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p21/Cyclin E pathway modulates anticlastogenic function of Bmi-1 in cancer cells

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Apart from regulating stem cell self-renewal, embryonic development and proliferation, Bmi-1 has been recently reported to be critical in the maintenance of genome integrity. In searching for novel mechanisms underlying the anticlastogenic function of Bmi-1, we observed, for the first time, that Bmi-1 positively regulates p21 expression. We extended the finding that Bmi-1 deficiency induced chromosome breaks in multiple cancer cell models. Interestingly, we further demonstrated that knockdown of cyclin E or ectopic overexpression of p21 rescued Bmi-1 deficiency-induced chromosome breaks. We therefore conclude that p21/cyclin E pathway is crucial in modulating the anticlastogenic function of Bmi-1. As it is well established that the overexpression of cyclin E potentially induces genome instability and p21 suppresses the function of cyclin E, the novel and important implication from our findings is that Bmi-1 plays an important role in limiting genomic instability in cyclin E-overexpressing cancer cells by positive regulation of p21.

Introduction

Bmi-1, a polycomb gene family member, is known to play key roles in stem cell self-renewal, embryonic development and proliferation.^{1–5} The expression of Bmi-1, strong in undifferentiated cells, declines during differentiation³ and diminishes in cells undergoing senescence.⁵ In addition, the overexpression of Bmi-1 is commonly detected in many types of human cancer.^{3,6–10} Knockdown of Bmi-1 inhibited the proliferation and development of various types of human

Key words: Bmi-1, p21, cyclin E, genomic instability, replicative stress

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tumors.^{11–13} In combination with other genetic alterations, Bmi-1 functions as a proto-oncogene, as exemplified by the pioneer finding that Bmi-1 collaborates with Myc in tumorigenesis in mice.¹⁴ One of the major functions of Bmi-1 is to suppress or prevent the overexpression of p16^{ink4a} and p19^{arf}, thus enabling cells to overcome cellular senescence.^{1,5} Yet, accumulating evidence has revealed other functions of Bmi-1. In mouse models of leukemia and glioma lacking expression of p16^{ink4a} and p19^{arf}, the expression of Bmi-1 is still required for malignancy development,^{15,16} indicating the ink4a/arf-independent function(s) of Bmi-1. In human mammary epithelial cells, Bmi-1 has been reported to induce telomerase activity to contribute to cellular immortalization.¹⁷ Bmi-1 has been found to regulate mitochondrial function; and Bmi-1 deficiency leads to increased intracellular accumulation of reactive oxygen species.^{18,19} Moreover, several recent studies have shown that Bmi-1 has anticlastogenic functions through promoting repair of DNA damage. In particular, Bmi-1 is rapidly recruited to DNA damage sites.^{20–22} Depletion of Bmi-1 resulted in radio-sensitivity of mammalian cells.^{20–22} In Bmi-1-knockout mouse cells, S- and G2-phase recovery was impaired.²² Moreover, Bmi-1 is involved in DNA homologous recombination repair in human cancer cells.²² These findings on the role of Bmi-1 in DNA damage response/repair suggest that Bmi-1 plays important roles in maintaining genome integrity. Indeed, the deficiency of Bmi-1 has been found to induce spontaneous chromatid breaks in 293T (an immortalized human embryonic kidney cell line) and HCT116 (a human colon cancer cell line).²² In the present study, we extended the above finding by demonstrating that Bmi-1 deficiency enhanced spontaneous chromatid

What's new?

The gene Bmi-1 is overexpressed in many cancers, and it has been implicated in preventing chromosomal breakage. In this paper, the authors discovered just how Bmi-1 may effect this protection. They observed that increasing or decreasing Bmi-1 expression also increased or decreased p21 expression; furthermore, adding p21 to cells that lack Bmi-1 prevented the expected chromosome breaks. It's known that p21 inhibits cyclin E, which is frequently overexpressed in cancer cells and can also induce chromatid breaks. Therefore, Bmi-1 and p21 appear to promote cancer cell survival by protecting them from chromosomal breakage caused by cyclin E.

breaks in multiple additional human cancer cell lines. We also found, for the first time, that the knockdown of Bmi-1 decreased the expression of p21^{WAF1/WIP1}, and the forced overexpression of Bmi-1 increased p21 expression, indicating a role of Bmi-1 in positive regulation of p21 in human cancer cells. We further demonstrated that the ectopic overexpression of p21 rescued Bmi-1-deficiency-induced chromatid breaks, suggesting that the decreased p21 expression after Bmi-1 knockdown played a critical role in causing chromatid breaks. One of the well-established functions of p21 is to inhibit the function of cyclin E.^{23,24} The decreased expression of p21 after Bmi-1 knockdown would allow over-activation of cyclin E, which is known to be a potent inducer of structural chromosome instability.^{23,25} Indeed, we found that Bmi-1 deficiency-induced chromatid breaks in cancer cells were dependent upon cyclin E overexpression. Based on these findings, we conclude that p21/cyclin E pathway is crucial in the manifestation of genomic instability induced by Bmi-1 knockdown in cancer cells. As both cyclin E and Bmi-1 are frequently overexpressed in cancer cells, the novel and important implication from our findings is that Bmi-1 plays a particularly important role in limiting genomic instability in cyclin E-overexpressing cancer cells.

Material and Methods**Cell lines**

A cervical cancer cell line (HeLa), an osteosarcoma cell line (U2OS) and a nasopharyngeal carcinoma cell line (HONE1) were used in our study. HeLa and U2OS cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM) medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS). HONE1 cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS. All cells were maintained at 37°C in 5% CO₂ incubators. An immortalized cervical epithelial cell line NC104-E6E7 was cultured in 1:1 mixture of EpiLife (Cascade Biologicals) and defined keratinocyte serum-free medium (Life Technologies) with the provided supplements.

RNA interference and ectopic p21 expression

Small interfering RNAs (siRNA) against human Bmi-1, cyclin E or control siRNA (scramble RNA sequence) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The siRNAs were resuspended in RNase-free water provided by Santa Cruz Biotechnology as recommended by the manufacturer. Ectopic p21 expression vector, pCEP-p21, and the

empty vector, pCEP, were obtained from Addgene (deposited by Prof. B. Vogelstein's Laboratory,²⁶ the Johns Hopkins University School of Medicine, Baltimore, MD). Before transfection, culture medium was changed to serum-free DMEM or RPMI-1640. Cells were transfected with siRNA, pCEP-p21, pMSCV-Bmi-1 or empty vectors with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 3 hr. Then the culture medium was refreshed with serum-containing medium, and the cells were incubated for 48 hr before harvest for analysis.

Ectopic Bmi-1 expression

Retroviral vector for ectopic Bmi-1 expression, pMSCV-Bmi-1 (also encoding puromycin-resistant gene), was kindly provided by Prof. Mu-Sheng Zeng at the State Key Laboratory of Oncology in Southern China, Sun Yat-sen University Cancer Center.⁷ The empty vector pMSCV (encoding puromycin-resistant gene) was obtained from Yingrun Biotechnologies, Changsha, China. The infectious viruses were produced from phoenix 293 cells. Two days after retroviral infection, the cells were selected with 1 µg/ml puromycin for 4 days. The first-passage cells after drug-selection were analyzed.

Chromosome aberration analysis

Metaphase chromosome spreads were prepared as previously described.²⁷ Chromosome were stained with DAPI (4',6-diamidino-2-phenylindole). Images under a 100× lens of Leica fluorescence microscope were captured by computer with SPOT software (Leica), and then analyzed on computer screen. Two hundred metaphases were analyzed for each datum of chromosome aberration frequency. Chromosome aberrations in HeLa cells were also analyzed using 24-color spectral karyotyping (SKY) as previously reported.²⁸ Aberrations were identified according to the criteria of the International System for Human Cytogenetic Nomenclature [1995, Mitelman F (ed); Karger, Basel].

Western Blotting analysis

Western Blotting was used to analyze the levels of protein expression. Briefly, 15 µg of the protein from the whole-cell extract was separated by using 10% SDS-PAGE. The resolved proteins in the gel were transferred onto a polyvinylidene fluoride membrane (Amersham, GE Health, Sweden) at room temperature for 1.5 hr. The membrane was washed with 1× TBST and blocked with 2% bovine serum albumin (BSA) (for p21 and

phospho-CHEK1 analysis) or 10% nonfat milk (for other protein analysis) in 1× TBST for 1 hr. After washing, the membrane was incubated with primary antibodies in 1% BSA (for p21 and phospho-CHEK1 analysis) or 5% nonfat milk (other protein analysis) in 1× TBST at 4°C overnight. The primary antibodies: Bmi-1 (F6; 1:600) was from Upstate, Millipore (Billerica, MA); p21^{WAF/Cip1} (1:200), cyclin E (1:1000) and actin (1:1000) were from Santa Cruz Biotechnology; p53 (1:1000), phospho-p53(ser15) (1:500), CHEK1 (1:500) and phospho-CHEK1(ser345) (1:500) were from Cell Signaling Technology (Danvers, MA). After washing twice with 1× TBST for 20 min, the membrane was incubated with appropriate secondary antibodies (1:3,000) in 5% nonfat milk in 1× TBST for 1 hr at room temperature. After washing, the membrane was incubated with ECL-Plus chemiluminescence kit (Amersham) for 5 min. The signals were visualized using Biomax X-ray film (Kodak, Rochester, NY).

BrdU (5-bromo-2-deoxyuridine) staining

Cells grown on coverslips were incubated with BrdU (10 μM) (Life Technologies, Grand Island, NY) for 1 hr, fixed with 100% methanol for 30 min on ice, washed with phosphate buffered saline and permeabilized with 0.3% Triton X-100 at room temperature for 20 min. After washing with 1× TBST, the cells were incubated in 2 M HCl at room temperature for 20 min, neutralize with 0.1 M borate buffer for 5 min, washed with 1× TBST, and incubated in 3% BSA in 1× TBST for 30 min. After washing, the cells were incubated with anti-BrdU (1:400) (Dako, Glostrup, Denmark) for 1 hr at room temperature, washed with 1× TBST, incubated with second antibody (1:2,000) Alexa Fluor 488 (Life Technologies) in 1× TBST for 1 hr at room temperature, and washed again. The cells were counterstained with DAPI containing antifade. The coverslips were mounted onto slides. The percentages of BrdU-positive cells out of 500 cells were scored under a Leica fluorescence microscope.

Immunofluorescence staining

Cells grown on coverslips were fixed with 4% paraformaldehyde for 10 min on ice, and permeabilized with 0.3% Triton X-100 at room temperature for 20 min. After washing with 1× TBST, the cells were incubated in 3% BSA (resuspended in 1× TBST) for 30 min. After washing, the cells were incubated with anti-γ-H2AX (Upstate, Millipore) (1:500) in 1% BSA for 1 hr at room temperature, washed, incubated with second antibody (1:2,000) Alexa Fluor 488 (Life Technologies) for 1 hr at room temperature, and washed again. Then the cells were counterstained with antifade-containing DAPI. The coverslips were mounted onto slides. Images were captured under a Leica fluorescence microscope by computer with SPOT software (Leica), and analyzed on computer screen.

Statistical analysis

Student's *t*-test was used to analyze differences between experimental groups. A *p* value ≤ 0.05 was considered statistically significant.

Results

Bmi-1 knockdown induced spontaneous chromatid breaks in multiple cancer cell lines

As Bmi-1 is commonly overexpressed in cancer cells, we reduced Bmi-1 expression by using siRNA against Bmi-1 to investigate the role of Bmi-1 in chromosomal instability in multiple cancer cell lines of different origins. We used HeLa and U2OS (a cervical cancer and an osteosarcoma cell line, respectively), which are widely used in chromosomal instability study, and HONE1 (a commonly used nasopharyngeal carcinoma cell line) as our cell models. The successful Bmi-1 knockdown was confirmed by Western Blotting results shown in Figure 1a. Of various forms of structural chromosome abnormalities analyzed using metaphase chromosome staining, the frequencies of spontaneous chromatid breaks were found significantly increased in cells after Bmi-1 knockdown (*p* < 0.05, Fig. 1b). Examples of Bmi-1 knockdown-induced chromatid breaks were shown in Figure 1c. There were no significant changes in total chromosome number or ploidy after Bmi-1 knockdown when compared to the cells transfected with scramble RNA sequences (data not shown). These results are in line with the previous report that Bmi-1 deficiency increased spontaneous chromatid breaks in 293T and HCT116 cell lines²² and extend the finding to other carcinoma cell lines.

The exhibition of chromatid breaks after Bmi-1 knockdown involved the function of cyclin E overexpression in cancer cells

It is known that spontaneous structural chromosome instability can be caused by endogenous replicative stress induced by hyperactivation of S-phase promoting oncogenes presumably through compromising prereplication complex assembly and licensing of origins of replication.^{29,30} Cyclin E is one of the typical oncogenes promoting S-phase progression to induce replicative stress and structural chromosome instability.^{23,25,29,30} We therefore first examined whether the chromatid breaks in Bmi-1 knockdown-cells were related to alteration in expression levels of cyclin E in cancer cells. Our Western Blotting analysis showed that Bmi-1 knockdown modestly increased the expression levels of cyclin E in U2OS and HONE1 cells but not significantly the HeLa cells. Of note, compared to the other two cell lines, HeLa cell line had a remarkably higher basal level of cyclin E expression, which may render the change in cyclin E expression less sensitive to the knockdown of Bmi-1 (Fig. 1a). As cyclin E is frequently overexpressed in cancer cells,³¹ we then knocked down cyclin E expression by siRNA in Bmi-1 knockdown cells to examine the potential involvement of cyclin E in chromatid breaks induced by the knockdown of Bmi-1 expression. The significant knockdown of cyclin E expression was confirmed by Western Blotting analysis (Fig. 2a). The cyclin E knockdown did not impair the effectiveness of Bmi-1 knockdown, as evidenced by the similar low level of or barely detectable

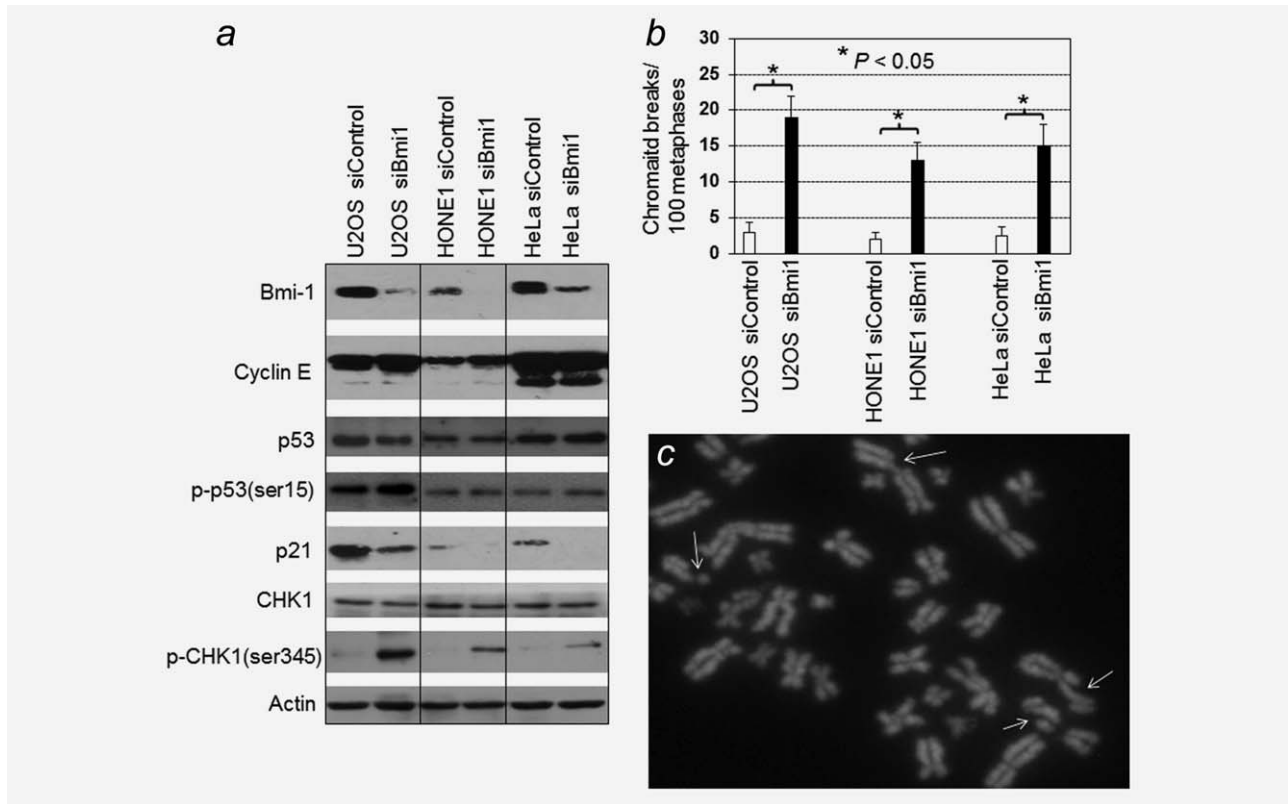


Figure 1. Western Blotting and chromosome aberration analysis. (a) Western Blotting analysis for protein expression; (b) Frequencies of chromatid breaks 48 hr after transfection with siRNA against Bmi-1 and scramble RNA (siControl). Two hundred metaphases were analyzed. Error bars indicate standard deviation (SD) and (c) Examples of chromatid breaks (indicated by arrows) in HeLa cells.

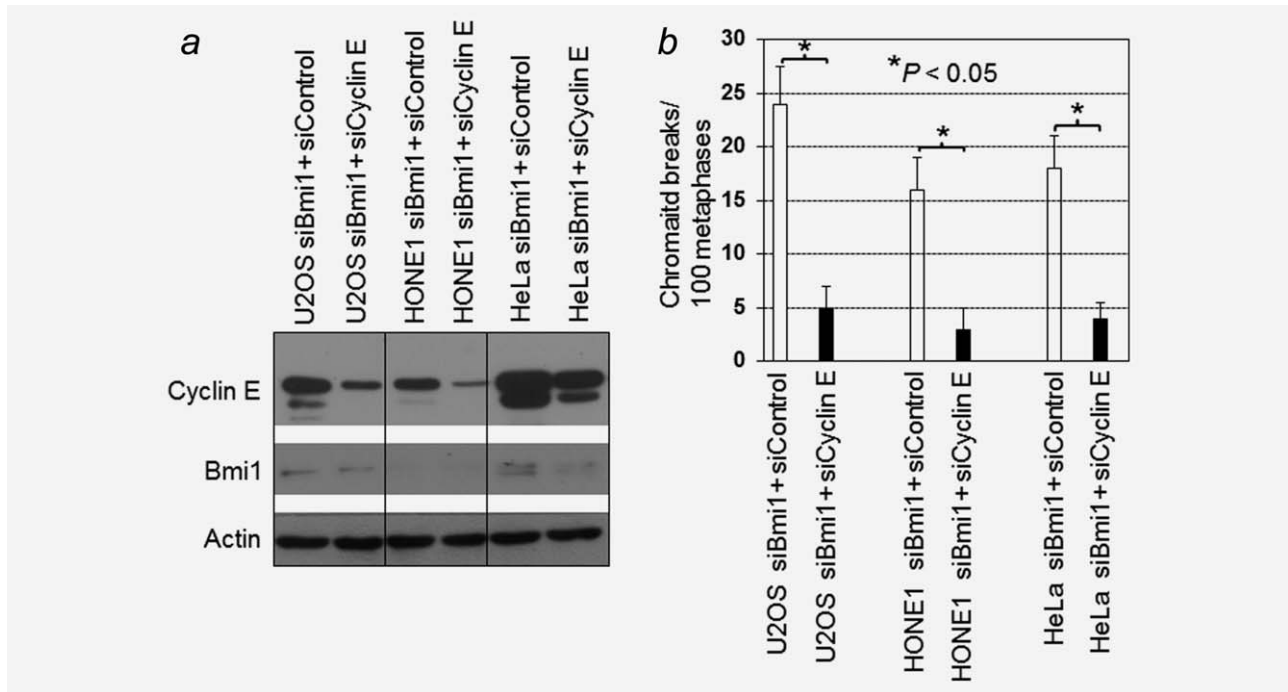


Figure 2. Bmi-1 knockdown-induced chromatid breaks were rescued by knockdown of cyclin E. (a) Western Blotting analysis confirmed the effective knockdown of cyclin E and (b) Frequencies of chromatid breaks 48 hr after transfection with siRNA against Bmi-1 together with siRNA against cyclin E or scramble RNA. Two hundred metaphases were analyzed. Error bars indicate SD.

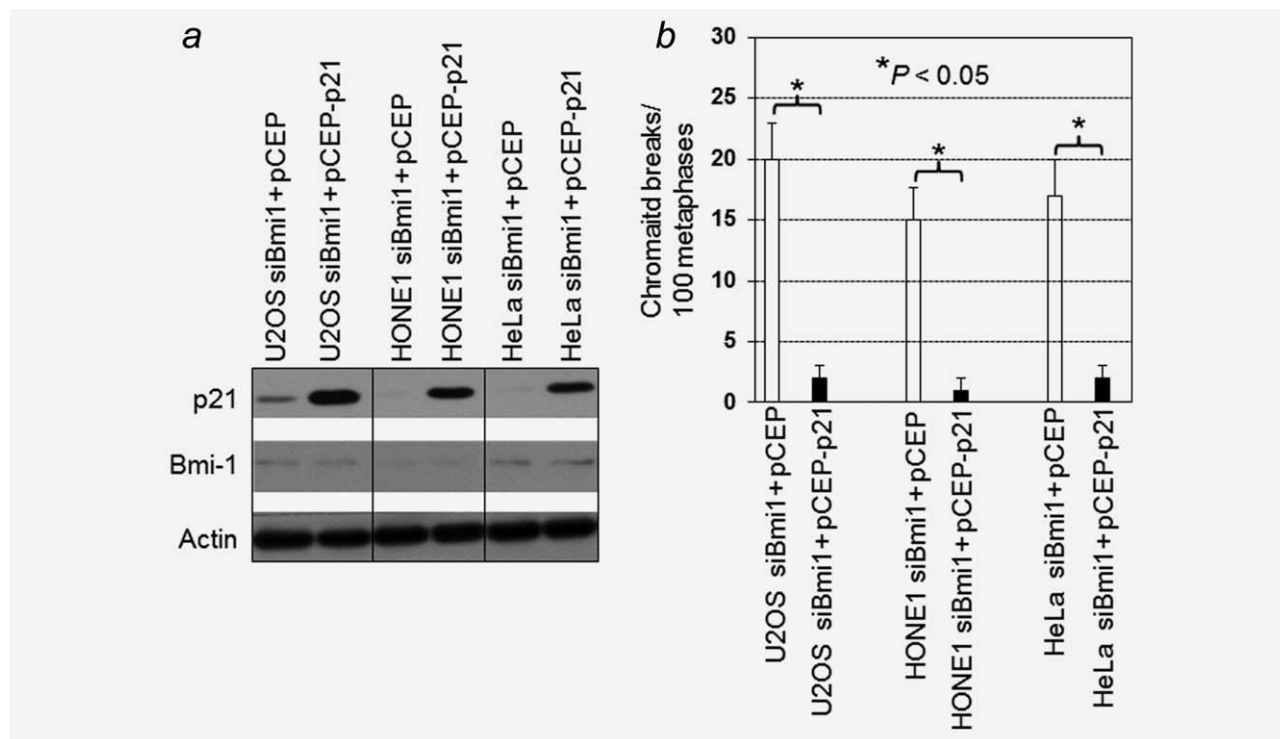


Figure 3. Bmi-1 knockdown-induced chromatid breaks were rescued by ectopic p21 overexpression. (a) Western Blotting analysis confirmed the effective p21 overexpression and (b) Frequencies of chromatid breaks 48 hr after transfection with siRNA against Bmi-1 together with pCEP-p21 or empty vector. Two hundred metaphases were analyzed. Error bars indicate SD.

expression of Bmi-1 compared to the Bmi-1 expression levels in the cells without cyclin E knockdown (Fig. 2a). Strikingly, chromosome aberration analysis showed that cyclin E knockdown significantly reduced the chromatid breaks induced by Bmi-1 knockdown ($p < 0.05$). This result demonstrated that Bmi-1 knockdown-induced chromatid breaks in the cancer cell lines involved the function of cyclin E overexpression.

Bmi-1 knockdown-induced chromatid breaks were dependent upon decreased p21 expression

The dependence of Bmi-1 knockdown-induced chromatid breaks on the original cyclin E overexpression indicates that other factor(s) regulating the active function of cyclin E may be involved. As p21 and p53 are known to suppress cyclin E function, we then examined whether Bmi-1 knockdown altered the expression levels of p21 and p53. Although the expression levels of p53 and phosph-p53(ser15) were not consistently changed across the three cell lines after Bmi-1 knockdown (Fig. 1a), we consistently detected the decreased expression of p21 in the cells with Bmi-1 knockdown compared to the cells transfected with scramble siRNA sequences. This finding was repeated by multiple authors of this manuscript in independent experiments. Therefore, the exhibition of chromatid breaks induced by Bmi-1 knockdown might involve the decreased expression of p21.

To address whether the reduced expression of p21 truly contributed to the induction of chromatid breaks after Bmi-1

knockdown, we performed rescue experiments by cotransfecting p21-expressing plasmids together with siRNA against Bmi-1 into the three cancer cell lines. Western Blotting results confirmed the successful overexpression of p21 (Fig. 3a). The ectopic p21 overexpression did not alter the effective knockdown of Bmi-1, as demonstrated by the similar low levels of Bmi-1 expression compared to those without p21 overexpression (Fig. 3a). Interestingly, chromosome aberration analysis revealed that the ectopic overexpression of p21 decreased the frequencies of chromatid breaks to the background levels (Fig. 3b) when compared to the frequencies in cells only transfected with siControl RNA (scramble siRNA) (Fig. 1b). These results demonstrated that the reduced p21 expression played a critical role in the induction of chromatid breaks after Bmi-1 knockdown in cancer cells.

Ectopic overexpression of Bmi-1 increased p21 expression

The result that Bmi-1 knockdown decreased p21 expression indicates that Bmi-1 may positively regulate p21 expression. We therefore directly tested whether overexpression of Bmi-1 could increase p21 expression. Among the three cancer cell lines we examined, HONE1 cell line has the lowest level of Bmi-1 expression as can be seen from the expression levels in siControl RNA-treated cells (Fig. 1a). We therefore further examined the effects of overexpression of Bmi-1 in HONE1 cells using Bmi-1-expressing vectors. The infected cells from the first passage after the drug selection were harvested for

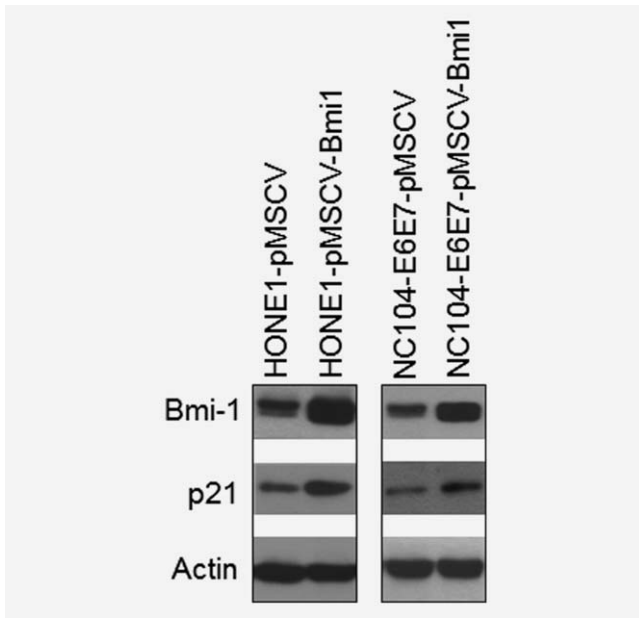


Figure 4. Overexpression of Bmi-1 increased p21 expression analyzed using Western Blotting. Actin showed equal protein loading.

Western Blotting analysis. Our result showed that p21 expression was clearly increased in the cells with the overexpression of Bmi-1 when compared to the cells infected with empty retroviral vectors (Fig. 4). We extended the experiment to an immortalized cervical epithelial cell line, NC104-E6E7, which was immortalized by expression of HPV16-E6E7.³² This cell line was chosen because of its low basal level of Bmi-1 expression among multiple immortalized and cancer cell lines we screened. Again, Bmi-1 overexpression clearly increased p21 expression in NC104-E6E7 cells. Our results from Bmi-1 knockdown or ectopic overexpression experiments support that Bmi-1 positively regulates p21 expression. In addition, the ectopic overexpression of Bmi-1 in HONE1 cells also decreased the frequency of spontaneous chromatid breaks by half (5 chromatid break/500 metaphases in Bmi-1 overexpressing HONE-1 cells) compared to 12 chromatid break/500 metaphases in control cells transfected with empty vectors. The immortalized NC104-E6E7 cells had a low frequency of spontaneous chromatid breaks (2 chromatid break/500 metaphases), and no further decrease in the frequency of spontaneous chromatid breaks could be observed after Bmi-1 overexpression.

Bmi-1 knockdown increased proportions of cells with DNA synthesis

One of the major functions of cyclin E is to promote S-phase entry, but the over-activation of cyclin E can cause hyper-replication-related replicative stress thus slows S-phase progression or completion, leading to increase of cell proportion in S-phase.²³ Considering the well-established role of p21 in inhibiting the function of cyclin E,^{23,24} we speculated that the reduced expression of p21 after Bmi-1 knockdown may lead

to functional activation of cyclin E, resulting in increased proportion of cells undergoing DNA synthesis. To test this hypothesis, we used bromodeoxyuridine (BrdU) labeling (which accurately reflects DNA synthesis in cells) to measure active S-phase proportion under normal growth condition after Bmi-1 knockdown. Two days after transfection with siRNA against Bmi-1 or scramble siRNA sequences, the cells were incubated with medium containing BrdU for 1 hr and harvested for nuclear staining of BrdU. Examples of BrdU staining were shown in Figure 5a. As predicted, the percentages of cells with nuclear BrdU staining in the three cancer cell lines after Bmi-1 knockdown increased significantly when compared to control cells transfected with scramble siRNA sequences (Fig. 5b).

Bmi-1 knockdown enhanced replicative stress in cancer cells

In mammalian cells, the presence of endogenous replicative stress (without challenge with exogenous DNA damage sources) can be detected by S-phase-restricted pan-nuclear staining of phosphorylated form of H2AX (γ -H2AX),^{33,34} which contrasts with the punctuate pattern (foci formation) of γ -H2AX induced by exogenous sources that cause DNA doubling strand breaks or endogenous DNA damage in non-S-phase cells.³⁵ The pan-nuclear staining of γ -H2AX has been used to detect cyclin E overexpression-induced replicative stress in a previous study.³⁴ We therefore used pan-nuclear γ -H2AX staining (exemplified in Fig. 5c) as one of the markers to indicate the degree of replicative stress in our study. Bmi-1 knockdown in the three cancer cell lines significantly increased the percentages of cells with pan-nuclear γ -H2AX staining, indicative of replicative stress, when compared to scramble siRNA transfection, (Fig. 5d). Notably, the percentages of Bmi-1-depleted cells with pan-nuclear γ -H2AX staining decreased significantly after ectopic overexpression of p21 (Fig. 5d).

CHK1 phosphorylation represents a classical and sensitive response to replicative stress.^{36,37} Our Western Blotting analysis detected increased expression levels of phosphorylated CHK1 in Bmi-1 knockdown cells compared to those in the cells transfected with scramble siRNA (Fig. 1a). This was in line with the notion that Bmi-1 knockdown increased replicative stress.

As fragile sites are hot-spots of breakage induced by replicative stress, we tested whether Bmi-1 knockdown preferentially induced chromatid breaks at fragile sites in cancer cells. Metaphases from HeLa cells after Bmi-1 knockdown were analyzed using 24-color SKY and DAPI staining to identify the breakpoint distribution on specific chromosome arms. HeLa cells were chosen because of their relatively simple chromosomal structural abnormalities which facilitated reliable identification of chromosome bands on most of their chromosome arms. Examples of SKY and DAPI images of HeLa cells with chromatid breaks are shown in Figures 6a and 6b. The list of all chromatid breakpoints in whole

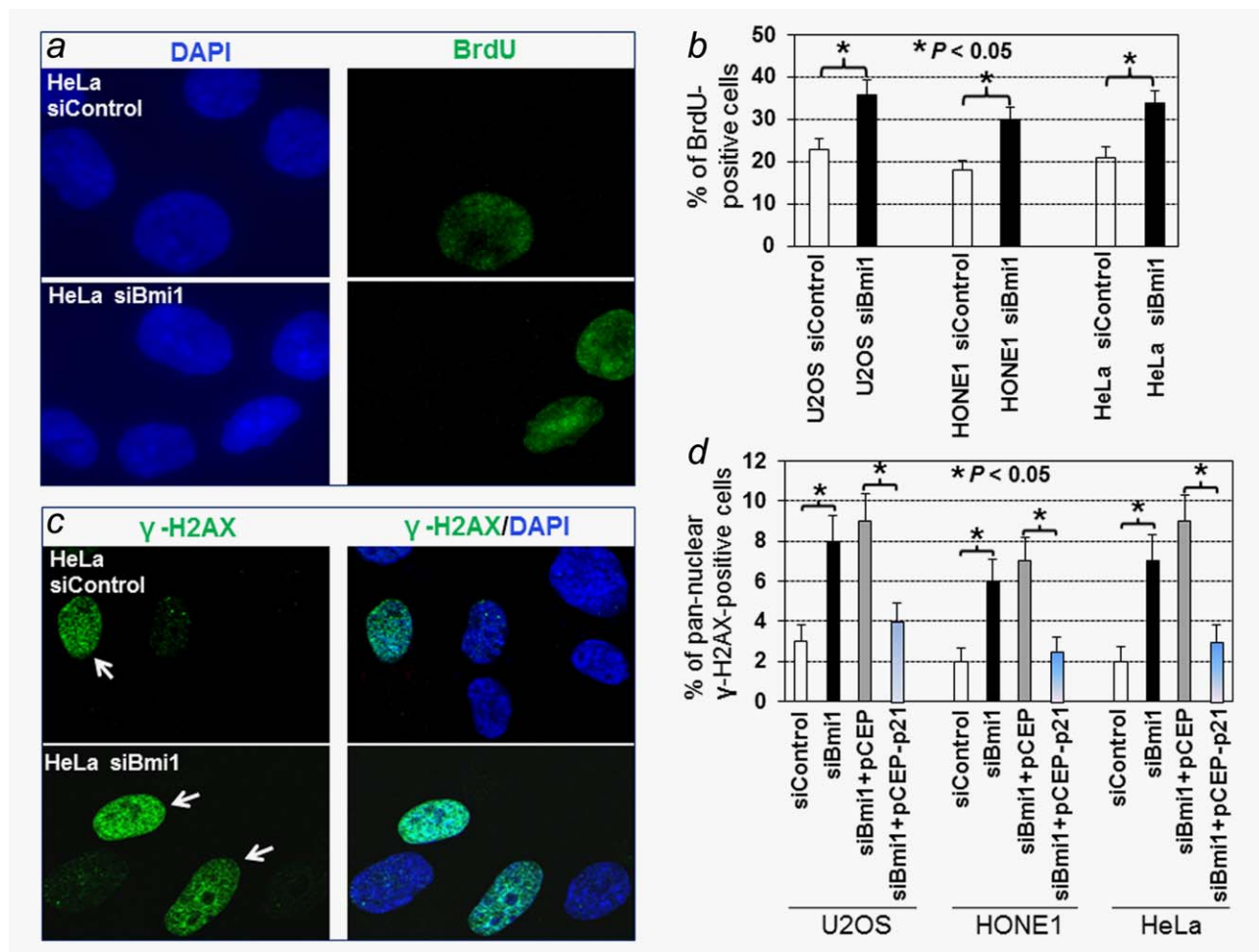


Figure 5. Bmi-1 knockdown increased the proportions of cells with DNA synthesis and replicative stress. (a) Examples of BrdU-staining to indicate the cells with DNA synthesis; (b) Frequencies of BrdU-positive cells after transfection with siRNA against Bmi-1 or scramble RNA; 500 cells were analyzed and error bars indicate SD; (c) Example of pan-nuclear staining of γ -H2AX (indicated by arrows) and (d) Frequencies of pan-nuclear γ -H2AX-positive cells after transfection with scramble siRNA, siRNA against Bmi-1, the combination of siRNA against Bmi-1 and empty vectors, or the combination of siRNA against Bmi-1 and p21 overexpression vectors. Five hundred cells were analyzed and error bars indicate SD. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

genome of 200 metaphases is shown in Figure 6c. About ~70% of breakpoints occurred at or near conventional fragile sites that were previously identified.³⁸ Although we did not accurately map the fine chromatid breakpoints, our data indicate that there may be an association between chromatid breakpoints and fragile sites.

Discussion

In elucidation of mechanisms underlying the Bmi-1 deficiency-induced chromosome instability, we found, for the first time, that Bmi-1-knockdown decreased the expression of p21, and the ectopic overexpression of p21 rescued Bmi-1 knockdown-induced chromatid breaks in three out of three cancer cell lines examined. These results demonstrated that the decreased p21 expression is crucial for the manifestation of chromatid breaks induced by Bmi-1 knockdown. Although the detailed mechanisms regarding how the decreased p21 expression induced by Bmi-1 knockdown leads to structural

chromosome instability remains to be elucidated, our results suggest that the functions of cyclin E may be involved. It has been well-established that p21 suppresses the functions of cyclin E.^{23,24} If unsuppressed, the activation of cyclin E can potentially induce structural chromosome instability by causing replicative stress.²³ The reduced p21 expression after Bmi-1 knockdown would lead to functional activation of cyclin E in cells. Although we found only modest increase in the protein expression levels of cyclin E after Bmi-1 knockdown in two out of three cancer cell lines, we demonstrated, for the first time, that knockdown of cyclin E abolished the Bmi-1 knockdown-induced chromatid breaks in cancer cells, implicating the involvement of cyclin E in mediating the Bmi-1 knockdown-induced chromatid breaks. One simple interpretation of the results from the chromatid break rescue experiments was that the ectopic p21 overexpression or cyclin E knockdown in Bmi-1-knockdown cells could lead to G1- or S-phase arrest, thus reducing replicative stress and decreasing

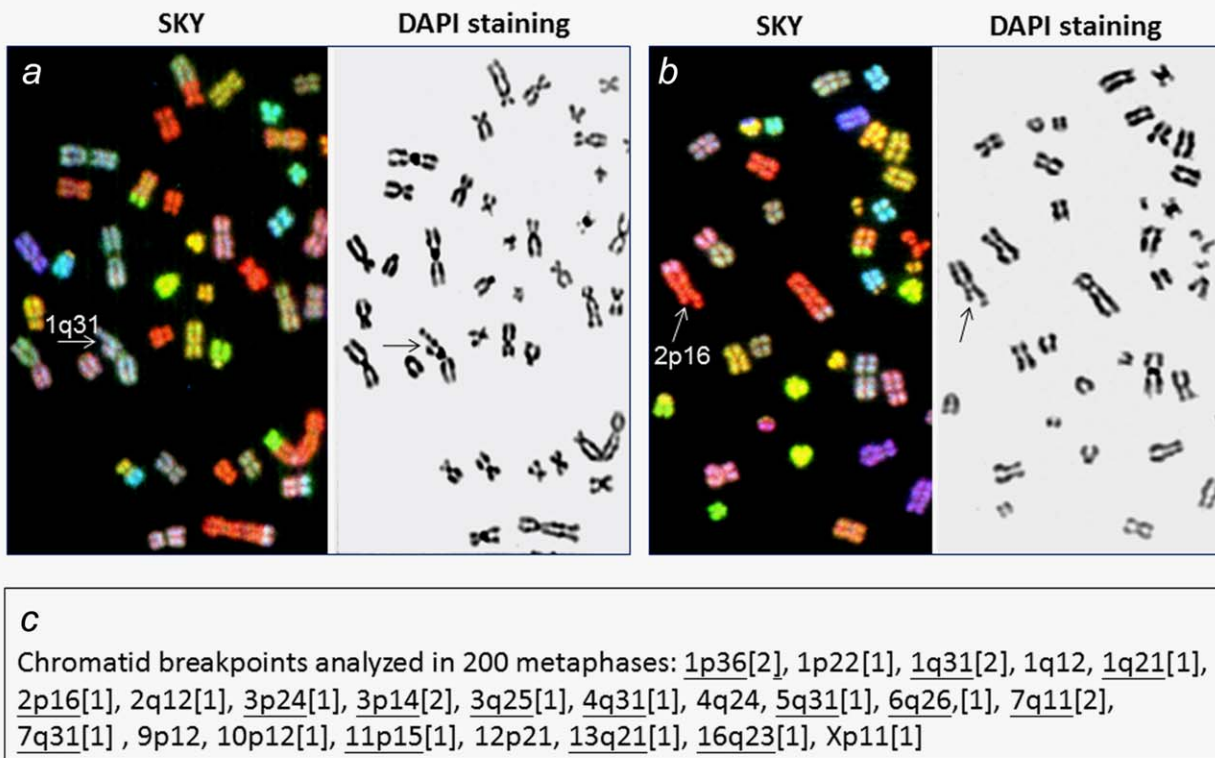


Figure 6. Bmi-1 knockdown induced chromatid breaks preferentially at fragile sites. (a) and (b) Two partial metaphases of HeLa cells with chromatid breaks at fragile sites after Bmi-1 knockdown. Arrows indicate breakpoints. (c) The list of all chromatid breakpoints in 200 metaphases of HeLa cells after Bmi-1 knockdown. The breakpoints at or near conventional fragile sites were underlined. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the formation of chromatid breaks in mitotic cells. In agreement with the notion that Bmi-1 knockdown led to replicative stress, we showed that the chromatid breaks after Bmi-1 knockdown might be associated with conventional fragile sites. Bmi-1 knockdown also increased the percentages of cells with pan-nuclear γ -H2AX staining (specific marker of replicative stress^{33,34}), and elevated the expression levels of phosphorylated CHK1, (classical and sensitive response to replicative stress). Although CHK1 may suppress extensive exhibition of replicative stress-induced chromosome instability,³⁹ it is evident that the response of CHK1 was not sufficient to completely suppress the formation of chromatid breaks after Bmi-1 knockdown in cancer cells. Noteworthy, we showed that the ectopic overexpression of p21 suppressed the Bmi-1 knockdown-induced replicative stress (Fig. 5d), which may explain the reduction in the frequencies of chromatid breaks in mitotic cells after ectopic p21 overexpression.

Our experiments further showed that Bmi-1 overexpression increased p21 expression. Combined with the results of decreased p21 expression after Bmi-1 knockdown, our results revealed that Bmi-1 positively regulates p21 expression in human cancer cells. This has been repeatedly confirmed by multiple experiments in the present study. Yet this finding is contrary to our initial expectation based on the data from mouse neural stem cells in which it was shown that Bmi-1 neg-

atively regulates p21 expression.⁴⁰ We have not unraveled the molecular mechanism of p21 regulation by Bmi-1 in our study. It has been reported that Bmi-1 binds to p21 promoter to regulate p21 transcription.⁴¹ However, it is known that Bmi-1 can differently regulate the expression levels of its target genes depending on cell contexts. For example, after Bmi-1 knockdown, E4F1 gene expression (one of the downstream target genes of Bmi-1) was downregulated in E11 but on the contrary upregulated in E16 neural progenitor cells.⁴⁰ Other undefined factors may be involved in p21 regulation by Bmi-1 in human cancer cell lines. The role of p53 in the response of p21 to Bmi-1 knockdown is also unclear in the present study. The p53 is one of the well-known upstream regulators of p21. In our study, we did not find consistent changes in expression levels of either total p53 or phosph-p53(ser15) (the activated form of p53) after Bmi-1 knockdown in the cell lines with different status of p53. HONE1 cells carry a heterozygous point mutation at codon 280 in exon 8 of p53 gene⁴²; U2OS and HeLa cells carry wild-type p53 gene.⁴³ However, HeLa cells are infected by HPV18. Presumably, the p53 function in HeLa cells is inactivated by the expression of HPV18-E6. Although p53 expression levels did not show consistent change after Bmi-1 knockdown, we could not absolutely exclude the involvement of p53 in the regulation of p21 after Bmi-1 knockdown. The involvement of p53 in p21 regulation by Bmi-1 warrants further investigation.

A previous study has shown that Bmi-1 depletion in mice also exhibit a high frequency of spontaneous chromatid breaks in primitive hematopoietic cells.²² In addition, the cultures initiated from *Bmi-1*^{-/-} hematopoietic stem cells exhibited a significant increase of cycling cells including increased S- and G2-phase cells when compared to *Bmi-1*^{+/+} hematopoietic stem cells. But eventually, higher frequencies of *Bmi-1*^{-/-} hematopoietic stem cells were succumbed to apoptosis than *Bmi-1*^{+/+} hematopoietic stem cells.²² Therefore, genomic instability induced by *Bmi-1* knockout was not favorable to cell survival. In agreement with this, several studies have shown that Bmi-1 knockdown suppressed the proliferation of various types of human tumors.^{11–13}

Although genomic instability is a hallmark of cancer and is believed to play critical roles in cancer development, yet high

levels of genomic instability can affect cancer cell viability, which is exploited by many chemotherapeutic reagents that induce severe genomic instability. Therefore, restriction of genomic instability at tolerable levels is beneficial for continued proliferation and progression of cancer cells. As cyclin E is commonly overexpressed in cancer cells,³¹ the important implication from our study is that Bmi-1 may exert its protective function of genome integrity in cyclin E-overexpressing cells at least in part through upregulation of p21, hence, limiting genomic instability at tolerable levels. In the light of the previous findings that Bmi-1 promotes the response/repair after DNA damage,^{20–22} the p21/cyclin E pathway is most unlikely the sole pathway that modulates the anticlastogenic function of Bmi-1. Yet our findings in this study add a new dimension to the functions of Bmi-1 in limiting spontaneous genomic instability in cancer cells.

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