and standard deviations were calculated from triplicates of a representative experiment. (E) WI38 cells were infected with an empty lentivirus vector (Mock) or lentivirus vectors encoding shRNA silencing individually human *RB*, *p107*, or *p130* genes. The efficiency of silencing and the effect of silencing on p16 expression were determined by direct immunoblotting.

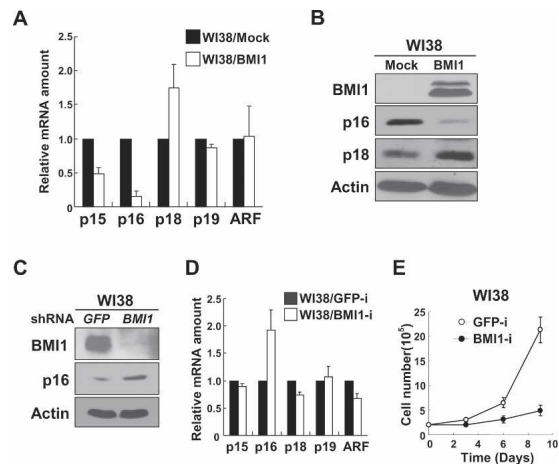
additional genetic changes that might occur during the extended life span of WI38 cells after E6 and/or E7 expression, we used WI38/E6 or WI38/E7 stable lines within 10 passages, well before they reach a senescent stage. Compared with parental WI38 cells, both p16 protein and mRNA were slightly increased in WI38/E6 cells and substantially increased in WI38/E7 cells (Fig. 1C,D), confirming the inverse correlation between *p16* gene expression and pRB function. The pRB family includes three pocket proteins, pRB, p107, and p130, with pRB specifically binding to E2F1/2/3/4 and p107 and p130 to E2F4/5 (Trimarchi and Lees 2002). Previous studies have used either virally transformed cells in which all three *RB* family genes are functionally inactivated or tumor-derived cells that have accumulated additional mutations, making it unclear which pRB family protein(s) may be involved in repressing *p16* expression. To determine this, we infected normal WI38 human primary fibroblasts with lentiviruses encoding short hairpin RNA (shRNA) molecules silencing each individual *RB* family gene and determined the consequences of this knock-down on the level of *p16* expression. Silencing of each gene was verified by direct Western blot and resulted in an increase of *p16* expression (Fig. 1E). These data indicate that all three pRB family proteins are normally involved in repressing *p16* expression.

#### The oncogene *BMI1* represses *p16* expression

To search for the mechanism underlying the repression of *p16* by the pRB family proteins, we examined the role of *BMI1* in *p16* regulation. *BMI1* ectopic expression extends the replicative life span of normal human fibroblasts and is associated with a decrease in *p16* expression (Jacobs et al. 1999a; Itahana et al. 2003). We first examined whether *BMI1* also represses other *INK4* genes. A WI38 stable cell line ectopically expressing *BMI1* was established after retroviral transduction and was used to

examine the expression of all four *INK4* genes. Confirming the negative regulation of *p16* by *BMI1*, quantitative RT-PCR (Q-RT-PCR) analysis showed that the level of *p16* mRNA in WI38/*BMI1* cells was substantially decreased, to <20% of that of cells infected with empty virus (WI38/Mock) (Fig. 2A). Associated with *p16* mRNA decrease, the steady-state level of p16 protein was also decreased substantially in WI38/*BMI1* cells (Fig. 2B). The level of *p15<sup>INK4b</sup>* mRNA decreased by ~50% in WI38/*BMI1* cells, while *p19<sup>INK4d</sup>* and *p14<sup>ARF</sup>* expression was not significantly affected by ectopic *BMI1* expression (Fig. 2A). Unexpectedly, *p18<sup>INK4c</sup>* mRNA was increased in WI38 cells expressing *BMI1*. The increase of *p18<sup>INK4c</sup>* mRNA was associated with an increase in the steady-state level of p18 protein (Fig. 2B). The mechanism underlying the *BMI1*-mediated *p18<sup>INK4c</sup>* increase is not clear at present.

We next examined conversely how loss of *BMI1* function affects *INK4* gene expression. We designed a retroviral vector encoding a shRNA that specifically targets human *BMI1*. Infection of WI38 cells with *BMI1* shRNA retroviruses reduced *BMI1* level to an undetectably low level (Fig. 2C). Associated with *BMI1* decrease is a substantial increase of p16 protein (Fig. 2C). Q-RT-PCR analysis showed that of the four *INK4* and *ARF* genes, only *p16* mRNA was significantly increased in WI38 cells when *BMI1* was silenced (Fig. 2D). The level of *p18<sup>INK4c</sup>* and *p14<sup>ARF</sup>* mRNA was actually decreased slightly by *BMI1* silencing. Associated with p16 in-



**Figure 2.** The oncogene *BMI1* represses *p16* gene expression. (A,B) WI38 cells were infected with empty (Mock) or *BMI1*-expressing retroviruses and selected by puromycin treatment. The levels of individual mRNA (A) and protein (B) levels were determined by Q-RT-PCR and direct immunoblotting, respectively. Q-RT-PCR results are expressed relative to the corresponding values for WI38/Mock cells, and mean values and standard deviations were calculated from triplicates of a representative experiment. (C) WI38 cells were infected with a retrovirus vector encoding shRNA against either *GFP* or *BMI1* and selected by puromycin treatment. The efficiency of *BMI1* silencing and the effect of *BMI1* silencing on p16 expression were determined by direct immunoblotting. (D) The effects of *BMI1* silencing on the expression of the four *INK4* and *ARF* genes were determined by Q-RT-PCR, and results are expressed relative to the corresponding values for WI38/*GFP-i* cells. The mean values and standard deviations were calculated from triplicates of a representative experiment. (E) The growth curves of WI38 cells infected with a retrovirus vector encoding shRNA against either *GFP* or *BMI1*. Viable cells were counted by Trypan Blue staining at indicated days after initial seeding of  $2 \times 10^5$  cells.

crease, BMI1 knockdown resulted in slower cell growth (Fig. 2E). Together, these results demonstrate that of the four *INK4* and *ARF* genes, *p16* seems to be a specific target of *BMI1* function in human primary cells. In *Bmi1*<sup>-/-</sup> MEFs, both *p16* and *Arf* mRNA levels are increased (Jacobs et al. 1999a,b), suggesting that the regulation of the mouse *p16-Arf* locus by *Bmi1* is different from that of the human locus.

#### BMI1-mediated *p16* repression requires the function of pRB proteins

Both BMI1 and pRB family proteins repress *p16* expression, which led us to determine whether they may functionally collaborate. We first infected WI38 primary fibroblasts, VA13, 293 cells that were transformed by adenovirus (inactivating pRB family proteins by E1A), and Saos-2 osteosarcoma cells that contain a mutation in the *RB* gene with retroviruses expressing *BMI1*. In contrast to *p16* repression by BMI1 in WI38 cells, ectopic BMI1 expression had no effect on p16 expression in VA13, 293, and Saos-2 cells despite high levels of p16 expression in these cells (Fig. 3A). Consistent with the notion that BMI1 promotes cell proliferation in part through the repression of *p16*, transduction by the BMI1 retrovirus increased the proliferation rate of WI38 cells but had no effect on the proliferation of VA13 and Saos-2 cells (Fig. 3B).

VA13, 293, and Saos-2 cells have sustained additional genetic changes or functional loss caused by viral transformation in addition to the inactivation of the *RB* gene or pRB family function. To confirm that the function of pRB family proteins is required for *p16* repression by BMI1, we infected WI38/E7 cells with a retrovirus ex-

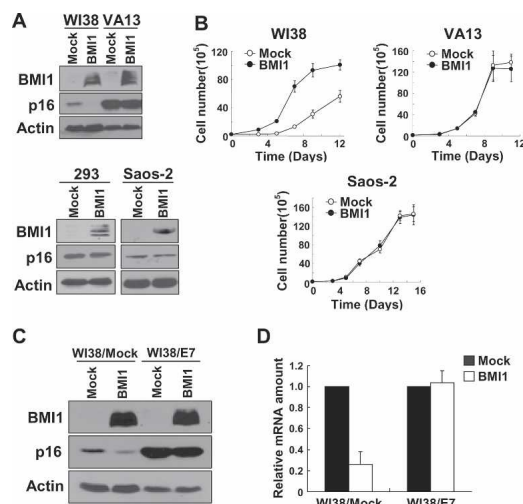
pressing BMI1 and compared the level of p16 with WI38 cells infected with control empty retrovirus. In spite of a high level of *p16* in WI38/E7 cells, ectopic expression of BMI1 had no detectable effect on p16 protein level (Fig. 3C). Confirming this result, Q-RT-PCR showed that ectopic BMI1 expression reduced *p16* mRNA by ~75% in WI38/Mock cells but had very little effect in WI38/E7 cells (<10% repression) (Fig. 3D). SUZ12, BMI1, and RING1 bind to the *HoxC13* locus and repress its expression in HeLa cells deficient for pRB protein function (Cao et al. 2005), and the expression of *HoxD10*, another target gene of *Bmi1*-deficient cells, remains unchanged after E7 expression, indicating that the link between pocket proteins is not broadly involved in the regulation of PcG target genes. Taken together, these data indicate that pRB family proteins are required for BMI1-mediated repression of *p16* expression.

#### BMI1 binds to the *p16* genomic region in an RB-dependent manner

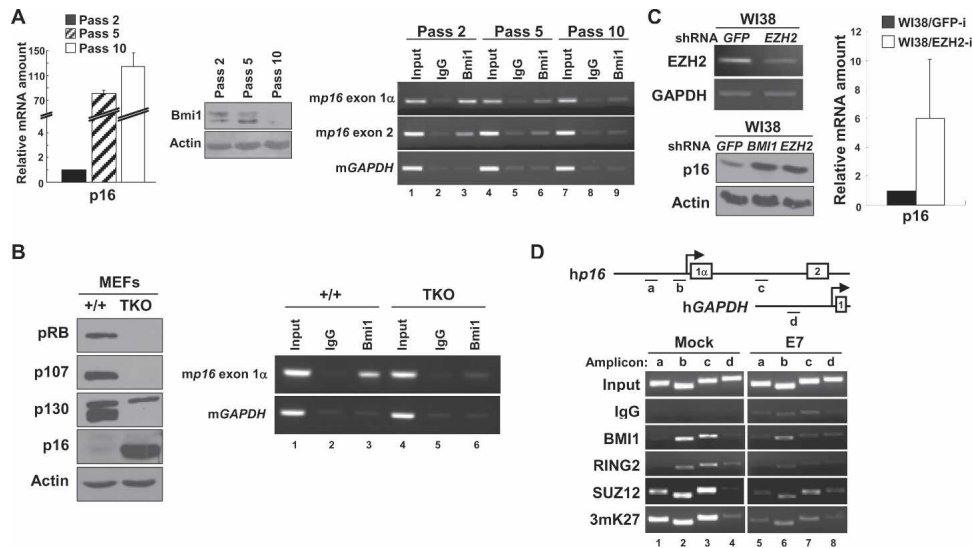
Previous genetic studies in mice have linked the function of *Bmi1* in controlling cell proliferation and stem cell renewal with repression of *p16* (Jacobs et al. 1999a; Smith et al. 2003). Whether *Bmi1* binds directly to and regulates the transcription of the *p16* gene has not been tested yet. To clarify this issue, we carried out ChIP assays to test whether *Bmi1* directly binds to the *p16* gene. Oligonucleotide primers were designed corresponding to a sequence within mouse *p16* exon 1 $\alpha$  and exon 2, where it was recently shown to be bound by the Phc2 protein (Isono et al. 2005), a member of the PRC1L (human Polycomb-repressive complex-1-like) E3 ubiquitin ligase complex, which also includes RING1, RING2, and BMI1 (H. Wang et al. 2004). A ChIP assay demonstrated that *Bmi1* binds directly to *p16* exon 1 $\alpha$  and exon 2 in early-passage young MEF cells (Fig. 4A, right panel, lane 3), providing the first direct evidence that *Bmi1* regulates *p16* expression by directly binding to regulatory sequences of the *p16* gene. The p16 protein level increased during in vitro passage of MEFs, and contributes at least in part to cellular senescence. Concomitant with the increase in the *p16* mRNA level, the *Bmi1* protein level decreased in a passage-dependent manner (Fig. 4A, left panel). Consistent with this observation, the binding of *Bmi1* to the *p16* gene also decreased in a passage-dependent manner (Fig. 4A, right panel, lanes 3,6,9). We noted that the decrease of *Bmi1* binding to p16 preceded the decrease of *Bmi1* level (Fig. 4A, passage 5), suggesting that *Bmi1* level may not be the rate-limiting factor in *p16* repression and another factor(s) may be involved.

To determine how the function of pRB family proteins may affect the regulation of *p16* by *Bmi1*, we carried out ChIP assays in parallel in both wild-type MEFs and MEFs triply deficient for all three pRB proteins (TKO). The expression of pRB, p107, and p130 is readily detected in wild-type MEFs, which express a very low level of p16. Associated with the loss of pRB family proteins, the level of p16 was substantially increased in TKO cells (Fig. 4B, left panel). ChIP assays demonstrated that *Bmi1* binds directly to *p16* in wild-type MEFs, but was not detected in TKO MEFs (Fig. 4B, right panel, lanes 3,6), indicating that the presence of pRB family proteins is required for *Bmi1* to bind with the *p16* gene.

Previous studies on the silencing of *Hox* genes by the PcG proteins suggest a hierarchical recruitment model



**Figure 3.** BMI1-mediated *p16* repression requires the function of pRB proteins. (A) WI38, VA13, 293, and Saos-2 cells were infected with empty or BMI1-expressing retroviruses and selected with puromycin. The levels of individual proteins were determined by Western blotting. (B) The growth curve of WI38, VA13, and Saos-2 cells infected with empty or BMI1-expressing retroviruses were determined by Trypan Blue staining at indicated days after initial seeding of  $2 \times 10^5$  cells (WI38) or  $1 \times 10^5$  cells (VA13 and Saos-2). (C,D) WI38/Mock and WI38/E7 (G418 resistant) were reinfected with empty or BMI1-expressing retroviruses and selected by puromycin treatment. The levels of individual proteins (C) and mRNA (D) were determined by direct immunoblotting and Q-RT-PCR (D).



**Figure 4.** BMI1 directly associates with the *p16* genomic region through pRB protein function. (A) The passage-dependent expression of the *p16* gene in MEFs was determined by Q-RT-PCR, and results are expressed relative to the corresponding values for MEFs (pass 2). (Left panel) The mean values and standard deviations were calculated from triplicates of a representative experiment. (Middle panel) The levels of Bmi1 protein in MEFs were determined by Western blotting. (Right panel) ChIP assays using antibodies against Bmi1 and IgG control in multiply passed MEFs. (B, left panel) The levels of pRB, p107, p130, and p16 proteins in wild-type (WT) and *RB*<sup>-/-</sup>; *p107*<sup>-/-</sup>; *p130*<sup>-/-</sup> (TKO) MEFs were determined by Western blotting. (Right panel) ChIP assays were done using antibodies against Bmi1 and IgG control in wild-type or TKO MEFs. (C) WI38 cells were infected with a retrovirus vector encoding shRNA against either GFP or *EZH2* and selected by puromycin treatment. (Top left panel) The efficiency of *EZH2* silencing was determined by RT-PCR. The effect of *EZH2* silencing on p16 expression was determined by Western blotting (bottom left panel) and by Q-RT-PCR (right panel). The results are expressed relative to the corresponding values for WI38/GFP-i cells. The mean values and standard deviations were calculated from triplicates of a representative experiment. (D) A schematic representation of the human *p16* and *GAPDH* gene loci and amplicons (a, b, c, and d) used for ChIP assays. WI38/Mock and WI38/E7 cells were reinfected with BMI1-expressing retroviruses. Antibodies against BMI1, RING2, SUZ12, and trimethyl-H3K27 and IgG control were used in the ChIP assays. PCR was carried out using primers for each amplicon.

wherein PRC2-mediated H3K27 methylation is required for recruitment of PRC1L, which causes H2A-K119 ubiquitination both in fly and human cells (L. Wang et al. 2004; Cao et al. 2005). Therefore, we examined whether *EZH2*, a catalytic component of PRC2, is also required to repress *p16* expression. Western blotting and Q-RT-PCR analysis showed that both p16 protein and mRNA were significantly increased in WI38 cells when *EZH2* was silenced (Fig. 4C), suggesting that PRC2 is also required for the *p16* repression.

We next examined BMI1 association with the human *p16* gene. For this purpose, we designed a panel of >30 pairs of primers covering a >8-kb region on both sides of human *p16* exon1 $\alpha$  and searched for the BMI1-binding site (data not shown). Positive binding of BMI1 was detected in a sequence within the human *p16* promoter close to the 5' end of exon 1 $\alpha$  and the intron between exon 1 $\alpha$  and exon 2 in WI38 cells (Fig. 4D, lanes 2,3). We also demonstrated that RING2, a component of the hPRC1L complex, and SUZ12, a component of PRC2, also bind to the *p16* gene (Fig. 4D, lanes 2,3), further supporting the notion that PRC2 and hPRC1L directly regulate *p16*.

Given that the *RB* genes are required for BMI1 recruitment (Fig. 4B), we investigated whether they are also required for PRC2 binding to and H3K27 methylation of the *p16* locus. As determined by ChIP assay, the *p16* locus is bound by SUZ12 and is methylated at Lys27 of H3 (Fig. 4D, lanes 1–3). Importantly, inactivation of the pRB family proteins by the expression of E7 completely abolished the SUZ12 binding to and H3K27 methylation of the human *p16* gene and disrupted the binding of

BMI1 and RING2 to the *p16* locus (Fig. 4D, lanes 6,7). H3K4 methylation, on the other hand, is slightly increased in WI38/E7 cells (data not shown), indicating that not all histone methylation marks on the *p16* locus are affected the same way by the inactivation of pocket proteins. These results suggest that pRB proteins function upstream of both the PRC2 and PRC1L complexes.

The results presented here provide the first biochemical evidence supporting a direct regulation of *p16* transcription by the PRC2 histone methyltransferase complex and the BMI1-RING2-containing PRC1 histone ubiquitin ligase complex. We further demonstrated that both H3K27 methylation at and BMI1/RING2 binding to the *p16* locus require the function of the pRB family proteins, linking for the first time H3K27 methylation and the function of BMI1 with the pRB proteins. The detailed biochemical mechanism by which pRB family proteins collaborate with BMI1 to repress *p16* transcription is yet to be determined. In repeated attempts, we have not been able to detect binding of pRB to the *p16* locus. The simplest model suggested by our results is that the pRB family proteins are either involved in regulating the enzymatic activity or the recruitment of PRC2 to the *p16* locus. H3K27 methylation by PRC2 would then facilitate recruitment of the BMI1-containing PRC1L complex to ubiquitinate H2A, leading to *p16* silencing.

Our results also suggest a regulatory loop between p16 and the pocket proteins, with p16 acting as an upstream activator of the pocket proteins and the pocket proteins repressing *p16* transcription as negative feedback. INK4 proteins are intrinsically stable and, once synthesized,

stably bind to and inhibit the activity of CDK4/6 by both interfering with ATP binding and by reducing the cyclin-CDK4/6 surface (Jeffrey et al. 2000). Without a mechanism for repressing *INK4* expression, mitogen-induced cyclin D synthesis would not be able to compete off *INK4* from CDK4/6, and displaced, monomeric cyclin D proteins would be rapidly degraded, leaving a constitutive activation of *RB* function and locking cells in a permanent G1-arrested state. Repression of *p16* expression by pRB family proteins thus also constitutes a feedback loop to set up a balance between *INK4*-mediated inhibition and cyclin D-mediated activation of G1 progression. This function of p16, however, must be repressed in stem cells, which undergo continuous proliferation and self-renewal in vivo. We speculate that one mechanism to achieve this is through expression of BMI1 in the stem cell compartment.

## Materials and methods

### Cell culture, Western analysis, and antibodies

MEFs carrying combined mutations in *RB* family genes, including *RB*<sup>-/-</sup>; *p107*<sup>-/-</sup>; *p130*<sup>-/-</sup> (TKO) MEFs were previously described (Sage et al. 2000), and early-passage WI38 and SV40-transformed VA13 cells were purchased from American Type Culture Collection (ATCC). MEF, WI38, VA13, and 293 cells were cultured in DMEM supplemented with 10% FBS, and Saos-2 cells were cultured in McCoy's 5A supplemented with 15% FBS. Cells were lysed with RIPA buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM DTT, 1 mM PMSF, and a cocktail of protease inhibitors containing 25 mg/L leupeptin, 25 mg/L aprotinin, 150 mg/L benzamide, and 10 mg/L trypsin inhibitor). Antibodies to BMI1 (F6; Upstate Biotechnology), p16 (Ab-4; Neomarkers), human pRB (Ab-1; Neomarkers), mouse pRB (G3-245; BD Biosciences), p107 (C-18; Santa Cruz Biotechnology), p130 (Rb2; BD Biosciences), and actin (C-11; Santa Cruz Biotechnology) were purchased commercially.

### Q-RT-PCR

Total RNA was extracted by RNeasy (Qiagen), and 1 µg was used for cDNA synthesis primed with Oligo(dT)<sub>20</sub> primers (Invitrogen). The cDNA was added to a Q-RT-PCR mixture that contained 1× SYBR Green PCR master mix (Applied Biosystems) and 500 nM gene-specific primers. Assays were performed in triplicate on a 7900 HT sequence detection system (Applied Biosystems). The PCR protocol comprised incubations for 2 min at 50°C and for 10 min 95°C, followed by 40 cycles, each consisting of 15 sec at 95°C and 1 min at 60°C. The expression level of each gene was normalized with glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The specific PCR pairs were as follows: *hGAPDH*, 5'-AGGTGAAGGTCGGAGTCAAC-3' and 5'-AGTTGAGGTCAATGAA GGGG-3'; *hp15*, 5'-GGACTAGTGGAGAAGGTGCG-3' and 5'-GGGC GTGCCCATCATCATG-3'; *hp16*, 5'-CACCGAATAGTTACGGTCC GG-3' and 5'-GCACGGTCCGGTGAGAGTG-3'; *hp18*, 5'-GGGGAC CTAGAGCAACTTAC-3' and 5'-GTAGCAGTCTCCTGGCAATC-3'; *hp19*, 5'-CTCAACCGCTTCGGCAAGAC-3' and 5'-GGACTGGTACC GGAGGTGTC-3'; *hARF*, 5'-GGCCCTCGTGCTGATGCTAC-3' and 5'-TGGAGCAGCAGCTCCGC-3'; *mGAPDH*, 5'-GGTGAAGGTCG GTGTGAACG-3' and 5'-TGTAGACCATGTAGTTGAGG-3'; and *mp16*, 5'-CCGAACCTTTCCGGTCTAC-3' and 5'-GAGAAGGTAGTGGG GTCCTC-3'. Normal RT-PCR was performed to confirm the efficiency of *EZH2* knockdown. The specific PCR pairs for *EZH2* and *hGAPDH* were as follows: *EZH2*, 5'-TGCACATCCTGACTTCTGTG-3' and 5'-AAGGGCATTACCAACTCC-3'; and *hGAPDH*, 5'-GAGTCAACGGA TTTGGTCTG-3' and 5'-GACAAGCTTCCCCTTCTCAG-3'.

### Retroviral production and transduction

WI38 cells were infected with retroviruses expressing E6, E7, or both (kindly provided by Dr. D. Galloway, Fred Hutchinson Cancer Research Center, Seattle, WA), and stable pools were selected in media containing 400 µg/mL G418 for 1 wk. Human *BMI1* cDNA was cloned into a pBabe-puro retrovirus vector. Retroviruses encoding shRNAs silencing *BMI1*,

*EZH2*, and control *GFP* were constructed by ligating respective oligonucleotides (*BMI1*, GTATTGTCCTATTTGTGAT; *EZH2*, AGACTCTG AATGCAAGTTGC; *GFP*, GCTACGTCAGGAGCGCAC) into a pSuper-retro vector. Lentivirus vectors carrying shRNAs silencing human *RB*, *p107*, and *p130* were constructed by ligating oligonucleotides into the pSicoR lentivirus backbone. The sequences of the oligonucleotides are GGAGATGCTTTACTATAAA for *p130*, GCGATTATGTGCCCAAATA and GAAGTTATATTCCCAAATA for *p107*, and GGACATGTGAAC TTATATA and GAACGATTATCCATT CAA for *RB*. Viral supernatants were produced after transfection of 293T cells with 4 µg of pSicoR plasmids and 3 µg of helper plasmids expressing the VSV-G envelope. For retrovirus production, individual expression vectors (4 µg) were cotransfected with pCI-VSV and pCI-PGZ (3 µg each) into 293T cells using the calcium phosphate method. Cells were incubated at 37°C in DMEM supplemented with 10% newborn calf serum for 18 h. The medium was replaced with DMEM supplemented with 2% newborn calf serum and incubated for an additional 24 h at 32°C. Viral supernatant was collected and filtered using a 0.45-µm syringe filter, supplemented with 10% FBS and 10 µg/mL polybrene. Cells were infected with 8 mL of the viral supernatant for 24 h at 37°C, and infection was repeated once to increase infection efficiency. After infection, cells were selected with 1 µg/mL (for WI38) or 2 µg/mL (for VA13, 293, and Saos-2) puromycin for 3 d.

### ChIP assay

WI38 or MEF cells (5 × 10<sup>6</sup>) were treated with 1% formaldehyde for 10 min at room temperature, then 0.125 M glycine was added and incubated for 5 min at room temperature. The cells were lysed with cell lysis buffer on ice (10 mM HEPES at pH 7.9, 0.5% NP-40, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM DTT, and protease inhibitor cocktail). After centrifugation at 4000 rpm for 5 min, the cell pellets were lysed by sonication on ice with nuclear lysis buffer (20 mM HEPES at pH 7.9, 25% glycerol, 0.5% NP-40, 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, and protease inhibitor cocktail). After centrifugation at 13,000 rpm for 10 min in a cold room, the lysates were diluted with an equal volume of dilution buffer (1% Triton X-100, 2 mM EDTA, 50 mM NaCl, 20 mM Tris-HCl at pH 7.9, and protease inhibitor cocktail). Immunoprecipitation was performed with an antibody specific to BMI1 (F6; Upstate Biotechnology), RING2, SUZ12, 3m-H3K27 (Cao et al. 2005), and normal mouse IgG as a control for 6 h or overnight at 4°C. After immunoprecipitation, 20 µL of salmon sperm DNA/protein G agarose (Upstate Biotechnology) were added and incubated for 1 h. Precipitates were sequentially washed with TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl at pH 8.1), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl at pH 8.1), and TSE III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl at pH 8.1), and washed twice with TE buffer before being eluted with 1% SDS and 0.1 M NaHCO<sub>3</sub>. The eluates were incubated for at least 6 h at 65°C to reverse the formaldehyde cross-linking. DNA fragments were purified by using the PCR Purification Kit (Qiagen). PCR was performed using Platinum Taq polymerase (Invitrogen) and the following pairs of primers: *mp16* exon 1a, 5'-CGAACTCGAGGAGAGCATC-3' and 5'-ACACTCCTT GCCTACCTGAA-3'; *mp16* exon 2, 5'-TCACGTAGCAGCTCTTCTG C-3' and 5'-CAGCGGAACGCAAATATCGC-3'; *mGAPDH*, 5'-CCC ACTTGCCCTCTGTATTGG-3' and 5'-CTGTGGGGAGTCTTTTCA G-3'; *hp16* (a), 5'-GGCATCAGCAAAGTCTGAGC-3' and 5'-CTGGG AGACAAGAGCGAAAC-3'; *hp16* (b), 5'-AGGGGAAGGAGAGAGCA GTC-3' and 5'-GGGTGTTTGGTGTATAGGG-3'; *hp16* (c), 5'-GGCT AGTTTTATGACCAAGACG-3' and 5'-GAGGATGGTGGTGTAAA GAGG-3'; and *hGAPDH* (d), 5'-GGTAGGGAGTTCGAGACCAG-3' and 5'-TCAACGCAGTTCAGTTAGGC-3'.

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