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# BAF180 Is a Critical Regulator of p21 Induction and a Tumor Suppressor Mutated in Breast Cancer

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## Abstract

**Screening for tumor suppressor genes in breast cancer revealed multiple truncating mutations of *PBI*, which encodes the BAF180 subunit of the PBAF chromatin remodeling complex. Mutation was associated with loss of heterozygosity of the wild-type allele. BAF180 complementation of BAF180-mutant tumor cells caused G<sub>1</sub> arrest that was dependent on increased expression of the cyclin/cyclin-dependent kinase inhibitor p21/WAF1/CIP1. Endogenous wild-type BAF180 bound to the p21 promoter and was required for proper p21 expression and G<sub>1</sub> arrest after transforming growth factor- $\beta$  and  $\gamma$ -radiation treatment. BAF180 thus functions on two tumor suppressor signaling pathways as a physiologic mediator of p21 expression. We conclude that BAF180 suppresses tumorigenesis, at least in part, through its ability to regulate p21. [Cancer Res 2008;68(6):1667–74]**

## Introduction

Many tumor suppressor genes that are commonly mutated in cancer are involved in the regulation of the cell cycle. These include genes that encode proteins such as Rb and p16, which are directly involved in the cell cycle, and proteins such as p53 and SMAD, which modulate the transcription of cyclin/cyclin-dependent kinase (CDK) inhibitors in response to specific signals (1–4). SWI/SNF complexes regulate gene expression in response to environmental stimuli through the remodeling of chromatin. Genetic alterations of two SWI/SNF subunits, hSNF5/INI1/BAF47 (hSNF5) and BRG1, are associated with the suppression of tumor development, which likely occurs through their ability to regulate cell cycle factors (5–13). hSNF5 and BRG1 are found in several distinct SWI/SNF complexes and have been shown to inhibit the cell cycle primarily via their regulation of CDK inhibitors (11, 12, 14). The BRG1 complexes are heterogeneous and can be separated into the SWI/SNF A and B complexes (15). The SWI/SNF B complex (also known as PBAF) is distinguished from the A complex because it contains BAF180 (16, 17). It has been shown that PBAF purified with an anti-BAF180 affinity column is required for nuclear receptor-mediated transcription *in vitro* (18). Recent analysis has further resolved PBAF to include BAF200 (19). Whereas BAF200, but not BAF180, is critical for the induction of IFN- $\alpha$  target genes *in vivo*, it has been

shown that BAF180 is critical for retinoic acid-dependent gene activation in cells (20).

We encountered the *PBI* gene, which encodes BAF180, during a screen for homozygous deletions in human breast tumors to identify novel tumor suppressor genes. Truncating mutations of *PBI* were mapped in different breast tumor samples. These findings encouraged us to pursue the mechanism through which BAF180 could suppress breast epithelial tumorigenesis. Complementation of BAF180 in a mutant tumor cell line reduced cell growth through inhibition of the cell cycle. Western blotting was used to screen for potential cell cycle factors affected by BAF180, which revealed induction of p21/WAF1/CIP1. RNAi, chromatin immunoprecipitation, quantitative reverse transcription-PCR (RT-PCR), and cell signaling were used to determine that BAF180 is a direct regulator of p21. We found that BAF180 binds to the p21 promoter and regulates baseline and signal-dependent p21 transcription, thus providing a plausible explanation for its genetic inactivation in tumors.

## Materials and Methods

**Cell culture and breast tumors.** Twenty-six breast cancer cell lines were obtained from American Type Culture Collection (ATCC). Eight breast cancer cell lines (HCC38, HCC1143, HCC1187, HCC1395, HCC1428, HCC1806, HCC1937, and HCC2157) and paired lymphoblastoid lines were provided by Dr. Adi Gazdar (University of Texas Southwestern Medical Center, Dallas, TX). Nine breast cancer cell lines (SUM44, SUM52, SUM149, SUM159, SUM185, SUM225, SUM229, SUM190, and SUM1315) were obtained from Dr. Stephen Ethier (Wayne State University School of Medicine, Detroit, MI). The original primary tumor tissue and paired normal DNA for SUM1315 were provided by Dr. Douglas Schwartzentruber (National Cancer Institute, Bethesda, MD). MCF10A cell line was purchased from ATCC and grown in DMEM/F-12 in the presence of 5% horse serum, 20 ng/mL epidermal growth factor (EGF), 10  $\mu$ g/mL insulin, 100 ng/mL cholera toxin, and 0.5  $\mu$ g/mL hydrocortisone. SUM1315 cells were grown in Ham's F-12 in the presence of 10 ng/mL EGF, 5  $\mu$ g/mL insulin, and 5% fetal bovine serum (FBS). HCC1143 and BT549 cells were grown in RPMI 1640 with 10% FBS. Breast tumor xenograft Bx41 (BCA-4) was provided by Rajeshwari R. Mehta (University of Illinois at Chicago, Chicago, IL). Genomic DNA samples from human breast primary tumors were provided by Dr. Hanina Hibshoosh (Department of Pathology, Columbia University Medical Center, New York, NY) with permission from the Institutional Review Board.

**Antibodies.** BAF180 polyclonal antibodies were generated against glutathione *S*-transferase fusion proteins of BAF180 fragments (amino acids 1–938 and 736–1,475). p21 antibodies were purchased from Santa Cruz and PharMingen (clone SXM30). Tubulin monoclonal antibody (Tu27) was purchased from Covance. p15 polyclonal antibody and cyclin E monoclonal antibody were obtained from Santa Cruz. p27 and cyclin D1 monoclonal antibodies were from NeoMarkers.

**Representational difference analysis.** Representational difference analysis (RDA) was done as previously described with some modifications (21). Briefly, tumor DNA (HCC1143) was used to drive the subtractions,

**Note:** Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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whereas corresponding normal DNA (HCC1143BL) was used as the tester. One microgram of DNA was digested with *Bgl*II and ligated to adaptors. The amplicons were by PCR to generate tester (normal lymphoblast) and driver (cancer cell line). After removing adaptors from amplicons and changing adaptors on tester amplicon, subtractive hybridization was done using 40 µg of driver and 500 ng of tester. First-round PCR with optimal cycles was done using 50 ng of hybridized DNA as a template. Remaining ssDNA was removed by digestion with mung bean nuclease (New England Biolabs). This produced the first-round RDA product. After the third round of RDA, the final PCR products were digested with *Bgl*II to remove adaptors and then cloned into pZero-2 vector (Invitrogen) for PCR and sequencing.

**Mutation screening.** Total cellular RNA and polyadenylate RNA were prepared using the Qiagen RNeasy Kit (Qiagen) and QuickPrep Micro mRNA Kit (Pharmacia), respectively, according to manufacturer's instructions. RNA was reverse transcribed using Superscript II (Life Technologies, Inc.) and the reaction was diluted to 100 µL. We used 2 µL of cDNA for PCR amplification with 40 cycles of 95°C for 30 s, 58°C for 1 min, 70°C for 2 min. Four sets of primers (set 1, 5'-GGATCCTAATACGACTCACTATAGGGAGAC-CACCATGGGTTCCAAGAGAAGAAGAGC and 5'-TTTTCTTTGAAGGCAAA-TGGTAA; set 2, 5'-GGATCCTAATACGACTCACTATAGGGAGACCACCAT-GGCCTTTATGGATGTTTCAAATCCT and 5'-GGAATTTCTGCTAAAGAAT-CGC; set 3, 5'-GGATCCTAATACGACTCACTATAGGGAGACCACCATGG-TTGAGGACTTTGTCATGAT and 5'-ATGGCTCCTTCTGAGGAACA; set 4, 5'-GGATCCTAATACGACTCACTATAGGGAGACCACCATGGACAAGCAGAT-GAAGAAATTCAAA and 5'-AATCTTTGTGTATTGATAAAGTC) were used to synthesize the entire BAF180 coding sequence. The PCR product was treated with exonuclease I and shrimp alkaline phosphatase and sequenced using PCR primers. The protein truncation test was done by TNT-quick coupled transcription/translation systems (Promega). The synthesized proteins were analyzed by SDS-PAGE and autoradiography. Exons were amplified from genomic tumor DNA and sequenced on both strands to identify somatic mutations (primers available on request).

**Screening for loss of heterozygosity.** Normal and tumor DNA sample pairs of human breast tissue were screened for loss of heterozygosity (LOH) by the Genome Center of Columbia University. Microsatellite markers flanked *PBI* (D3S1578 and D3S3640). The data were analyzed using the program Genotyper 2.0.

**Colony suppression assay.** Cells were transfected with pBabepuro-BAF180 or pBabepuro and selected with puromycin for 2 wk. Colonies were stained with crystal violet and counted. All experiments were done in triplicate. A two-tailed *t* test was used to test for significant differences between means of colony numbers.

**Chromatin immunoprecipitation assay.** Ten million cells were collected and cross-linked with formaldehyde at 37°C, neutralized with glycine, and sonicated using Misonix 3000 sonicator. After incubation with Salmon Sperm DNA/Protein A beads, the sonicated lysate was diluted and incubated with polyclonal antibody against BAF180 and protein A beads. The beads were washed and eluted. The elution was incubated at 65°C for 4 h to reverse the cross-linking after adjustment of NaCl concentration. The DNA was purified with Qiagen PCR Purification Kit and subjected to PCR. The sequences of the primer pair that span the region -879/-593 of p21 promoter were adopted from the publication of Giraud et al. (22): 5'-TTCAGGAGACAGACAACCTCACTC and 5'-GACACCCCAACAAAGCA-TCTTG. The product was amplified for 35 cycles. Primers used for other regions are as follows: -297F/+8R, 5'-CAGATTTGTGGCTCACTTCGTG and 5'-GACTTCGGCAGCTGCTACACC (11); -2,760/-2,486, 5'-TTGTGC-CACTGCTGACTTTGTC, 5'-AGCCTGAAGAAGGAGGATGTGAGG; +7,786/+7,987 (exon 3), 5'-TGTTGAGCCTTTCCCTCTTT and 5'-GGTGCCCTTC-TTCTGTGTGT (22).

**Cell cycle analysis and sorting.** Cells were either stained with Hoechst 33342 (Sigma; 10 µg/mL) or fixed with 80% ethanol in PBS and then incubated with propidium iodide (Sigma; 0.05 mg/mL in PBS with 0.3% Igepal CA-630) plus DNase-free RNase A (Roche; 1 mg/mL). Stained cells were subjected to fluorescence-activated cell sorting (FACS) analysis using BD LSRII. The results were analyzed with the FlowJo software (Tree Star). Sorting was done using BD FACSARIA.

**Quantitative RT-PCR.** Total RNA was extracted using Qiagen RNeasy Mini Kit and quantified with Nanodrop (ND-1000 UV-Vis) spectrophotometer for the purpose of normalization. Reverse transcription was carried out according to the manufacturer's instruction using SuperScript II reverse transcription and random primer from Invitrogen. Quantitative real-time PCR was done on Stratagene Mx3000P system. The following primers were used for PCR reactions: tubulin, 5'-GGTCAGTGTGGCAACCAGAT and 5'-AGGATGGCACGAGGAACATA; p21, 5'-CCGAAGTCAGTTCTTGTGG and 5'-GCCATTAGCGCATCACAG; and MXA, 5'-CGTGGTGATTAGCAG-GAAG and 5'-TGCAAGGTGGAGCGATTCTG. MXA and p21 were normalized to tubulin levels.

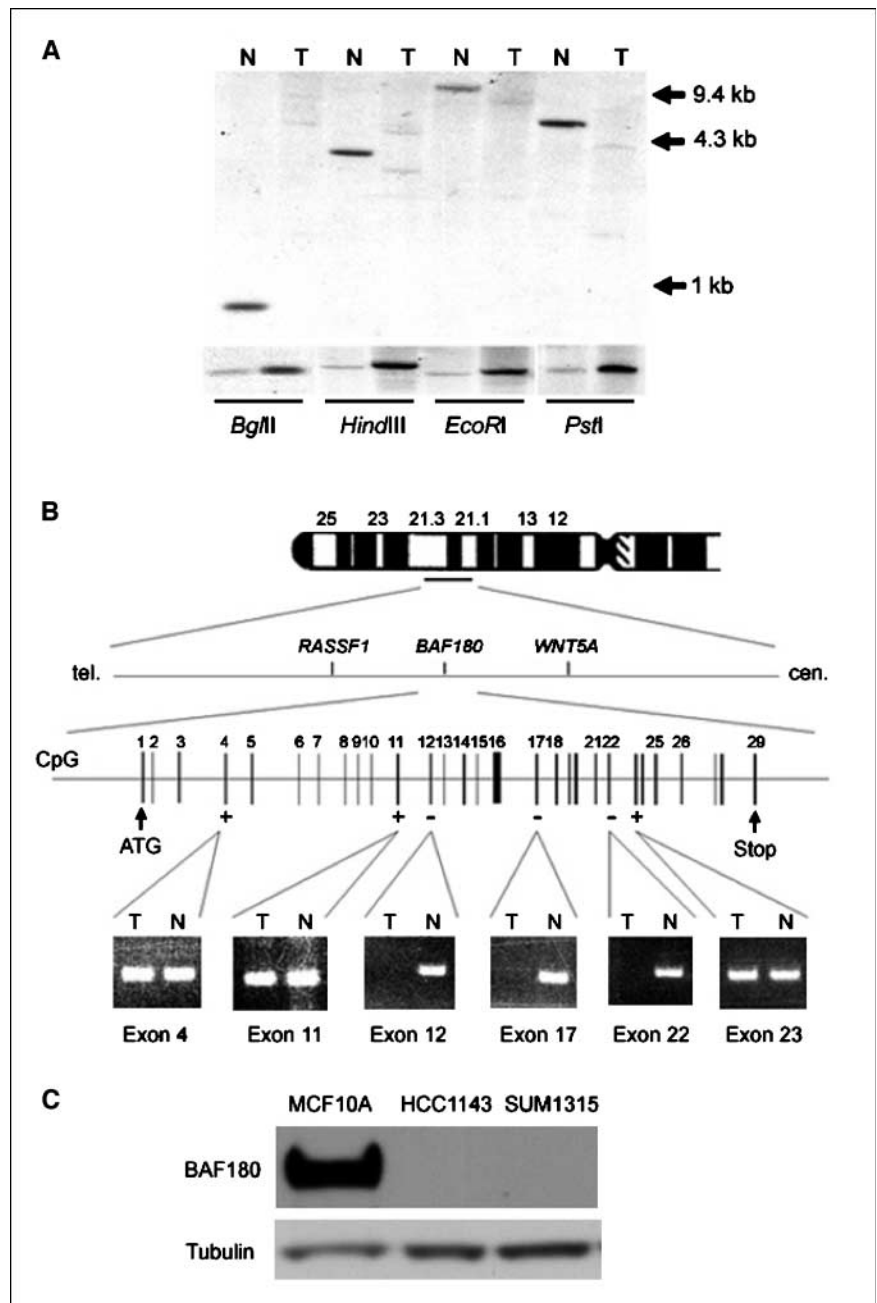
**Transfection.** Full-length cDNA was cloned into pBABEpuro, pIRES-EGFP, and pQBI25. Cells were transfected with either Nucleofector (Amaxa) or Lipofectamine 2000 (Invitrogen). siControl Nontargeting siRNA #1 and siGenome SMARTpool Upgrade siRNA oligos for BAF180 (*PBI*) were obtained from Dharmacon (D-008692). SignalSilence p21 siRNA was obtained from Cell Signaling. A second siRNA targeted to p21 (*CDKN1A*) at 5'-CTGGCATTAGAATTATTTAA-3' was obtained from Qiagen. BAF180 and p21 siRNAs were tested for their ability to activate the IFN response by testing transfected cells for MXA expression using quantitative RT-PCR. No evidence of MXA activation was detected in either HCC1143 or MCF10A (data not shown).

## Results and Discussion

**Genetic alterations of *PBI* leading to truncations of BAF180 occur in human breast cancer.** To identify a candidate tumor suppressor gene through the mapping of homozygous deletions, we performed genomic subtraction using representational difference analysis on a human breast cancer cell line, HCC1143, and a paired Epstein Barr virus-transformed lymphoblastoid cell line derived from the same patient, HCC1143BL (21). After three rounds of subtraction, one cloned fragment was amplified in the lymphoblastoid but not the tumor line and was located within the *PBI* gene on chromosome 3p21. To confirm the potential homozygous deletion, genomic Southern blot analysis was done on the paired normal and tumor lines with the cloned fragment serving as the probe. As shown in Fig. 1A, RDA clone, 1143-75, was homozygously deleted in HCC1143 but not in the corresponding peripheral blood cell line, HCC1143BL. PCR analysis of the *PBI* locus showed that the homozygous deletion was circumscribed, generating an intragenic deletion including exons 12 to 22 (Fig. 1B). Lack of full-length BAF180 protein in HCC1143 line was confirmed by Western blot with polyclonal antibodies generated against BAF180 (Fig. 1C).

Mutation screening of breast cancer cell lines with four overlapping wild-type BAF180 RT-PCR products beginning in the first coding exon (exon 3) and spanning the entire open reading frame identified two novel truncating mutations. Case Bx41 derived from an early-passage xenograft sample showed an aberrant RT-PCR transcript with primer pair 1 (exons 3-13) that produced a truncated protein when translated *in vitro*; no wild-type transcript was detected in this sample although this primer pair readily detected the wild-type transcript in other samples (Supplementary Fig. S1). Sequencing of the Bx41 transcript revealed a duplication of exons 5, 6, and 7, leading to the formation of a stop codon (TGA) at codon 222 (Fig. 2A). The second case, breast cancer cell line SUM1315, had an abnormal transcript using primer set 2 (exons 13-18) with no detectable wild-type expression in this sample, which was detected in the other samples examined (Fig. 2B). SUM1315 was verified to have a duplication of exons 16 and 17, which generated a stop codon (TAA) at codon 646 (Fig. 2D), and expressed no detectable full-length BAF180 protein (Fig. 1C). To

**Figure 1.** Intragenic homozygous deletion within *PB1* encoding BAF180 in the breast cancer cell line HCC1143. **A**, Southern blot of human breast cancer cell line HCC1143 DNA showing homozygous deletion of RDA clone 1143-75. RDA fragment, 1143-75, is used as a probe. *N* and *T*, the DNA of Epstein Barr virus-transformed lymphoblastoid cell line HCC1143BL and breast cancer cell line HCC1143, respectively. DNAs were digested by *Bgl*II, *Hind*III, *Eco*RI, and *Pst*I. Bottom, loading control using another RDA fragment as a probe. **B**, HCC1143 has an intragenic homozygous deletion of *PB1/BAF180*. A schema of normal 3p21 region is shown with genes (not to scale) represented by top horizontal line. Blowup of exon structure is shown below with exons indicated with vertical bars. PCR analysis of exons using DNA of HCC1143 and HCC1143BL as templates (–) from tumor DNA and showed homozygous deletion. Tumor DNA was amplified (+) in exons 4, 11, and 23. **C**, Western blot confirming the absence of BAF180 full-length protein in HCC1143 and SUM1315 cells, with MCF10A used for positive control.

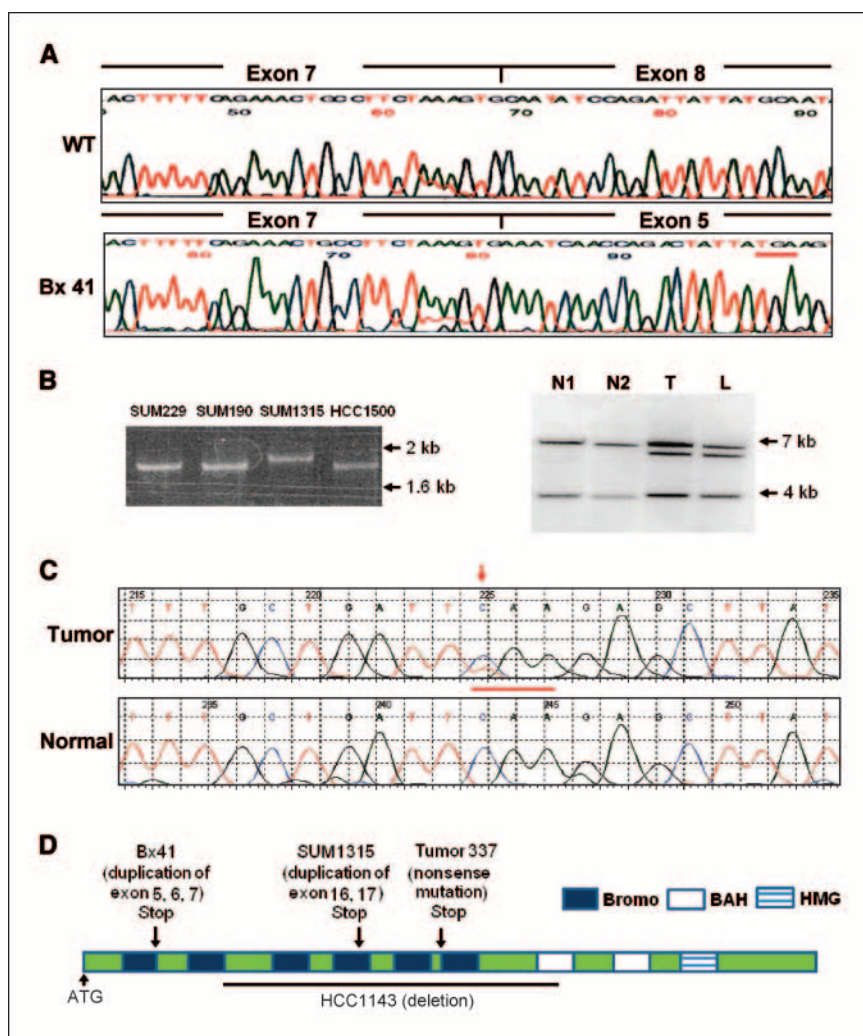


investigate whether this rearrangement occurred in the metastatic lymph node from which the cell line was derived, genomic DNA blot analysis was carried out on the tumor biopsy (Fig. 2B). Using the duplicated exons as a probe, an extra band was detected in the metastatic lymph node as well as the tumor cell line DNA, but not in the paired nontumor DNA sample from the same patient. These data showed that the rearrangement was a somatic alteration that occurred in the patient. We then examined HCC1143, SUM1315, and BX41 for LOH using high-density (250K) single nucleotide polymorphism arrays with dChip software and found that all three lines had strong evidence for LOH of BAF180 (Supplementary Fig. S2; ref. 23). For primary tumors, LOH analysis was done using paired DNA samples from normal and tumor tissue. Fifty-two pairs of genomic DNA samples were screened using two microsatellite

markers that flank the locus of BAF180 on 3p21. Of these 52 tumor samples, 25 (48.1%) had LOH, which suggests that loss of BAF180 could contribute to tumor progression. To look for further evidence for the involvement of BAF180 in tumorigenesis, we sequenced the exons of BAF180 in these tumors to screen for mutations. A nonsense mutation was found in exon 18, which encodes the last two bromo domains of BAF180 (Fig. 2C). Thus, we have identified four truncating mutations of BAF180, all of which occur in the bromo domains and are associated with loss of wild-type BAF180 (Fig. 2D).

**Reexpression of BAF180 in BAF180-deficient cells induces cell growth arrest in G<sub>1</sub> phase.** The genetic data shown above suggested that BAF180 may have tumor suppressor activity. To test the growth inhibition potential of BAF180, exogenous BAF180





**Figure 2.** Genetic alterations of BAF180 in breast cancer. *A*, genetic alteration of BAF180 in Bx41. Sequence analysis indicates that Bx41 has duplication of exons 5, 6, and 7, producing stop codon at codon 222. The stop codon is underlined. *B*, genetic alteration of BAF180 in breast cancer and its derived cell line SUM1315. *Left*, the RT-PCR product of SUM1315 showed an aberrant transcript. The set 2 primers were used. *Right*, Southern blot of tumor genomic DNA shows duplication of exons in tumor biopsy taken from lymph node (lane 3) and in the cell line from the same patient SUM1315 (lane 4). Rearranged fragment was detected in lanes 3 and 4, but not in lane 1 (unrelated normal blood DNA) and lane 2 (normal blood DNA from the same patient). DNAs were digested with *Bgl*II. The RT-PCR product of exon 15 through exon 17 was used for a probe. *C*, genetic alteration of BAF180 in case 337. Exon sequencing revealed a nonsense mutation on exon 18 in a primary tumor sample (case 337). A corresponding normal sequence (case 336) is shown for comparison. *D*, domains and mutations of BAF180. The BAF180 cDNA encodes a protein of 1,635 amino acids. Mutations are indicated with arrows. Homozygous deletions are indicated with black bars.

was reexpressed in mutant BAF180 HCC1143 cells. Reexpression of BAF180 reduced colony number compared with the empty vector control ( $P < 0.01$ ) and the colony size as well (Fig. 3A and B).

To understand the mechanism through which BAF180 inhibited the colony formation of breast tumor cells, flow cytometry was done on fused GFP-BAF180-transfected HCC1143 cells. Cells that were positive for green fluorescence from either GFP vector or GFP-BAF180 were subjected to cell cycle analysis. It was found that the expression of GFP-BAF180 caused a significant increase of  $G_1$  population in HCC1143 cells compared with the controls (Fig. 3C and D). We included two controls, the empty vector control that produces GFP in the cytoplasm and an H2B-GFP fusion control that localizes in the nucleus (Fig. 3C; data not shown). Taken together, these data indicate that BAF180 plays a role in the regulation of the  $G_1$ -S transition of the cell cycle when reintroduced into mutant cells.

**p21 is induced by BAF180 reexpression in mutant breast tumor lines and is required for  $G_1$  arrest.** To determine the signaling pathway through which BAF180 mediates cell cycle regulation, we checked the protein levels of several cyclins (cyclins A, D1, and E) and cyclin-dependent kinase inhibitors (p15, p16, p21, and p27) in BAF180-transfected cells. Reexpression of BAF180 in mutant HCC1143 cells up-regulated the protein level of p21

(Fig. 4A). BAF180 reexpression also up-regulated p21 in another BAF180 mutant line SUM1315 (Fig. 4D). p16 was not expressed in SUM1315 and HCC1143 because of deletion of exon 1 in SUM1315 and methylation in HCC1143 (data not shown). There were no significant changes in the cyclins, p15, or p27 at the protein level on BAF180 reexpression. To determine whether p21 is required for BAF180-mediated cell cycle inhibition, p21 was knocked down with siRNA in GFP-BAF180-expressing HCC1143 cells. As expected, p21 knockdown using two different RNAi oligonucleotide duplexes specific for p21 partially rescued the cell cycle arrest induced by BAF180 reexpression (Fig. 4B and C; Supplementary Fig. S3).

**BAF180 regulates p21 mRNA and binds the p21 promoter.** Using three different RNAi oligonucleotide duplexes for BAF180, we knocked down BAF180 in a normal human breast epithelial cell line, MCF10A. As predicted, the p21 protein decreased (Fig. 5C). To determine whether BAF180 regulates p21 at the mRNA level, quantitative RT-PCR was done to measure mRNA levels of p21 in the presence or absence of the BAF180 knockdown. Total RNA from MCF10A cells that were transiently transfected with either nontargeting or BAF180 siRNA oligos was subjected to reverse transcription reaction. The products of reverse transcription reaction were quantified by quantitative RT-PCR. We showed that knockdown of BAF180 led to a reduction in the level of p21 mRNA (Fig. 5B), suggesting that BAF180 could regulate the transcription

of p21 at its promoter. The protein lysates corresponding to the quantitative RT-PCR results showed decreased protein levels of p21 commensurate with the reduction in p21 mRNA (Supplementary Fig. S4), which suggests that BAF180 regulates p21 solely at the level of mRNA expression.

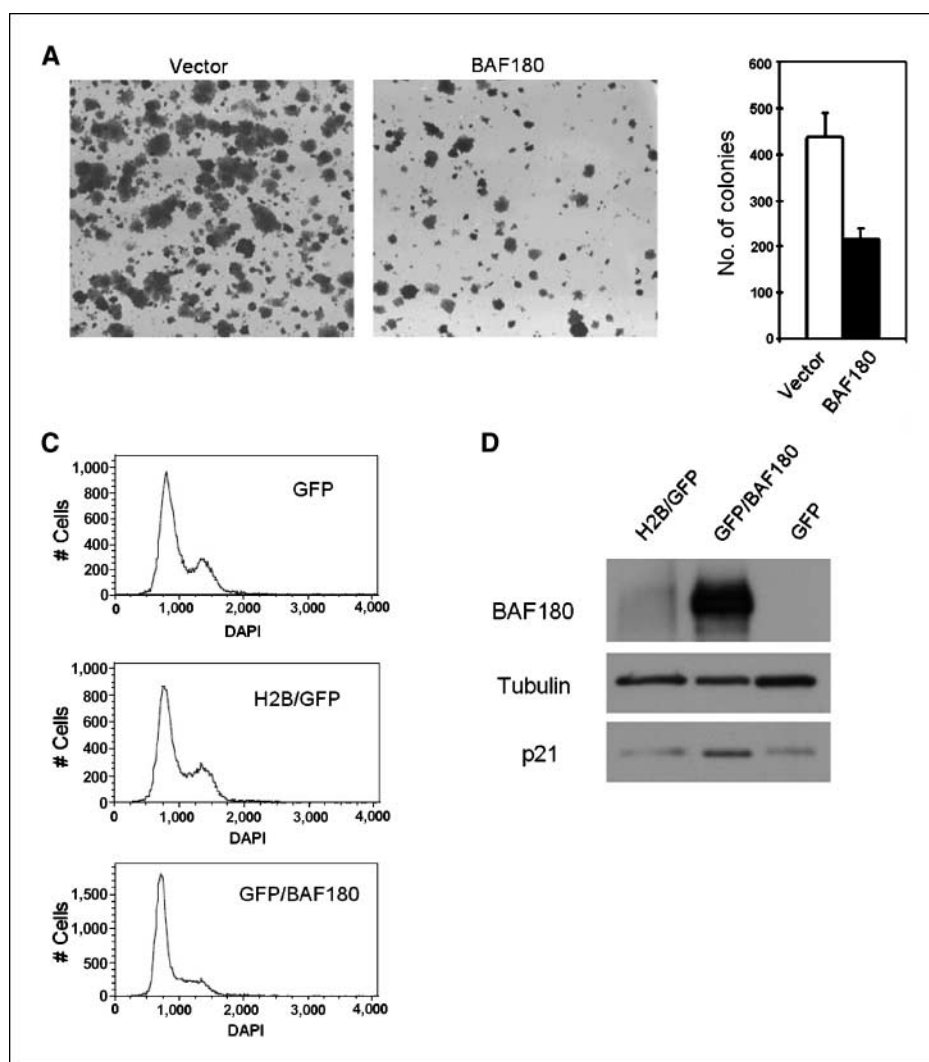
To determine whether BAF180 interacts with the p21 promoter, we performed chromatin immunoprecipitation assays with the breast tumor cell line MDA-MB-468, which expresses full-length BAF180. After cross-linking and sonication, endogenous BAF180 was immunoprecipitated. The genomic DNA fragments associated with BAF180 were subjected to PCR using several pairs of primers that amplify different regions of the p21 promoter, including primers used for mapping Brg1-binding sites (11, 22). The primer pair that amplified the region -879/-593 of the p21/WAF1 promoter generated the brightest band and the primer pairs that flanked this region (-297/+8 and -2,760/-2,486) also amplified a band of expected size albeit with much weaker intensity (Fig. 5A). No amplified products were seen in samples precipitated with preimmune serum. These data show that BAF180 binds to the p21 promoter and contributes to the physiologic expression of p21 in cells grown in serum.

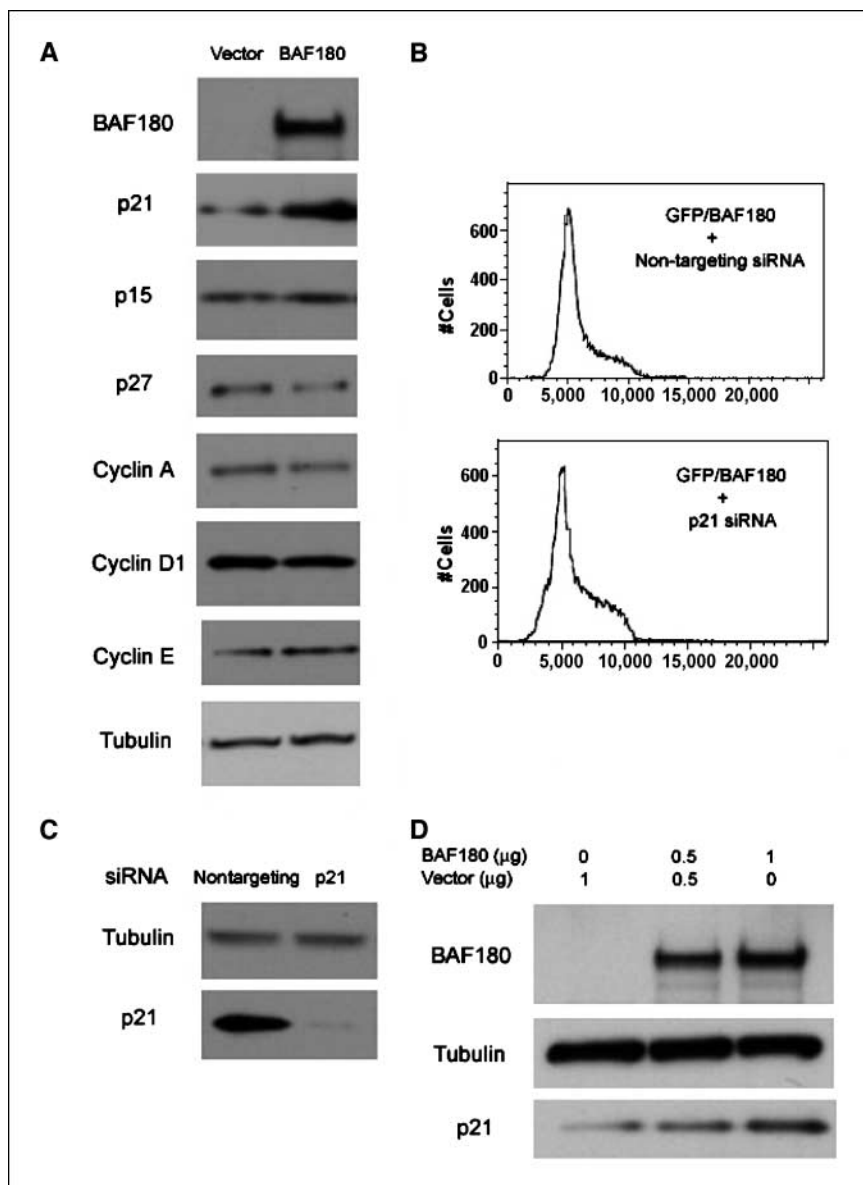
**BAF180 is required for p21 up-regulation on  $\gamma$ -irradiation and transforming growth factor- $\beta$  treatment.** The p21 promoter

is activated by a wide range of signals via signal-regulated transcription factors such as p53, SMAD2/3/4, signal transducer and activator of transcription 3, vitamin D<sub>3</sub> receptor, retinoid X receptor  $\alpha$ , and peroxisome proliferator-activated receptor  $\gamma$  (1, 2, 11, 12, 22, 24-28). Because BAF180 is the defining member of the PBAF-BAF180 complex, our data suggested that the PBAF-BAF180 complex could be involved in the induction of p21 due to the activation of one or more of these signal-dependent transcription factors. Because p53 and SMAD transcription factors are well-known mediators of the ataxia telangiectasia mutated/ $\gamma$ -radiation and transforming growth factor- $\beta$  (TGF- $\beta$ ) signaling pathways, as well as being tumor suppressors in their own right, we sought to dissect the contribution of BAF180 to p21 induction and cell cycle inhibition due to these signals in the normal cell line MCF10A (29, 30).

When p21 decreased on BAF180 knockdown in MCF10A cells, the distribution of cells in the cell cycle changed correspondingly, with fewer cells in the G<sub>1</sub> phase and more in S-G<sub>2</sub> (Fig. 6A). The effect of BAF180 knockdown on p21 and the cell cycle was magnified when MCF10A cells were challenged with extracellular stimuli known to induce p21 expression with TGF- $\beta$  and  $\gamma$ -irradiation treatment (29, 30). In BAF180 knockdown cells, p21 up-regulation and the consequent cell cycle response due to

**Figure 3.** BAF180 reexpression inhibits proliferation of HCC1143 cells. *A*, colony suppression assay of HCC1143 cells. HCC1143 cells were transfected with pBabeBAF180 and selected with puromycin. *B*, quantitation of the colony assay results. *C*, FACS analysis on GFP-positive HCC1143 cells 24 h after transfection with pCX-H2B-EGFP, pQBI25 (GFP), or pQBI25-BAF180 (GFP-BAF180). *D*, p21 induction in GFP-BAF180-transfected, but not pQBI25-transfected, GFP-positive cells, shown by Western blot. GFP-positive HCC1143 cells were sorted at 24 h after transfection and lysed for Western blotting.





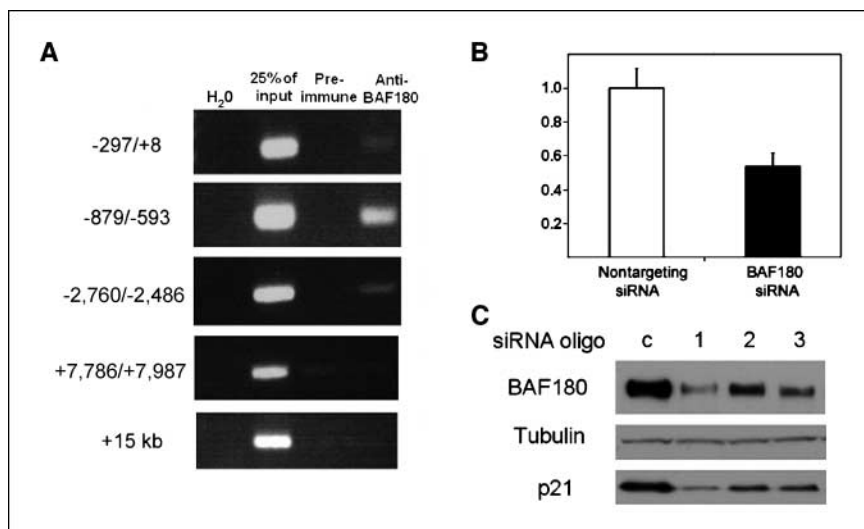
**Figure 4.** p21 is the downstream target of BAF180 signaling. **A**, p21 induction in HCC1143 cells shown by Western blots. HCC1143 cells were transfected with pIRES2-EGFP or BAF180pIRES2-EGFP and protein was harvested 16 h later for immunoblot analysis with indicated antibodies. **B**, knockdown of p21 partially rescues the G<sub>1</sub> arrest induced by GFP-BAF180 reexpression. HCC1143 cells were cotransfected with nontargeting siRNA or BAF180 siRNA and GFP-BAF180. GFP-positive cells were subjected to cell cycle analysis. **C**, p21 RNAi knockdown shown by Western blotting. **D**, BAF180 reexpression in SUM1315 induces p21 in a dosage-dependent manner. Cells were transfected and, 22 h later, harvested for immunoblot analysis.

either stimulus were compromised (Fig. 6). The reduction of p21 activation was detected by Western blotting after 24-hour treatment with TGF- $\beta$  or 3 hours after  $\gamma$ -irradiation (Fig. 6B and C). In response to TGF- $\beta$  treatment, the arrested G<sub>1</sub> population of BAF180 knockdown cells became much smaller relative to control siRNA cells (Fig. 6B). The decrease of G<sub>1</sub> population caused by BAF180 knockdown corresponded to the reduction of TGF- $\beta$ -induced p21 elevation (Fig. 6B), showing that BAF180 plays a role in TGF- $\beta$ -induced G<sub>1</sub> arrest. Similar results were obtained when exposing BAF180 knockdown cells to  $\gamma$ -irradiation. As a consequence of BAF180 knockdown, more cells shifted out of G<sub>1</sub> and into G<sub>2</sub> arrest induced by  $\gamma$ -irradiation (Fig. 6C). This change was also associated with reduced p21 activation in spite of a normal increase in the level of p53 (Fig. 6C). Taken together, our data suggest that the BAF180-mediated p21 activation is required for G<sub>1</sub>, but not G<sub>2</sub>, arrest. The remaining p21 activation seems to be sufficient for the  $\gamma$ -irradiation-induced G<sub>2</sub> arrest as reported (30).

We conclude that BAF180 plays an important role in the regulation of the cell cycle due, at least in part, to its ability to modulate the expression of p21 in response to different environmental stimuli such as treatment with TGF- $\beta$  or DNA damage. Our results support a model in which BAF180 assists in the induction of p21 promoter activity after transcription factors such as SMAD2/3/4 and p53 bind to the promoter. It is important to note that elevated MYC expression has the ability to block TGF- $\beta$  and  $\gamma$ -radiation induction of p21 expression, and it has been reported for MCF10A that TGF- $\beta$  induces cell cycle arrest in the absence of p21 up-regulation (31, 32). We surmise that our ability to detect p21 regulation in these cells is likely to be a consequence of our culture conditions or our stock of MCF10A, which may have relatively lower MYC expression. We suggest that BAF180 is also likely to function as an intermediary in the activation of p21 in response to vitamin D<sub>3</sub> receptor, which is known to induce p21, suppress breast cell growth, and require PBAF-BAF180 for ligand-mediated *in vitro* transcription (18, 25, 33). At this point, we do not understand how



**Figure 5.** BAF180 regulates p21 transcription. **A**, chromatin immunoprecipitation of BAF180 on p21 promoter. Immunoprecipitation of BAF180 cross-linked to chromatin detects BAF180 binding to p21 promoter with serum from rabbits immunized with BAF180 but not preimmune serum. No BAF180 binding is detected 7 to 15 kb outside of the promoter region. **B**, BAF180 knockdown decreases the level of p21 mRNA. RNAs from siRNA-transfected MCF10A cells were subjected to reverse transcription. Quantitative PCR was done using reverse transcription products as the templates and p21 and tubulin primers. The p21 results were normalized to tubulin and quantified with MxPro from Stratagene. **C**, BAF180 knockdown decreases the level of p21 protein. MCF10A cells were transfected with siRNA using Nucleofector (Amaxa) and then lysed on the next day. *c*, nontargeting oligo as a control; *1*, oligo from Dharmacon, D-008692-01; *2*, oligo from Dharmacon, D-008692-02; *3*, oligo from Dharmacon, D-008692-03.

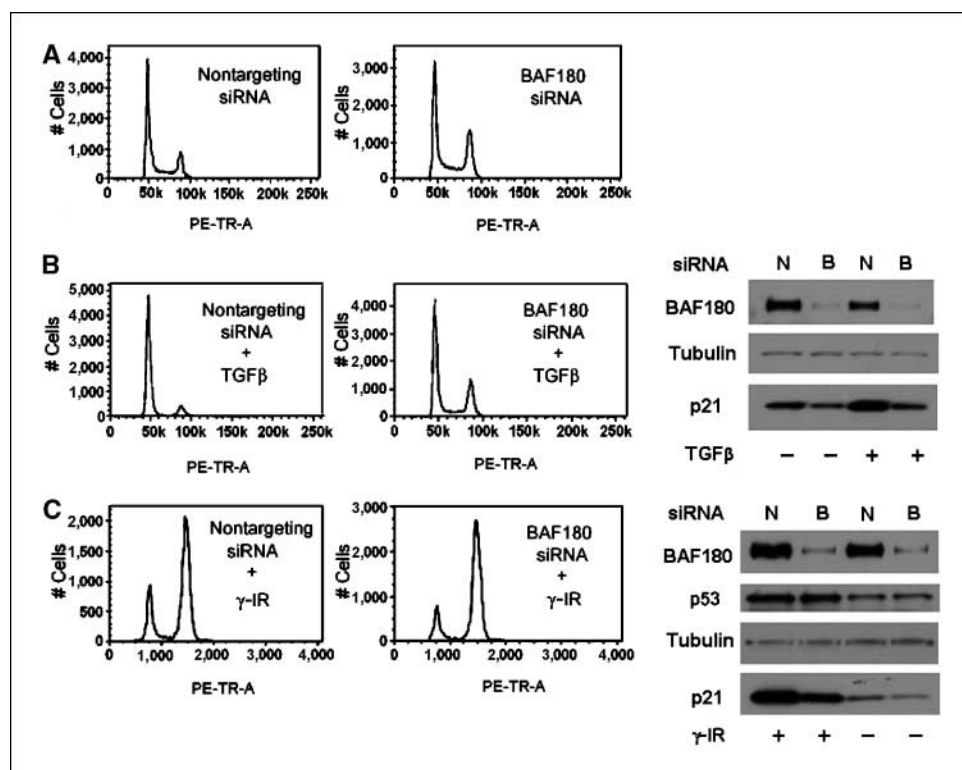


BAF180 contributes to baseline p21 transcription, but suggest that an unidentified ligand present in cell growth media could be activating a transcription factor that requires PBAF-BAF180 to transcribe p21. The important role of BAF180 in the regulation of p21 and the cell cycle is underscored by the identification of frequent LOH and truncating mutations in breast cancer. We presume that tumor-acquired mutations of BAF180 contribute to proliferation due to reduced baseline expression of p21 and reduced responsiveness to growth inhibitory tumor suppressor pathways that regulate the expression of p21. It also seems that BAF180 regulates the expression of additional cell cycle factors because (a) p21 RNAi only partially rescued the cell cycle arrest

due to BAF180 overexpression (Fig. 4B) and (b) RNAi to BAF180 reduced the magnitude of CDC25A down-regulation in response to TGF- $\beta$  (data not shown).

Because tumor cell lines (HCC1143 and SUM1315) that contain BAF180 mutations also have mutant p53 and inactive p16, we suggest that BAF180 mutation may cooperate with mutations in these genes to stimulate the cell cycle. In addition, given the important contribution of BAF180 to generating cell cycle arrest in response to diverse growth inhibitory signals (TGF- $\beta$  and  $\gamma$ -radiation), we suggest that BAF180 may be a critical regulator of cell cycle exit in response to a wide variety of additional external antimitogenic signals. It is even possible that the high

**Figure 6.** G<sub>1</sub> arrest and p21 induction by TGF- $\beta$  or  $\gamma$ -radiation requires BAF180. **A**, BAF180 knockdown causes cell population shift. MCF10A cells were transfected with BAF180 or nontargeting siRNA and collected 24 h later. **B**, MCF10A cells transfected with BAF180 siRNA (B) or nontargeting siRNA (N) were treated with 100 pmol/L TGF- $\beta$  (+) for 24 h and analyzed by cytometric and Western blotting. **C**, MCF10A cells transfected with BAF180 siRNA (B) or nontargeting siRNA (N) were  $\gamma$ -irradiated and subjected to cytometric and Western blot analyses. The cell cycle arrest and p21 induction elicited by either TGF- $\beta$  or  $\gamma$ -radiation is markedly attenuated by knockdown of BAF180 due to RNAi.





frequency LOH of BAF180 that occurs in cancer could weaken the ability of growth inhibitory signals to arrest cells in G<sub>1</sub> of the cell cycle.

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