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ARTICLE

The transcription factor PBX3 promotes tumor cell growth through transcriptional suppression of the tumor suppressor p53

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Pre-B-cell leukemia transcription factor 3 (PBX3) is a member of the PBX family and contains a highly conserved homologous domain. PBX3 is involved in the progression of gastric cancer, colorectal cancer, and prostate cancer; however, the detailed mechanism by which it promotes tumor growth remains to be elucidated. Here, we found that *PBX3* silencing induces the expression of the cell cycle regulator p21, leading to an increase in colorectal cancer (CRC) cell apoptosis as well as suppression of proliferation and colony formation. Furthermore, we found that *PBX3* is highly expressed in clinical CRC patients, in whom p21 expression is aberrantly low. We found that the regulation of *p21* transcription by PBX3 occurs through the upstream regulator of *p21*, the tumor suppressor p53, as PBX3 binds to the *p53* promoter and suppresses its transcriptional activity. Finally, we revealed that PBX3 regulates tumor growth through regulation of the p53/p21 axis. Taken together, our results not only describe a novel mechanism regarding PBX3-mediated regulation of tumor growth but also provide new insights into the regulatory mechanism of the tumor suppressor p53.

Keywords: Pre-B-cell leukemia transcription factor 3 (PBX3); p53; cyclin-dependent kinase inhibitor p21; apoptosis; cell proliferation

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INTRODUCTION

The cell cycle is a tightly controlled process that determines the rate of cell proliferation. Accurate cell cycle progression is crucial for maintaining the balance of cell proliferation and cell death, thereby maintaining the appropriate cell number, and dysregulation can result in various diseases, such as tumors [1, 2]. Previous studies have shown that the occurrence and development of breast, ovarian, prostate, sarcoma, and other cancers can be largely prevented by effectively inhibiting cell cycle progression [3]. *p53* is the most important tumor suppressor gene and was discovered 40 years ago [4]. p53 regulates various aspects of tumorigenesis, including cell number homeostasis, tumor cell metabolism, tumor cell senescence, drug resistance, and tumor angiogenesis [5–7]; furthermore, its expression level is closely related to tumor prognosis [8–10]. p53 regulation of its downstream gene, the cell cycle regulator *p21* (also known as *cyclin-dependent kinase inhibitor p21*), is considered one of the most critical components in maintaining an appropriate cell number and thus preventing the initiation of tumorigenesis. The p53/p21 axis is crucial for regulating cell cycle progression, leading to the regulation of cell proliferation and cell death [4]. Abnormal p53 expression leads to the deviation of cells from normal cell cycle regulation and is closely related to diseases, including cancer [5]. However, despite the importance of p53, the molecular mechanisms regulating its expression have not been fully elucidated.

In an effort to unravel the regulatory mechanisms underlying the p53/p21 axis in tumor growth, we previously performed high-throughput screening using a short hairpin RNA (shRNA) vector library for genes that regulate p21 transcription [11–13]. Our previous screening results suggested that pre-B-cell leukemia transcription factor 3 (PBX3), a member of the PBX family of three amino acid loop extension homeodomain transcription factors [14], is a potential regulator of p21, as its silencing leads to a significant increase in *p21* promoter activity. PBX3 is correlated with the growth and progression of various cancers, including gastric cancer [15], colorectal cancer (CRC) [16], cervical cancer [17] and prostate cancer [18]; however, the detailed mechanism of its regulation remains unknown.

In this study, we revealed that in CRC cells, PBX3 directly binds to the *p53* promoter and suppresses its transcriptional activity. This leads to significant downregulation of p21 expression, which results in a decrease in apoptosis and an increase in cell proliferation. These events subsequently increase the tumor cell number and colony formation. Furthermore, we showed that PBX3 expression exhibited a negative correlation with p21 expression in clinical CRC tissues. These findings not only reveal a novel p53 regulatory mechanism but also elucidate the molecular mechanism by which PBX3 regulates tumorigenesis.

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MATERIALS AND METHODS

Plasmids and constructs

Two shRNA expression vectors targeting different sites of PBX3 were constructed as described previously [19]. The target sites were designed using the algorithm previously reported [20], and the sequences were as follows: shPBX3-1, 5′-GGT CAA GGT TTA ATA TTG T-3′; shPBX3-2, 5′-GGG GAA ATG TGA ATA GGC A-3′. An shRNA expression vector targeting p53 was constructed as described previously [21], and the target sequence was as follows: 5′-GCA AGA AGG GAG ACA AGA T-3′. Reporter vector bringing the *p21* promoter (p21-luc) and *p53* promoter (p53-luc) were constructed as described previously [11]. For construction of the *PBX3* overexpression vector, a Takara Ex Taq Kit (Takara Bio, Dalian, China) was used to amplify the coding region of human *PBX3* from human cDNA obtained by reverse transcribing total RNA from HCT116 cells using a PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio). The amplicon was inserted into the *Bam*HI and *Eco*RI sites in pcDNA3.1(+) (Invitrogen Life Technologies, Carlsbad, CA).

Cell lines and cell cultures

Wild-type HCT116 and p53-null HCT116 ($HCT116^{p53null}$) cells were kindly provided Dr. Bert Vogelstein at the Johns Hopkins University School of Medicine and maintained in McCoy's 5A medium (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Biological Industries, Beith Haemek, Israel) and 1% penicillin–streptomycin. The human hepatocarcinoma cell line HepG2, breast carcinoma cell line MCF-7, and colon cancer cell line LoVo were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HepG2 and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (Gibco) with 10% FBS (Biological Industries) and 1% penicillin–streptomycin, while LoVo was maintained in Ham's F12K (Kaighn's) medium (Gibco) supplemented with 10% FBS (Biological Industries) and 1% penicillin–streptomycin. All cell lines were routinely tested using a Mycoplasma Detection Kit-QuickTest (Biotool, Houston, TX) and found to be negative for mycoplasma contamination. Transfection was performed using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's protocol.

For gene silencing experiments, cells were seeded in six-well plates and transfected with 2 μg of the indicated shRNA expression vector using Lipofectamine 2000. To eliminate untransfected cells, cells were selected with puromycin (final concentration: 1.2 μg/mL).

For actinomycin D and cycloheximide treatment, 1×10^6 cells were seeded in six-well plates and cultured for 24 h. Protein was harvested 1 h after the cells were treated with actinomycin D (final concentration: 1 μM) or cycloheximide (final concentration: 30 μg/mL) for 1 h.

Clinical human colon carcinoma specimens

Human colon carcinoma specimens were obtained from colon carcinoma patients undergoing surgery at Chongqing University Cancer Hospital (Chongqing, China) and stored in the Biological Specimen Bank of Chongqing University Cancer Hospital. Patients did not receive chemotherapy, radiotherapy, or other adjuvant therapies prior to surgery. The specimens were snapfrozen in liquid nitrogen. Prior patient's written informed consents were obtained, and the experiments were approved by the Institutional Research Ethics Committee of Chongqing University Cancer Hospital and conducted in accordance with the Declaration of Helsinki.

Animal experiments

For the in vivo tumor study, BALB/c-*nu/nu* mice (male, body weight: 18–22 g, 6 weeks old) were purchased from Chongqing Medical University (Chongqing, China). Animal studies were approved by the Institutional Ethics Committee of Chongqing Medical University and carried out at Chongqing Medical University. All animal experiments conformed to the approved Guidelines for the Care and Use of Laboratory Animals of Chongqing Medical University. All efforts were made to minimize suffering.

For the xenograft experiment, BALB/c-*nu/nu* mice were randomly divided into three groups ($n = 6$ mice per group), and each group was injected subcutaneously with 5×10^6 cells of the indicated stable cell lines. Tumor volumes (V) were evaluated by measuring the tumors with a caliper every 2 days and were calculated using the following equation: $V = a \times b^2/2$, where a and b are the major and minor axes of the tumor, respectively. The investigator was blinded to the group allocation and during the assessment.

RNA extraction and quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted using TRIzol (Invitrogen Life Technologies) according to the manufacturer's instructions. Total RNA (1 μg) was then reverse transcribed into cDNA using a PrimeScript Reagent Kit with gDNA Eraser (Takara Bio). qRT-PCR was performed using SYBR Premix Ex Taq (Takara Bio). The sequences of the primers used are listed in Supplementary Table S1.

Western blotting

Whole cells were lysed with RIPA lysis buffer supplemented with a protease inhibitor and phosphatase inhibitor cocktail (cOmplete™ cocktail; Roche Applied Science, Mannheim, Germany). Samples with equal amounts protein were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels before being transferred to a polyvinylidene fluoride membrane with 0.45-μm pores (Millipore, Billerica, MA). The membrane was then incubated with primary antibodies followed by secondary antibodies. The antibodies used are listed in Supplementary Table S2, and immunoblotting with an anti-β-actin antibody was conducted to ensure equal protein loading. Signals were measured using the Super Signal West Femto Maximum Sensitivity Substrate detection system (Thermo Scientific, Waltham, MA).

Caspase 3 and caspase 7 activity assay

Cells were transfected with the indicated shRNA expression vectors, and puromycin selection was performed as described above. After puromycin selection, cells were reseeded into 96-well plates for 24 h. Caspase 3 and caspase 7 activities were measured using a Caspase-Glo 3/7 Assay (Promega, Madison, WI) according to the manufacturer's instructions.

Dual luciferase assay

Cells were seeded into 24-well plates (8×10^4 cells/well). Twenty-four hours later, cells were cotransfected with the indicated shRNA expression vectors, the reporter vector, and the *Renilla* luciferase expression vector (pRL-SV40, Promega) as the internal control. Twenty-four hours after cotransfection, luciferase activity was measured with a Dual Luciferase Assay System (Promega).

Cell counting assay

Cells were transfected with the indicated vectors, and 24 h after transfection, puromycin selection was performed as described previously. Cells were then reseeded into 96-well plates at a density of 5×10^3 cells/well. Cells were counted at the indicated time points by colorimetric assays with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS, Promega) in accordance with the manufacturer's instructions.

Colony formation assay

Cells were transfected with the indicated shRNA expression vectors and selected using puromycin as described above. Cells were then reseeded into six-well plates at a density of 500 cells/well, cultured for 8 days, fixed with 30% paraformaldehyde and stained with methylene blue. Quantification was then performed

by counting the number of colonies formed. The investigator was blinded during the assessment.

Chromatin immunoprecipitation (ChIP) assay

Chromatin was immunoprecipitated using a ChIP Assay Kit (Beyotime, Shanghai, China) according to the manufacturer's instructions. Briefly, cells were lysed, and chromatin was immunoprecipitated using protein A + G agarose/salmon sperm DNA and an anti-PBX3 antibody or normal rabbit IgG, uncrosslinked for 4 h at 65 °C, and treated with 0.5 M EDTA, 1 M Tris (pH 6.5) and 20 mg/mL proteinase K. Immunoprecipitated chromatin was then subjected to PCR with PrimeSTAR Max (Takara Bio). The primer sequences for amplifying the p53 promoter region containing the predicted PBX3-binding site were 5′-CCT GTA ATA CCA GCT ACT TGT GAG G-3′ (forward primer) and 5′-CAC ATT TAA GTC CTT CGT GGA GA-3′ (reverse primer).

Cell apoptosis assay

Cells were transfected with the indicated shRNA expression vectors and selected with puromycin as described above. Cells were then reseeded in a six-well plate (3×10^5 cells/well), and 24 h later, cells were stained with Annexin V/PI (NeoBiosciences, Shanghai, China). The apoptosis rate was determined using flow cytometry.

5-Ethynyl-2′-deoxyuridine (EdU) incorporation assay

Cells were transfected with the indicated shRNA expression vectors and selected using puromycin as indicated above. EdU incorporation and staining were performed using a Cell Light EdU Apollo 488 In Vitro Imaging Kit (RiboBio, Guangzhou, China) according to the manufacturer's instructions. Nuclei were stained with DAPI. Images were acquired with a DMI6000B microscope (Leica, Heidelberg, Germany). Quantification of EdU-positive and DAPI-positive cells was performed using a Leica Microsystems LAS AF-TCS MP5 microscope (Leica), and the results are shown as the ratio of EdU-positive cells to DAPI-positive cells.

Statistical analysis

All quantification results are presented as the mean ± SD of triplicates. Statistical analysis was performed using the Student's *t* test, except for clinical sample data, which were analyzed by one-way ANOVA in SPSS Statistics v. 17.0. A value of $P < 0.05$ was considered statistically significant.

RESULTS

PBX3 negatively regulates *p21* transcription

Despite its importance in suppressing tumorigenesis, the regulatory mechanism of the p53/p21 axis has not been completely elucidated. In our previous study, we performed high-throughput screening for novel p21 regulators using an shRNA expression library with 3354 shRNA expression vectors covering 2065 genes [11] and identified PBX3 as a novel potential regulator of p21 transcriptional activity. However, the detailed mechanism by which PBX3 regulates the p53/p21 axis remains unknown. To reveal the mechanism by which PBX3 regulates the p53/p21 axis, we first investigated the effect of *PBX3* expression on p21 expression levels in colorectal carcinoma HCT116 cells. For this purpose, we constructed two shRNA expression vectors targeting different sites of PBX3 mRNA and confirmed their suppressive effect (Supplementary Fig. S1a, b). Next, using a dual luciferase assay, we examined the effect of silencing *PBX3* on the transcriptional activity of *p21*. *PBX3* silencing markedly enhanced the activity of the firefly luciferase reporter driven by the *p21* promoter (Fig. 1a), suggesting that PBX3 might negatively regulate *p21* transcriptional activity. Furthermore, p21 mRNA levels were significantly increased in *PBX3*-silenced HCT116 cells (Fig. 1b), while *PBX3* overexpression (Supplementary Fig. S2) strongly suppressed p21 mRNA expression (Fig. 1c). Concomitantly, *PBX3* silencing significantly increased p21 protein expression, whereas overexpression of *PBX3* suppressed p21 protein expression (Fig. 1d, e). Collectively, these results confirm that PBX3 is a novel negative regulator of p21 transcriptional activity.

Correlation between PBX3 and p21 expression in clinical colorectal cancer

To further elucidate the correlation between PBX3 and p21 expression as well as the possible role of PBX3 in tumorigenesis, we performed bioinformatic analysis using the NCBI GEO Database (GSE20916) [22]. The analysis results showed a negative correlation between PBX3 and p21 expression in clinical colorectal carcinoma tissue (Fig. 2a). Furthermore, high expression of PBX3 exhibited a positive correlation with decreased patient survival time (Fig. 2b). We next confirmed the expression of PBX3 and p21 in clinical CRC tissues. As shown by the immunohistochemical results, PBX3 expression was higher in tumor tissue than in tumor-adjacent tissue, while the expression of p21 showed the opposite trend (Fig. 2c). These trends were further confirmed by analyzing the protein levels of PBX3 and p21 in clinical CRC tissues (Fig. 2d). Taken together, these results indicate that PBX3 expression levels are negatively correlated with p21 expression levels in CRC tissue and that high PBX3 expression might be related to poor prognosis.

PBX3 inhibits apoptosis and promotes cell proliferation

Given that PBX3 negatively regulates p21 expression, we next attempted to elucidate whether PBX3 can regulate CRC cell proliferation and apoptosis. *PBX3* silencing significantly suppressed the increase in the total number of HCT116 cells (Fig. 3a). Furthermore, *PBX3* silencing suppressed the colony formation potential of HCT116 cells (Fig. 3b). Collectively, these results indicate that PBX3 might be involved in promoting an increase in the number as well as the colony formation potential of HCT116 cells.

The increase in cell number might be due to the increase in the cell proliferation potential, decrease in apoptosis, or both. Thus, we next examined the effects of *PBX3* silencing on the proliferation potential of HCT116 cells as well as their apoptosis rate. The results of the EdU incorporation assay showed that *PBX3* silencing significantly decreased the ratio of EdU-positive cells to DAPI-positive cells (Fig. 3c), indicating that PBX3 can increase tumor cell proliferation potential. Furthermore, the Annexin V/PI staining results revealed that *PBX3* silencing significantly increased the apoptosis rate of HCT116 cells (Fig. 3d). During the apoptosis cascade, executioner caspases (caspase 3 and caspase 7) are activated by cleavage. Hence, to further confirm the regulatory effect of PBX3 on apoptosis, we examined the levels of cleaved caspase 3 and caspase 7 in HCT116 cells. We found that the levels and activity of cleaved caspase 3 and 7 were strongly increased in *PBX3*-silenced HCT116 cells (Fig. 3e, f).

Collectively, these results clearly show that PBX3 is a negative regulator of tumor cell apoptosis. It is also notable that the increase in the apoptosis rate in *PBX3*-silenced HCT116 cells was even greater than the decrease in their proliferation potential, indicating that while both the increase in apoptosis and decrease in proliferation contribute to the suppressive effect of *PBX3* silencing on the increase in the cell number, the increase in apoptosis might make the greater contribution.

PBX3 regulates *p53* transcriptional activity by directly binding to the *p53* promoter

p53 is a transcriptional activator of *p21* and is involved in the regulation of cell proliferation and apoptosis. Therefore, we next investigated whether PBX3-mediated regulation of p21 occurs through p53. For this purpose, we knocked down *PBX3* and examined the effect on p53 expression. Both the mRNA

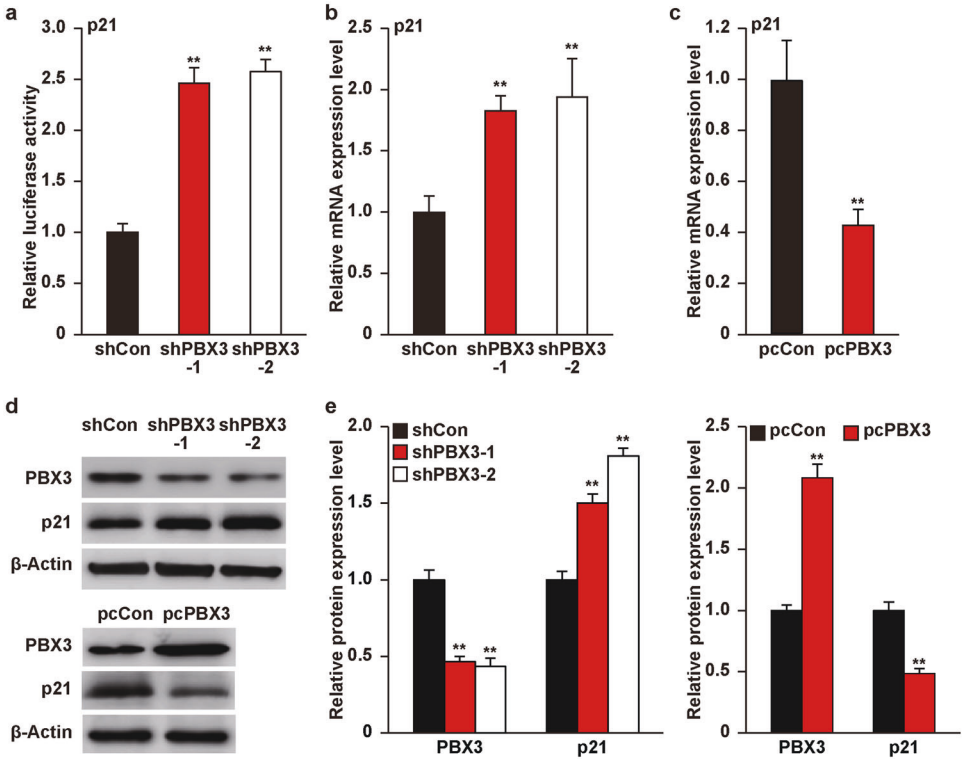

Fig. 1 PBX3 regulates p21 expression in colorectal cancer cells. a Relative luciferase activity of the firefly luciferase reporter vector with the *p21* promoter (p21-luc) in *PBX3*-silenced HCT116 cells, as analyzed using a dual luciferase reporter assay. **b** Relative p21 mRNA expression level in *PBX3*-silenced HCT116 cells, as examined using qRT-PCR. **c** Relative p21 mRNA expression level in *PBX3*-overexpressing HCT116 cells, as examined using qRT-PCR. **d**–**e** p21 protein expression levels in *PBX3*-silenced and *PBX3*-overexpressing HCT116 cells, as examined using Western blotting. Representative images (**d**) and quantification results (**e**) are shown. β-Actin was used for qRT-PCR normalization and as the loading control for Western blotting. Cells transfected with shCon or pcCon were used as controls. Quantitative data are shown as relative values with respect to controls and are expressed as the means ± SDs ($n = 3$). shPBX3: shRNA expression vector targeting *PBX3*; pcCon: pcDNA3.1(+); pcPBX3: *PBX3* overexpression vector; $^{**}P < 0.01$.

and protein levels of p53 increased when *PBX3* was silenced (Fig. 4a, b). Consistent with this result, PBX3 overexpression strongly suppressed the expression of p53 mRNA and protein (Fig. 4c, d). Furthermore, *PBX3* silencing significantly enhanced the levels of p53 and p21 in another colon cancer cell line, LoVo (Supplementary Fig. S3), as well as in the hepatocarcinoma cell line HepG2 and breast cancer cell line MCF-7 (Supplementary Fig. S4), suggesting that PBX3-mediated regulation of the p53/p21 pathway might be a general phenomenon in various types of carcinomas. Collectively, these results indicate that PBX3 is a novel regulator of p53 transcription.

Next, we investigated whether p53 is involved in the regulation of *p21* transcription by PBX3. For this purpose, we first knocked down *PBX3* in HCT116^{p53null} cells (Supplementary Fig. S5) and examined the effect on *p21* transcriptional activity. Our dual luciferase assay results demonstrated that unlike in HCT116 cells with wild-type p53, silencing *PBX3* in HCT116^{p53null} cells did not significantly affect the activity of the *p21* promoter (Fig. 4e). Concomitantly, silencing *PBX3* in HCT116^{p53null} cells did not significantly alter p21 expression at either the mRNA or protein level (Fig. 4f, g). Collectively, these results clearly indicate that p53 is critical for PBX3-mediated regulation of *p21* transcription.

The fact that PBX3 regulates *p21* transcription through p53 indicates that p53 might also be involved in PBX3-regulated HCT116 cell proliferation and apoptosis. Therefore, we next examined the effect of *PBX3* silencing on the proliferation and apoptosis of HCT116^{p53null} cells. *PBX3* silencing still suppressed the increase in total HCT116^{p53null} cell numbers (Supplementary Fig. S6); however, it did so to a significantly lower extent than in wild-type HCT116 cells. In line with this finding, the EdU incorporation assay results revealed that while *PBX3* silencing still decreased HCT116^{p53null} cell proliferation, it did so to a lower extent than in wild-type HCT116 cells (Fig. 5a). Furthermore, the effects of *PBX3* silencing on the apoptosis rate (Fig. 5b), as well as the levels and activity of cleaved caspase 3 and 7 (Fig. 5c, d), were substantially lower than those in wild-type HCT116 cells.

Collectively, these results suggest that the regulatory effect of PBX3 on tumor cell proliferation and apoptosis occurs mainly through regulation of the p53/p21 axis.

Knockdown of p53 restores the effect of *PBX3* silencing on tumor cell proliferation and apoptosis

To confirm the role of p53 in the decrease in tumor cell proliferation and the increase in tumor cell apoptosis induced by *PBX3* silencing, we next examined the effect of silencing both *PBX3* and *p53* in HCT116 cells (Supplementary Fig. S7a) on the total cell number. While *PBX3* silencing increased p21 transcriptional activity, *p53* silencing abolished it (Fig. 6a). Concomitantly, the increases in the expression levels of p53 and p21 in *PBX3*-silenced HCT116 cells were partially restored in HCT116 cells with double silencing of *PBX3* and *p53* (Fig. 6b).

Next, we examined the effect of silencing both *PBX3* and *p53* on HCT116 cell proliferation and apoptosis. Silencing of both *PBX3* and *p53* abolished the effect of *PBX3* silencing on the decreases in the total cell number and colony formation potential of HCT116 cells (Supplementary Fig. S7b, c). Compared to *PBX3* silencing in HCT116 cells, silencing of both *PBX3* and *p53* significantly suppressed the apoptosis rate and restored the proliferation potential (Fig. 6c, d). The fact that *PBX3* and *p53*-double silencing

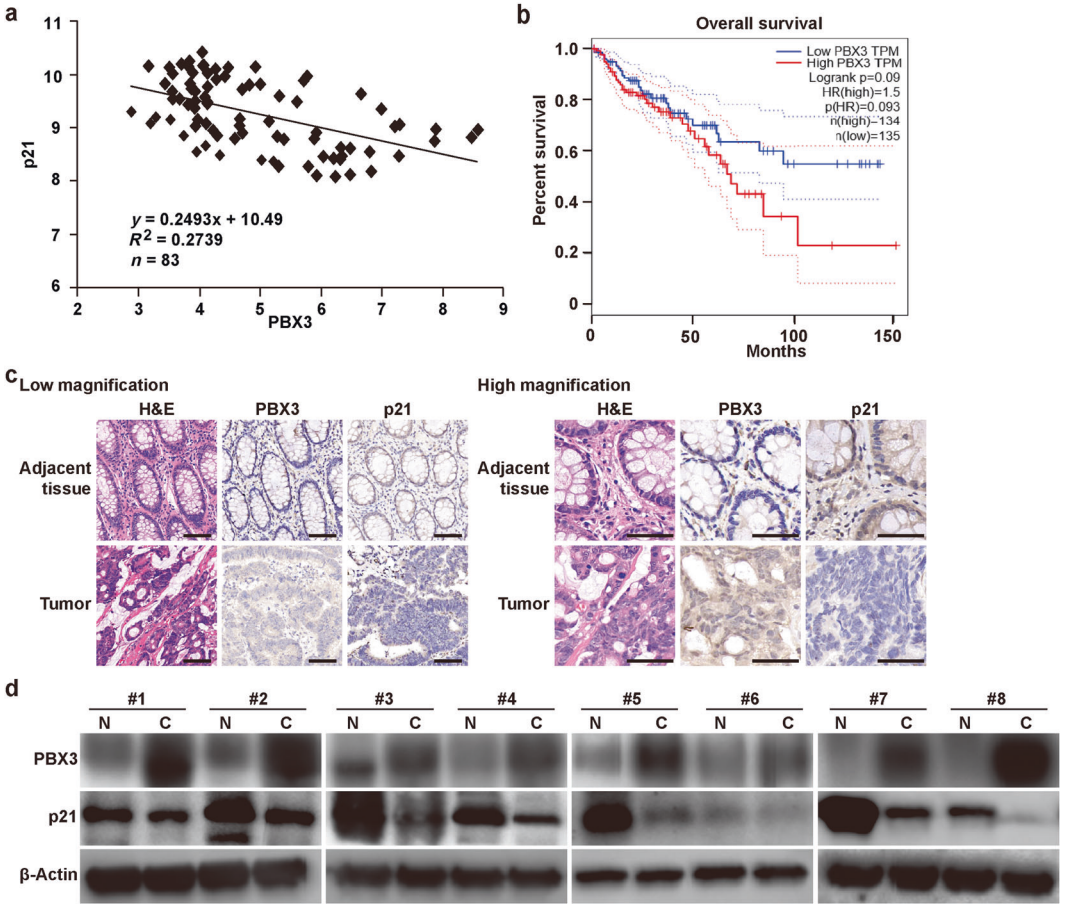

Fig. 2 PBX3 negatively correlates with p21 and prognosis in clinical colorectal cancer specimens. a Correlation analysis of PBX3 and p21 mRNA expression levels in colorectal carcinoma specimens (GEO dataset: GSE20916; $n = 83$). **b** Kaplan–Meier survival plots of colorectal carcinoma patients with low (black) or high (red) PBX3 expression. The default cutoff was 50%. High: samples with a PBX3 expression level within the top 50%; low: samples with a PBX3 expression level below the top 50%; dotted lines: confidence intervals; TPM transcripts per million. **c** Immunohistochemical staining showing the expression levels and localization of PBX3 in clinical human colorectal carcinoma and adjacent tissue samples. Low-magnification (left; scale bars, 100 μm) and high-magnification (right; scale bars, 25 μm) images are shown. **d** PBX3 and p21 protein expression levels in clinical colorectal carcinoma and adjacent normal tissues, as determined by Western blotting. β-Actin was used as the loading control for Western blotting.

abolished the increase in the levels of cleaved caspase 3 and 7 (Fig. 6e) as well as the activity of caspase 3 and 7 (Fig. 6f) further highlights the important roles of p53 in the regulation of CRC cell proliferation and apoptosis by PBX3.

PBX3 directly binds to the *p53* promoter and regulates its transcriptional activity

Subsequently, we attempted to elucidate the molecular mechanism of PBX3-mediated regulation of p53. To determine whether PBX3 regulates p53 expression at the transcriptional or translational level, we treated cells with actinomycin D or cycloheximide to inhibit de novo transcription or protein synthesis, respectively. Inhibition of both de novo transcription and protein synthesis abolished the upregulation of p53 induced by *PBX3* silencing (Fig. 7a, b), indicating that PBX3-mediated regulation of p53 occurs at the transcriptional level. Indeed, using JASPAR (http://jaspar.genereg.net) [23], we predicted a PBX3-binding site encompassing nucleotides −2445 to −2428 in the *p53* promoter. To confirm PBX3-mediated regulation of *p53* transcription, we constructed a firefly luciferase reporter vector with the full-length *p53* promoter (p53-luc) and a firefly luciferase reporter vector with a mutant *p53* promoter in which the predicted PBX3-binding site was deleted (p53-luc-del) (Fig. 7c). We found that while silencing *PBX3* significantly increased *p53* promoter activity, deletion of the predicted PBX3-binding site abolished this effect (Fig. 7d), suggesting that PBX3 can regulate the transcription of *p53* by suppressing its promoter activity and that the −2787 to −2389 region in the *p53* promoter is crucial for this regulation.

Next, to confirm whether PBX3 can bind directly to the *p53* promoter, we performed a ChIP assay using an anti-PBX3 antibody and primers flanking the −2542 to −2188 region in the *p53* promoter. The DNA fragment immunoprecipitated by the anti-PBX3 antibody was amplified by the above-mentioned primers, indicating that PBX3 can bind directly to the −2542 to −2188 region in the *p53* promoter (Fig. 7e). Collectively, our results reveal that PBX3 can bind directly to the promoter of *p53*, thereby suppressing its transcriptional activity.

PBX3 silencing suppresses tumorigenesis through p53

Finally, to determine the role of PBX3 in tumorigenesis, we performed xenograft experiments using stable HCT116 cells with silencing of *PBX3* and double silencing of *PBX3* and *p53* (Supplementary Fig. 8a) transplanted subcutaneously into BALB/c-*nu/nu* mice. The growth curves and morphological appearance of the xenografted tumors are shown in Fig. 8a, b, respectively. These results demonstrated that *PBX3* silencing significantly suppressed tumor growth, while *p53* silencing partially reversed this effect. Furthermore, examination of the weight of the tumors showed similar results (Fig. 8c). Collectively, these results further

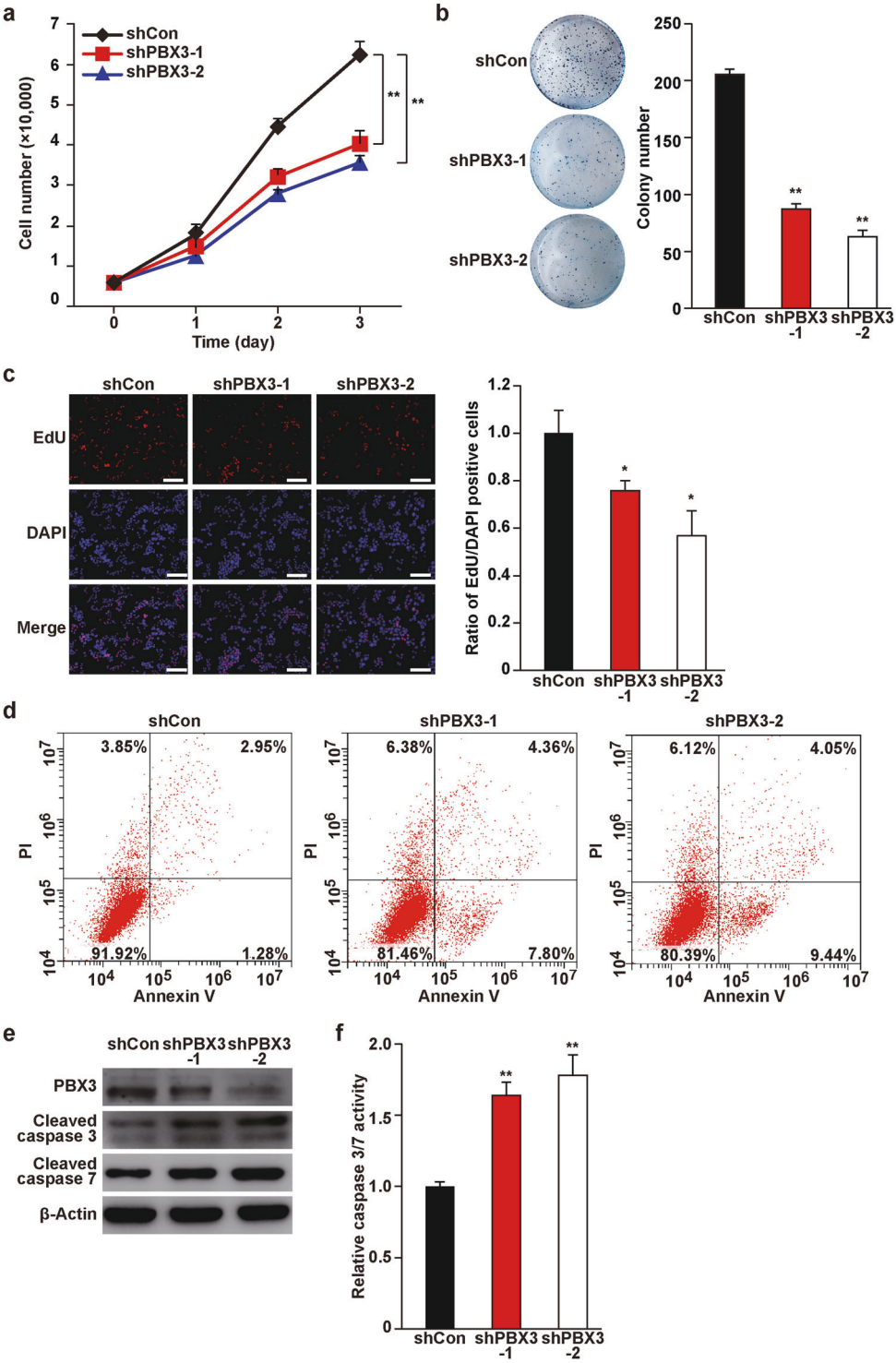

Fig. 3 PBX3 inhibits tumor cell apoptosis and promotes tumor cell proliferation. a The total number of *PBX3*-silenced HCT116 cells was counted at the indicated time points. **b** Colony formation potential of *PBX3*-silenced HCT116 cells. **c** Proliferation potential of *PBX3*-silenced HCT116 cells, as examined using an EdU incorporation assay. Representative images (left) and quantification results (right, $n = 6$) are shown. **d** Apoptosis rate of *PBX3*-silenced HCT116 cells, as examined using Annexin V/PI staining and flow cytometry. **e** Protein levels of cleaved caspase 3 and cleaved caspase 7 in *PBX3*-silenced HCT116 cells, as examined using Western blotting. **f** Relative activity of executioner caspases in *PBX3*-silenced HCT116 cells. β-Actin was used as the loading control for Western blotting. Cells transfected with shCon were used as controls. Quantitative data are shown as relative values with respect to controls and are expressed as the means ± SDs ($n = 3$). shPBX3: shRNA expression vector targeting *PBX3*; $^*P < 0.05$; $^{**}P < 0.01$.

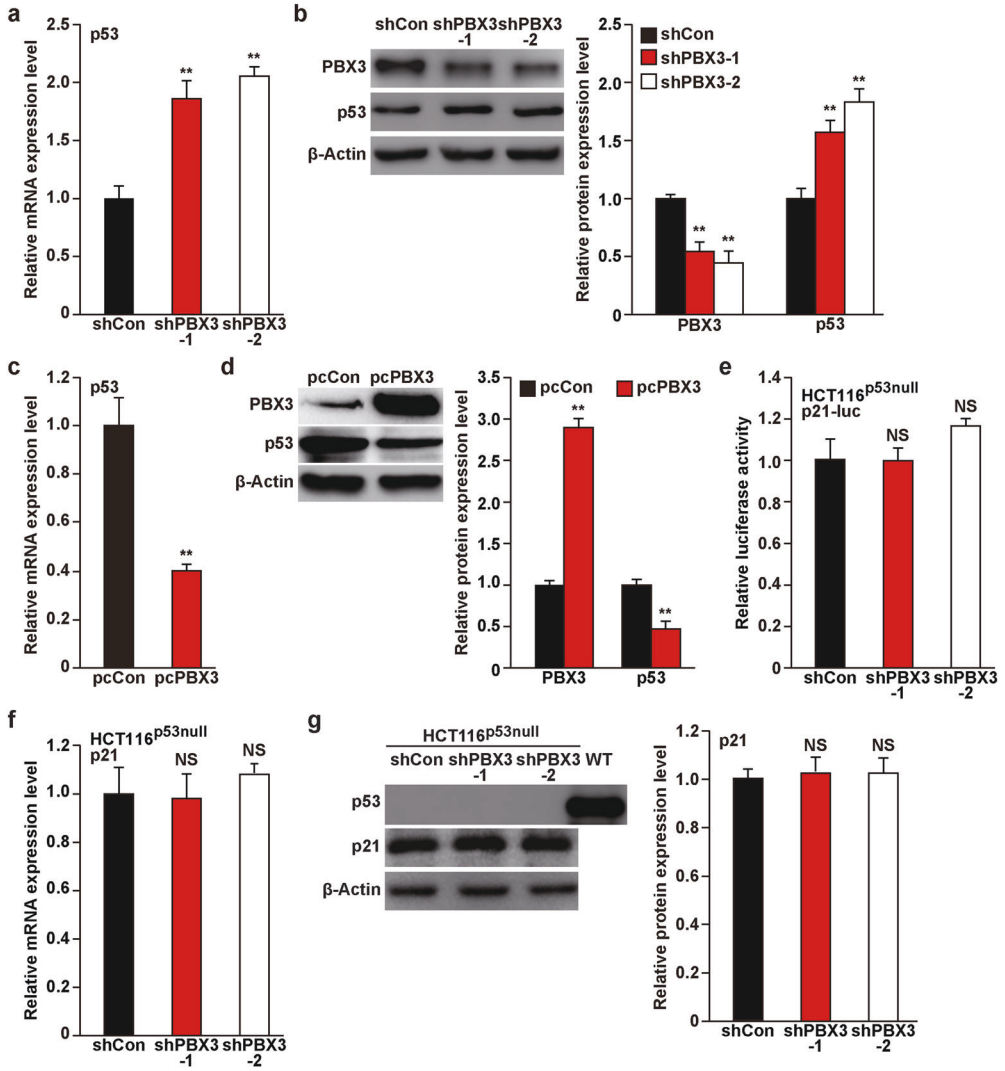

Fig. 4 PBX3 regulates p53 transcriptional activity. a Relative p53 mRNA expression level in *PBX3*-silenced HCT116 cells, as analyzed using qRT-PCR. **b** p53 protein expression level in *PBX3*-silenced HCT116 cells, as examined using Western blotting. **c** Relative p53 mRNA expression level in *PBX3*-overexpressing HCT116 cells, as analyzed using qRT-PCR. **d** p53 protein expression level in *PBX3*-overexpressing HCT116 cells, as examined using Western blotting. **e** Relative activity of the firefly luciferase reporter vector with the *p21* promoter (p21-luc) in *PBX3*-silenced HCT116^{p53null} cells, as analyzed using a dual luciferase reporter assay. **f** Relative p21 mRNA expression level in *PBX3*-silenced HCT116^{p53null} cells, as analyzed using qRT-PCR. **g** p21 protein expression level in *PBX3*-silenced HCT116^{p53null} cells, as determined using Western blotting. β-Actin was used for qRT-PCR normalization and as the loading control for Western blotting. Cells transfected with shCon or pcCon were used as controls. Quantitative data are shown as relative values with respect to controls and are expressed as the means ± SDs ($n = 3$). shPBX3: shRNA expression vector targeting *PBX3*; pcCon: pcDNA3.1(+); pcPBX3: *PBX3* overexpression vector; $^{**}P < 0.01$; NS: not significant.

confirmed that PBX3 is an oncogenic factor that exerts its effects at least partially by negatively regulating the expression of p53.

In summary, our results uncovered a novel role of PBX3 in regulating the p53/p21 axis by binding directly to the *p53* promoter and suppressing p53 expression, leading to enhanced apoptosis resistance, cell proliferation, and subsequent promotion of tumorigenesis (Fig. 8d).

DISCUSSION

PBX3 is a member of the PBX3 family with highly conserved homologous domains [24, 25]. PBX proteins bind specifically to target DNA sequences by interacting with homeobox (HOX) proteins, leading to transcriptional activation or suppression of target genes [26, 27]. Previous studies have revealed that PBX3 is crucial in promoting tumor growth and progression [28]. PBX3 is highly expressed in various tumors, including gastric cancer, CRC, and prostate cancer [18, 29, 30]. PBX3 can be regulated by tumor suppressor microRNAs, including miR-495, miR-129-5p, and miR-302, which leads to the inhibition of tumor cell proliferation, migration, and invasion as well as the induction of tumor cell apoptosis [31–33].

Despite the importance of PBX3 in regulating tumor cell proliferation and tumorigenesis, the role of PBX3 in tumorigenesis, particularly the molecular mechanism by which it regulates tumor cell apoptosis and proliferation, remains unclear. In this study, we found that PBX3 is highly expressed in patients with CRC and can bind directly to the *p53* promoter. Suppression of PBX3 expression increases the promoter activity and the transcription level of the *p53* gene, which in turn upregulates the transcription level of its downstream target gene *p21*. Activation of the p53/p21 axis by *PBX3* silencing leads to an increase in tumor cell apoptosis and a decrease in tumor cell proliferation, reduces the number and colony formation potential of tumor cells and, subsequently,

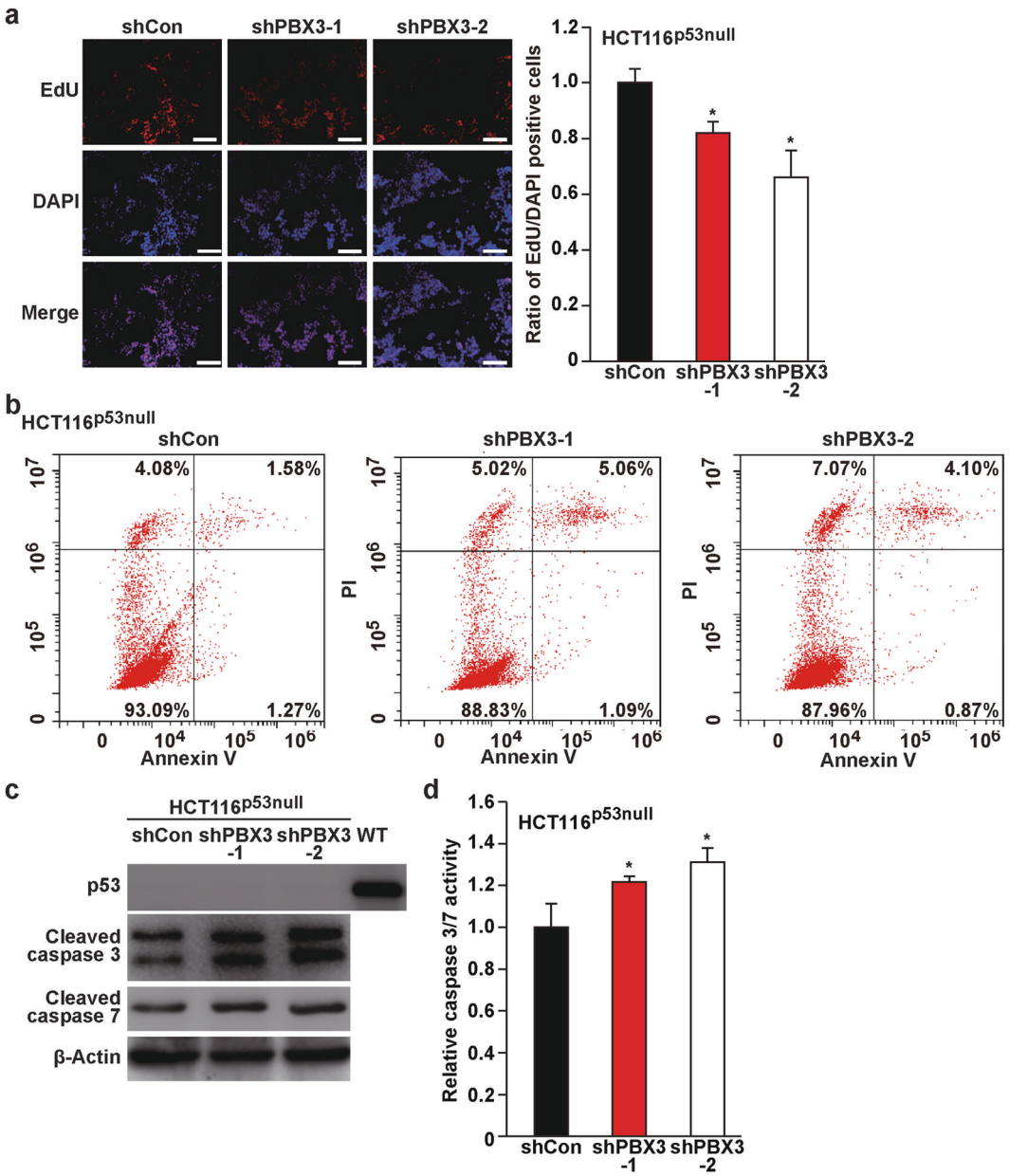

Fig. 5 p53 is crucial for PBX3-mediated regulation of tumor cell apoptosis and proliferation potential. a Proliferation potential of *PBX3*-silenced HCT116^{p53null} cells, as examined using an EdU incorporation assay. Representative images (left) and quantification results (right, $n = 6$) are shown. **b** Apoptosis rate of *PBX3*-silenced HCT116^{p53null} cells, as examined using Annexin V/PI staining and flow cytometry. **c** Protein levels of cleaved caspase 3 and cleaved caspase 7 in *PBX3*-silenced HCT116^{p53null} cells, as examined using Western blotting. **d** Relative activity of executioner caspases in *PBX3*-silenced HCT116^{p53null} cells. β-Actin was used as the loading control for Western blotting. Cells transfected with shCon were used as controls. Quantitative data are shown as relative values with respect to controls and are expressed as the means ± SDs ($n = 3$). shPBX3: shRNA expression vector targeting *PBX3*; *$P < 0.05$.

suppresses tumorigenesis. Hence, our findings suggest a novel molecular mechanism by which PBX3 enhances tumor growth.

Apoptosis evasion and uncontrolled cell proliferation are two of the key hallmarks of cancer, which lead to the loss of cell number homeostasis, an increase in the cell number, and the formation of tumor cell colonies [34, 35]. Furthermore, these hallmarks are closely related to the aberrant expression of oncogenes such as *c-myc*, *KRas*, and *YY1* [36, 37] as well as tumor suppressor genes such as *p53* and *Rb* [38, 39]. Expression of tumor suppressor genes can trigger the activation of caspases, a family of factors that activate the irreversible process of apoptosis, subsequently resulting in an increase in apoptosis. While evasion of apoptosis plays a crucial role in enhancing tumorigenic potential, the regulation of this process has not been fully elucidated. Here, we revealed that suppression of PBX3 expression activated the p53/p21 axis, leading to cleavage and increased activity of executioner caspases 3 and 7. Furthermore, suppression of PBX3 decreased tumor cell proliferation. Hence, our study revealed for the first time that PBX3 regulates tumor cell apoptosis through regulation of the p53/p21 axis. Interestingly, while *PBX3* silencing did not enhance the p21 expression level in *p53*-deficient cells, it still induced caspase cleavage and tumor cell apoptosis while suppressing tumor cell proliferation, although to a significantly lower extent than in tumor cells with wild-type p53. Indeed, while the p53/p21 axis is crucial for regulating tumor cell apoptosis, previous reports have shown that tumor cell apoptosis can also be induced in a

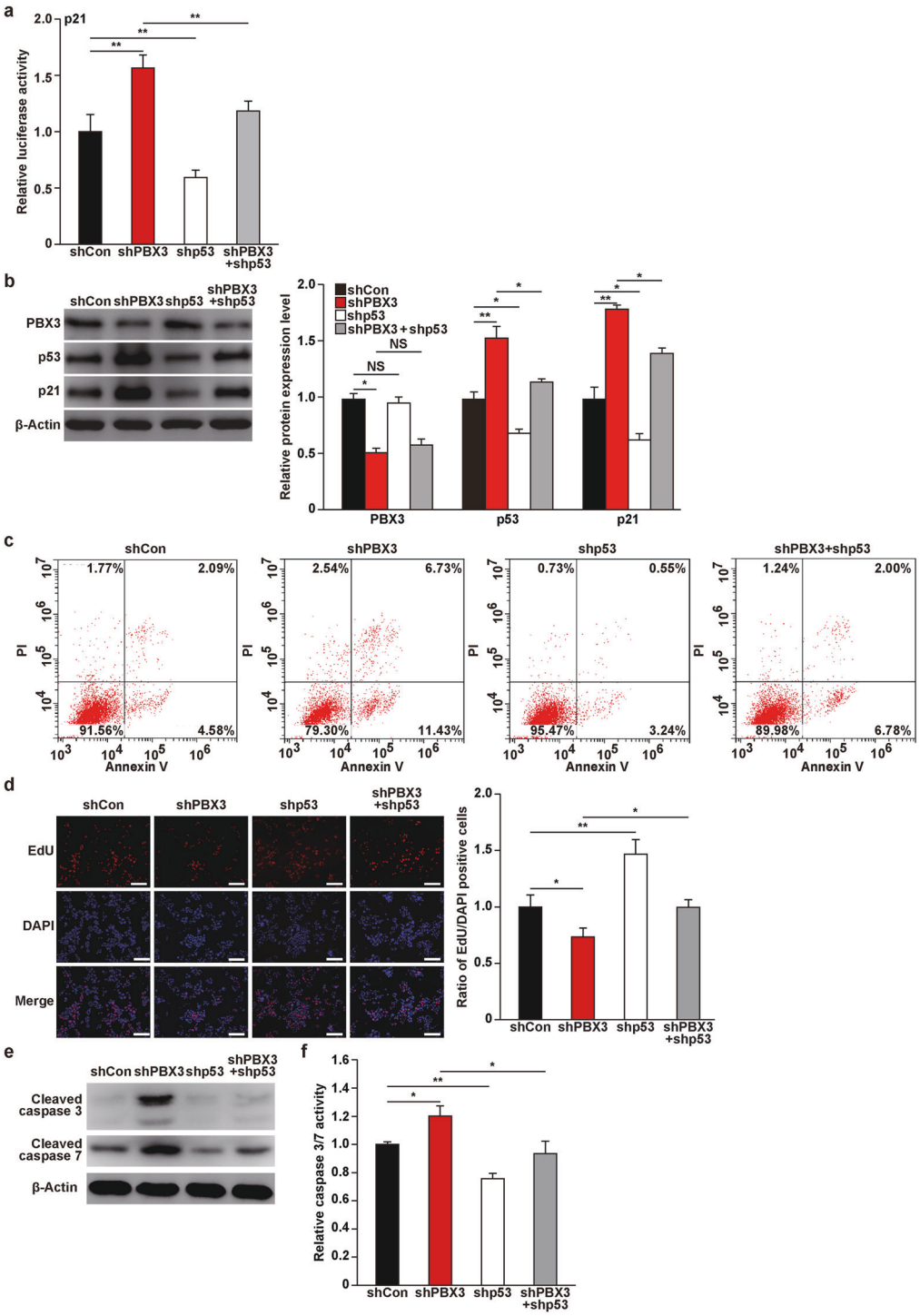

Fig. 6 PBX3 regulates tumor cell apoptosis and proliferation potential through the p53/p21 axis. a Relative luciferase activity of the firefly luciferase reporter vector with the *p21* promoter (p21-luc) in HCT116 cells with double silencing of *PBX3* and *p53*, as analyzed using a dual luciferase reporter assay. **b** p21 protein expression level in HCT116 cells with double silencing of *PBX3* and *p53*, as examined using Western blotting. **c** Apoptosis rate of HCT116 cells with double silencing of *PBX3* and *p53*, as examined using Annexin V/PI staining and flow cytometry. **d** Proliferation potential of HCT116 cells with double silencing of *PBX3* and *p53*, as examined using an EdU incorporation assay. Representative images (left) and quantification results (right, $n = 6$) are shown. **e** Protein levels of cleaved caspase 3 and cleaved caspase 7 in HCT116 cells with double silencing of *PBX3* and *p53*, as examined using Western blotting. **f** Relative activity of executioner caspases in *PBX3* and *p53*-double knockdown HCT116 cells. β-Actin was used as the loading control for Western blotting. Cells transfected with shCon were used as controls. Quantitative data are shown as relative values with respect to controls and are expressed as the means ± SDs ($n = 3$). shPBX3: shRNA expression vector targeting *PBX3*; shp53: shRNA expression vector targeting *p53*; $^{*}P < 0.05$; $^{**}P < 0.01$; NS: not significant.

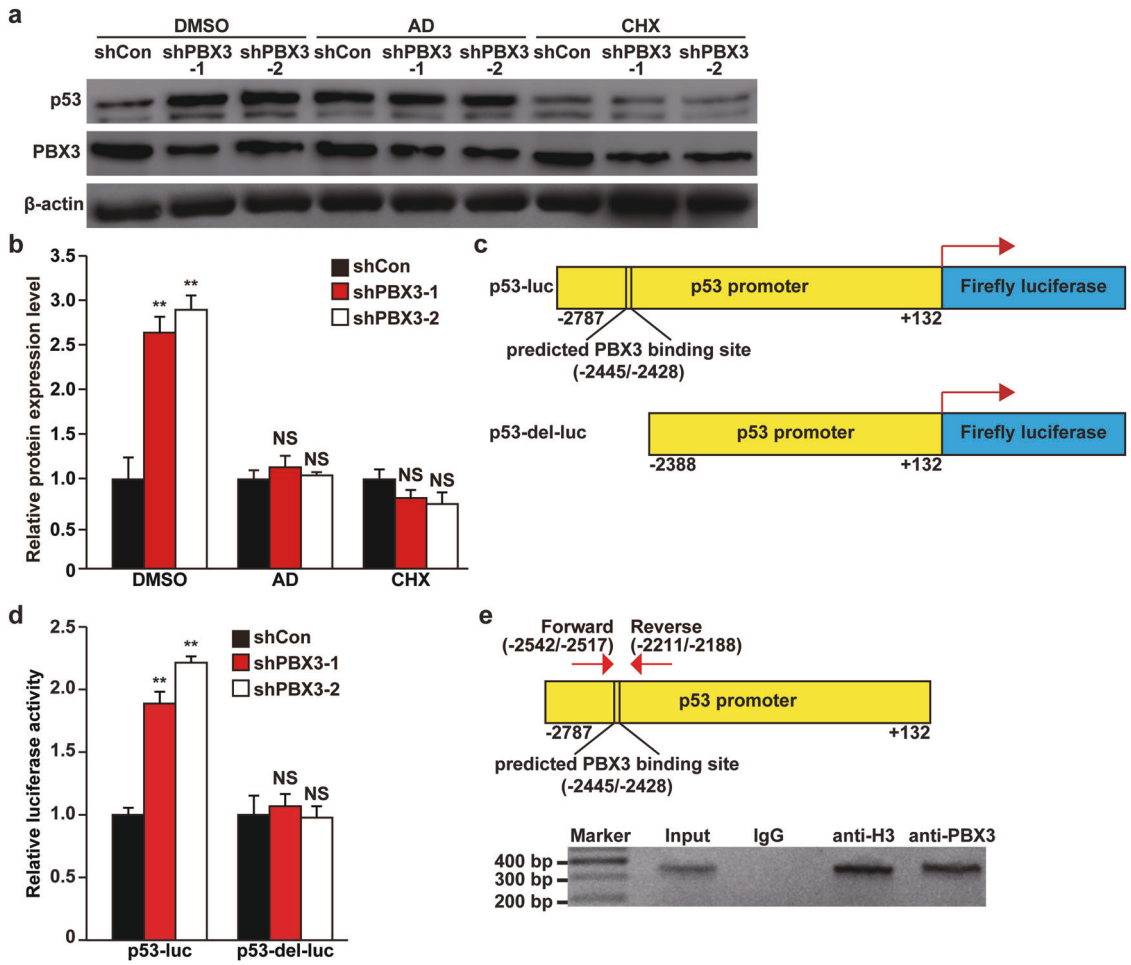

Fig. 7 PBX3 suppresses p53 transcription by binding directly to the p53 promoter and regulating its activity. a, **b** PBX3 and p53 protein expression levels in *PBX3*-silenced HCT116 cells treated with actinomycin D or cycloheximide, as determined using Western blotting. Cells treated with DMSO were used as controls. Representative images (**a**) and quantification results (**b**) are shown. **c** Schematic diagram of the *p53*-promoter reporter vector (p53-luc) and *p53*-promoter reporter vector lacking the predicted PBX3-binding site ($p53^{del}$-luc). **d** Relative luciferase activity of p53-luc and $p53^{del}$-luc in *PBX3*-silenced HCT116 cells. **e** The binding capacity of PBX3 to the predicted region in the *p53* promoter region in HCT116 cells was determined using a ChIP assay with an anti-PBX3 antibody followed by PCR. The predicted PBX3-binding site in the *p53* promoter and the location of the primer pair used for PCR are shown. Quantitative data are shown as relative values with respect to the control and are expressed as the means ± SDs ($n = 3$). shPBX3: shRNA expression vector targeting *PBX3*; $^{**}P < 0.01$; NS: not significant.

p53/p21-independent manner [40, 41]. Collectively, these facts suggest that while p53 is necessary in PBX3-mediated regulation of p21 expression and that PBX3 mainly exerts its regulatory function on apoptosis resistance and tumor cell proliferation by regulating the p53/p21 axis, it might also be able to regulate these processes in a p53-independent manner.

p53, which was discovered in 1979, was the first tumor suppressor gene identified and is one of the most important tumor suppressors identified to date [42]. p53 is crucial for determining cell fate during cell cycle progression by regulating p21, the key regulator of cell cycle progression that suppresses the activity of cyclins and cyclin-dependent kinases at each stage of the cell cycle [43–45]. Increased p53 protein accumulation upon exposure to genotoxic stress activates *p21* transcriptional activity, which in turn induces cell cycle arrest and facilitates DNA repair or initiation of apoptosis if damaged DNA cannot be repaired [8]. Thus, aberrant p53 expression is closely related to uncontrolled proliferation and apoptosis evasion in tumor cells. While p53 mutations can be found in more than 50% of tumors and are closely related to tumor progression and poor prognosis [10, 46–48], more recent studies have shown that the expression of p53 is also frequently downregulated in patients with the wild-type *p53* gene. This indicates the importance of aberrant regulation of p53 expression in promoting tumorigenesis [10–12, 49]. Regulation of p53 expression can occur not only at the transcriptional and translational levels but also at the posttranslational level through ubiquitination, which induces p53 protein degradation, and phosphorylation, which leads to p53 activation [50–52]. Herein, our results revealed that PBX3 regulates p53 expression at the transcriptional level by binding directly to the *p53* promoter, resulting in the suppression of its transcriptional activity. This suppression in turn enhances tumor cell proliferation, apoptosis evasion and, subsequently, tumorigenesis. Furthermore, recent studies revealed that p53 is also crucial for promoting other hallmarks of cancer, including angiogenesis, metabolic reprogramming, epithelial-to-mesenchymal transition, invasion, and metastasis [4, 9, 53]. Therefore, while further detailed investigations are needed, our findings suggest the possibility that PBX3 might also promote tumorigenesis by regulating other hallmarks of cancer.

Overall, in this study, we identified PBX3 as a novel regulator of the p53/p21 axis and revealed, for the first time, its mechanism in promoting tumor growth by regulating tumor cell apoptosis resistance and proliferation through the p53/p21 axis. Our findings are the first to reveal the relationship between PBX3 and p53, providing new insights into the regulation of *p53* transcriptional

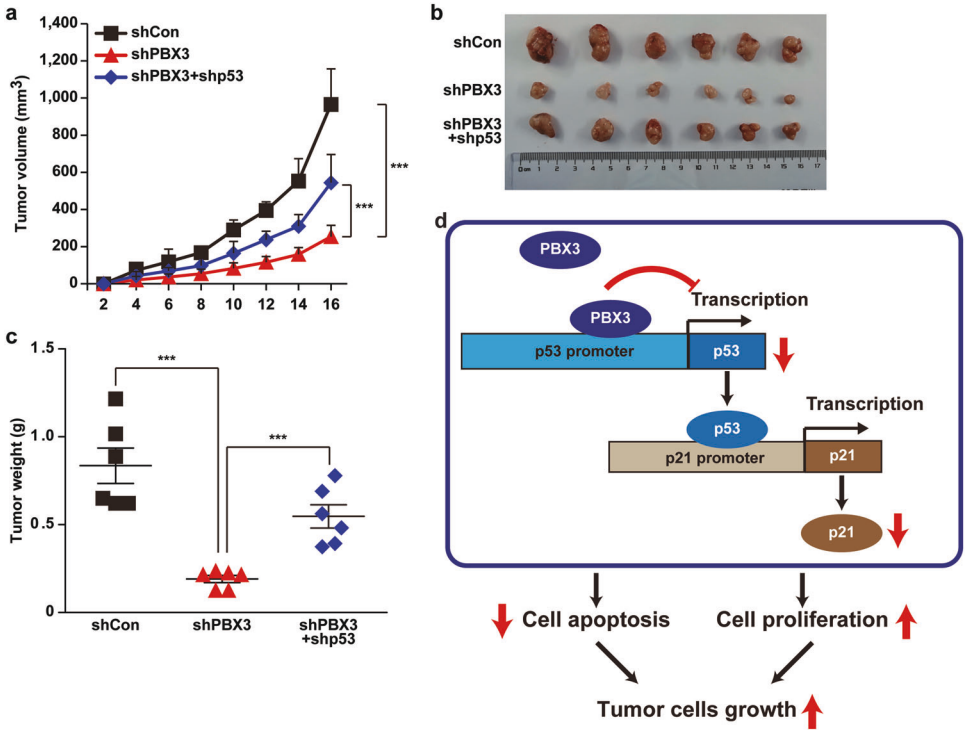

Fig. 8 PBX3-mediated regulation of p53 is crucial for its tumorigenic potential. a–c The tumorigenic potential of *PBX3*-silenced HCT116 cells and HCT116 cells with double silencing of *PBX3* and *p53* was examined in vivo by subcutaneous injection of these cells into BALB/c-*nu/nu* mice ($n = 6$). Tumor volumes at the indicated time points (**a**) and morphological images of the tumors generated (**b**) are shown. **c** Tumors were weighed on day 16 after transplantation. **d** Schematic diagram of PBX3-mediated regulation of tumor cell apoptosis and proliferation potential through the p53/p21 axis. Cells transfected with shCon were used as controls. shPBX3: shRNA expression vector targeting *PBX3*; ***$P < 0.001$

activity as well as into the physiological and pathological functions of PBX3. Furthermore, our findings demonstrate a potential strategy for targeting PBX3 in cancer therapy.

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AUTHOR CONTRIBUTIONS

VK and SRW conceived the project, analyzed and interpreted the experimental results; VK designed the most experiments of the project and wrote the manuscript. WFL and AH performed most of the experiments. YT and PH performed part of plasmid constructions and cell counting assay. GBS analyzed part of the data and provided part of the material. MM designed shRNA target sites and analyzed part of the data.

ADDITIONAL INFORMATION

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Competing interests: The authors declare no competing interests.

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