

# An EBF3-Mediated Transcriptional Program That Induces Cell Cycle Arrest and Apoptosis

Lisa Y. Zhao,<sup>1</sup> Yuxin Niu,<sup>1</sup> Aleixo Santiago,<sup>1</sup> Jilin Liu,<sup>1</sup> Sara H. Albert,<sup>1</sup> Keith D. Robertson,<sup>2,3</sup> and Daiqing Liao<sup>1,3,4</sup>

Departments of <sup>1</sup>Anatomy and Cell Biology and <sup>2</sup>Biochemistry and Molecular Biology, <sup>3</sup>Program in Cancer Genetics, Epigenetics and Tumor Virology, and <sup>4</sup>Program in Signaling, Apoptosis and Cancer, Shands Cancer Center, University of Florida College of Medicine, Gainesville, Florida

## Abstract

**In a genome-wide screen for putative tumor suppressor genes, the EBF3 locus on the human chromosome 10q26.3 was found to be deleted or methylated in 73% of the examined cases of brain tumors. EBF3 is expressed in normal brain but is silenced in brain tumors. Therefore, it is suggested that EBF3 is a tumor suppressor. However, it remains unknown whether inactivation of EBF3 locus also occurs in other types of tumors and what functions of EBF3 underlie EBF3-mediated tumor suppression. We show here that expression of EBF3 resulted in cell cycle arrest and apoptosis. The expression of cyclin-dependent kinase inhibitors was profoundly affected with early activation and then repression of p21<sup>cip1/waf1</sup> and persistent activation of both p27<sup>kip1</sup> and p57<sup>kip2</sup>, whereas genes involved in cell survival and proliferation were suppressed. EBF3 bound directly to p21<sup>cip1/waf1</sup> promoter and regulated transcription from both p21<sup>cip1/waf1</sup> and p27<sup>kip1</sup> promoters in reporter assays. Apoptosis occurred 48 hours after EBF3 expression with caspase-3 activation. Silencing of the EBF3 locus was observed in brain, colorectal, breast, liver, and bone tumor cell lines and its reactivation was achieved on treatment with 5-aza-2'-deoxycytidine and trichostatin A in a significant portion of these tumor cells. Therefore, EBF3 regulates a transcriptional program underlying a putative tumor suppression pathway. (Cancer Res 2006; 66(19): 9445-52)**

## Introduction

The early B-cell factors (EBF; also known as Olf, COE, or O/E; this family of proteins is hereafter called EBF) are a group of DNA-binding transcription factors with the basic helix-loop-helix (bHLH) domain. These factors were discovered in 1993 independently as proteins that are involved in regulating expression of genes in two specific cell types: B lymphocyte (1) and olfactory cell (2). Later studies revealed that these factors are expressed in B lymphocytes, adipocytes, neuronal cells, and several other cell types and that they have specific roles in regulating differentiation in cells originating from all three embryonic germ layers (3). The human and mouse genomes each carry four genes of EBF family (EBF1-EBF4) and multiple alternatively spliced transcripts from

each gene exist (3). These factors have well-recognized bHLH domains. The two amphipathic helices of 15 amino acids, separated by a loop of 7 amino acids, are strikingly similar, which distinguishes the EBF family from other known bHLH families of proteins that usually contain two dissimilar helices (4). This family of proteins bind directly to DNA sequences with a consensus of 5'-CCCNNGGG-3' as homodimers or heterodimers (5, 6). Within the highly conserved DNA-binding domain, there is a sequence motif that defines the signature of the EBF family (termed as COE). This signature sequence is an atypical Zn<sup>2+</sup> finger (H-X<sub>3</sub>-C-X<sub>2</sub>-C-X<sub>5</sub>-C) and is absolutely required for DNA binding (7). The COOH-terminal serine/threonine-rich sequence is the transactivation domain, although other sequences also contribute to transactivation (7).

These proteins exist only in the animal kingdom from *Caenorhabditis elegans* to the humans. The EBF orthologues in *Drosophila melanogaster* (Collier) and *C. elegans* (Unc-3) are involved in neurogenesis, which might be regulated through genetic interactions between EBF and the hedgehog or notch pathway (8). In the mouse, all four members of the EBF family are expressed in olfactory receptor neurons, where they regulate the expression of olfactory genes (5, 9). During mouse embryogenesis, EBF members are expressed in early postmitotic neurons from the midbrain to the spinal cord and at specific sites in the embryonic forebrain, suggesting that they may regulate neuronal maturation in the central nervous system (CNS; refs. 8, 10, 11). EBF2 is expressed in the embryonic CNS (12) and targeted inactivation of EBF2 has revealed roles for EBF2 in peripheral nerve morphogenesis, migration of hormone-producing neurons, and projection of olfactory neurons (9, 13). During neuronal differentiation, the members in the EBF family might have distinct roles. For example, the mouse EBF2 seems to act earlier than EBF1 or EBF3. In *Xenopus*, EBF3 is implicated in promoting differentiation of specific neuronal subtypes (3, 8).

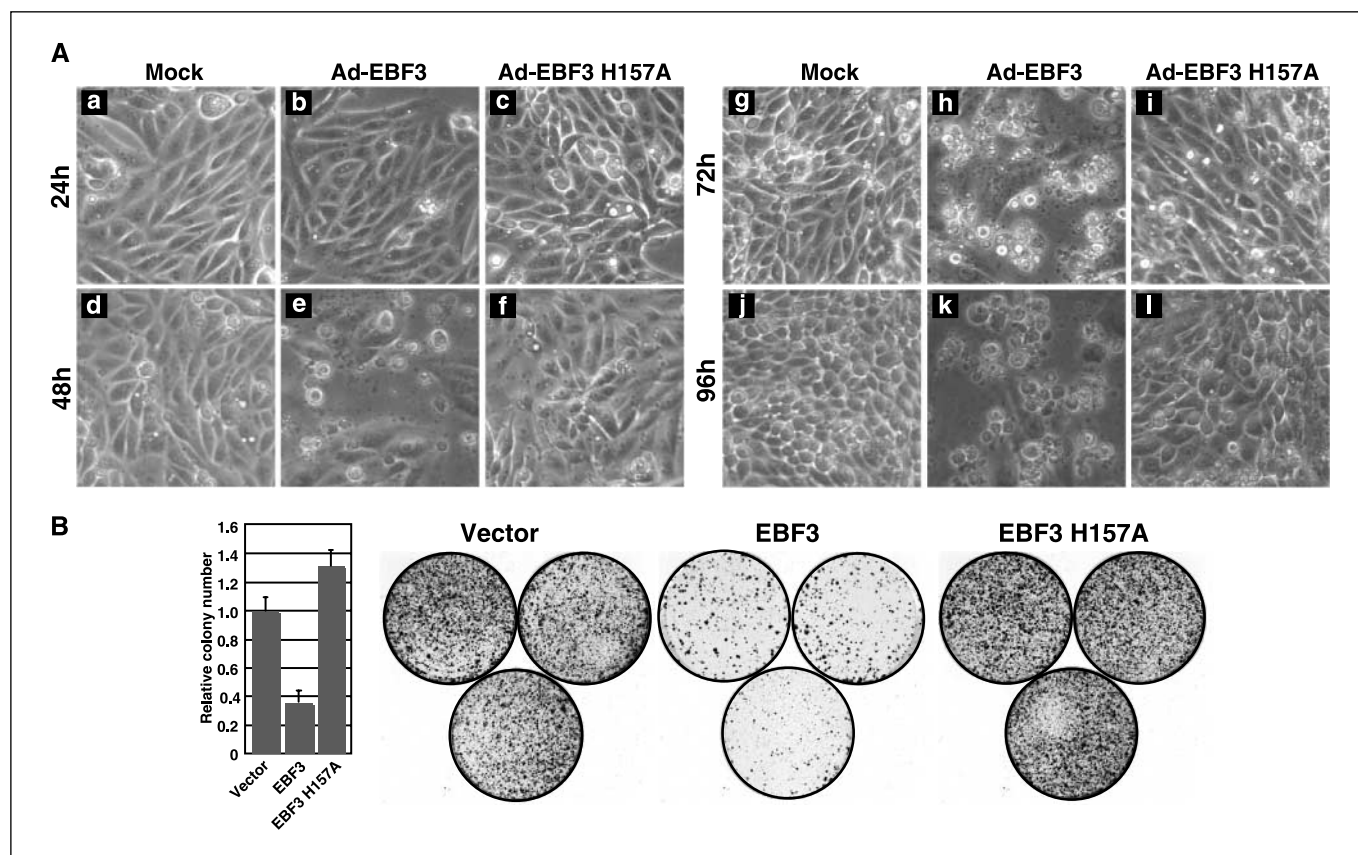
In addition to roles in neuronal differentiation, the EBF family of transcription factors are also implicated in other developmental pathways. It has been extensively documented that EBF1 is essential for B-cell development (14, 15). EBF1-deficient mice produce only B-biased progenitor cells but not mature B cells (16). EBF2 is a regulator of osteoblast-dependent differentiation of osteoclasts and targeted disruption of EBF2 resulted in reduced bone mass (17). Although mice deficient for EBF1 and EBF2 are viable (9, 13, 16, 17), homozygous EBF3 knockout mice exhibited neonatal lethality before postnatal day 2, suggesting that EBF family members are not functionally redundant (9).

A genome-wide screen using integrated genomic and epigenetic analyses revealed that the EBF3 locus at the human chromosome 10q26.3 is biallelically altered by methylation and/or deletion in most high-grade brain tumor cases (18). Whereas EBF3 was found to be inactivated in 50% of grade II tumors, 83% of grade III and 90% of grade IV brain tumors have mutated (deleted) or silenced

**Note:** Current address for J. Liu: Applied Genetic Technologies Corp., 11801 Research Drive, Suite D, Alachua, FL 32615. Current address for S.H. Albert: Department of Biology, University of Louisiana at Lafayette, P.O. Box 42451, Lafayette, LA 70504-2451.

**Requests for reprints:** Daiqing Liao, Department of Anatomy and Cell Biology, Shands Cancer Center, University of Florida College of Medicine, P.O. Box 103633, 1376 Mowry Road, Gainesville, FL 32611-3633. Phone: 352-273-8188; Fax: 352-273-8285; E-mail: dliao@ufl.edu.

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**Figure 1.** EBF3 suppresses tumor cell growth. *A*, Saos2 cells were grown in six-well plates and were either untreated (*Mock*; *a*, *d*, *g*, and *j*) or infected with recombinant adenoviruses for EBF3 (*Ad-EBF3*; *b*, *e*, *h*, and *k*) or EBF3 H157A (*Ad-EBF3 H157A*; *c*, *f*, *i*, and *l*). Cells were photographed at 24, 48, 72, and 96 hours after infection. *B*, HCT116 cells were cotransfected with a vector for puromycin-resistant gene along with the empty vector, EBF3, or EBF3 H157A expression vector. Cells were selected in medium containing puromycin and grown until visible colonies appeared. The colonies were stained and counted and relative numbers are plotted.

EBF3 locus (18). Consistently, EBF3 is expressed in normal brain cells but is silenced in brain tumor cells (18). These data suggest that EBF3 is a potential tumor suppressor in brain tumors, although what functions of EBF3 at biochemical or cellular levels are implicated in tumor suppression remain unknown. Furthermore, whether EBF3 inactivation is involved in the development of tumors of other tissue origins has not been examined. In this study, we show that inactivation of the EBF3 locus occurs not only in brain tumors but also in breast, colorectal, liver, and bone tumor cells. Strikingly, expression of EBF3 in tumor cells resulted in growth suppression and apoptosis. In cells with EBF3 expression, genes involved in growth suppression were activated, whereas those involved in cell growth and proliferation were suppressed. Therefore, EBF3 regulates a transcriptional program that may underpin its tumor suppression function.

## Materials and Methods

**Antibodies.** Antibodies to Bax, caspase-3, CDC2, cyclin-dependent kinase (CDK) 2, cyclin A, Daxx, EBF, p16 (CDKN2A), p21<sup>cip1/waf1</sup> (hereafter called p21), p27<sup>kip1</sup> (hereafter called p27), p57<sup>kip2</sup> (hereafter called p57), p107 (Rbl1), and poly(ADP-ribose) polymerase (PARP) were from Santa Cruz Biotechnology (Santa Cruz, CA); those to AKT, Bcl-xL, and myeloid cell leukemia-1 (Mcl-1) were from Cell Signaling (Danvers, MA); and antibodies to extracellular signal-regulated kinase 1/2 (ERK1/2), FLAG, and  $\alpha$ -tubulin were from Sigma (St. Louis, MO). The anti-Janus kinase 1 (JAK1) antibody was from BD Biosciences (San Jose, CA).

**Recombinant adenoviruses.** A mouse EBF3 cDNA clone was provided by Dr. Randall Reed (Johns Hopkins University). The EBF3 open reading frame encoding 596 amino acids was preceded by codons for FLAG epitope and cloned into pShuttle-CMV. The EBF3 H157A mutant was generated using QuickChange protocol (Stratagene, La Jolla, CA). Recombinant adenoviruses for EBF3 and its mutant were generated with the AdEasy system (Stratagene). Near confluent cell culture was infected with viruses with a multiplicity of infection of  $\sim 10$ . Infected cells were harvested at 24, 48, 72, and 96 hours postinfection. The infected cells were processed either for Western blot analysis or flow cytometry as described (19).

**Electrophoresis mobility shift assays.** Synthetic oligonucleotides were annealed to form dsDNA and their 5' ends were labeled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase. FLAG-EBF3 and FLAG-EBF3 H157A were purified from Saos2 cells transfected with a relevant expression plasmid using affinity gel in which monoclonal anti-FLAG M2 is conjugated to agarose beads (Sigma) according to the manufacturer's protocol. Purified FLAG-EBF3 or the H157A mutant was preincubated in a reaction volume of 20  $\mu$ L containing 10 mmol/L HEPES (pH 7.5), 70 mmol/L KCl, 1 mmol/L EDTA, 2.5 mmol/L MgCl<sub>2</sub>, 1 mmol/L ZnCl<sub>2</sub>, 5% glycerol, 0.1  $\mu$ g/ $\mu$ L poly(deoxyinosinic-deoxycytidylic acid), 5  $\mu$ g/ $\mu$ L bovine serum albumin, 0.05% NP40, and 1 mmol/L DTT on ice for 20 minutes. The labeled oligonucleotides (0.066 pmol) was then added to the reaction mix and further incubated at 30°C for 20 minutes. For antibody-mediated supershift, 0.5  $\mu$ g anti-FLAG antibody was added to the reaction and incubated further for 10 minutes at 30°C. For competition using nonradioactive DNA, 0.5 pmol nonradioactive DNA was added in the binding reaction. The reaction mix was separated on a 6% native polyacrylamide gel with 0.5 $\times$  Tris-borate-EDTA (45 mmol/L Tris-borate, 2 mmol/L EDTA). The gel was dried and subjected to autoradiography.

**Chromatin immunoprecipitation.** Saos2 cells grown on 10-cm dishes were infected with recombinant adenovirus encoding EBF3, and 24 hours after infection, the cells were fixed with 1% formaldehyde for 10 minutes at room temperature. The rest of steps in chromatin immunoprecipitation were done according to a typical protocol using 2  $\mu$ g anti-EBF (Santa Cruz Biotechnology). PCR on immunoprecipitated templates was done with one step at 95°C for 5 minutes and 35 cycles of 95°C, 55°C, and 72°C each for 95 seconds and a final step at 72°C for 2 minutes. The PCR primers used were (from 5' to 3') p21 promoter forward GGTAATCCTTGCCTGCCA-GAG and reverse ACTTCCCTCCTCCCCAGTC and  $\beta$ -actin promoter forward ACGCAAAACTCTCCCTCCTCTC and reverse CATAAAAGG-CAACTTTCGGAACGGC.

**Luciferase promoter reporter assays.** The 2.4 kb p21 promoter was progressively deleted from the 5' end and all promoter fragments were cloned into pGL3-Basic (Promega, Madison, WI). The set of p27 promoter reporter constructs was obtained from Dr. Toshiyuki Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan; ref. 20). Saos2 cells were seeded in 48-well plates and were transfected with the indicated expression plasmid, promoter reporter construct, and SV40-*Renilla* control reporter vector. Lysates were assayed at 24 hours after transfection using the Dual Luciferase Reporter System (Promega).

**Assessing EBF3 expression in tumor cells and its reactivation.** Reverse transcription-PCR (RT-PCR) was carried out according to standard protocols. Briefly, untreated and treated cells were homogenized in Trizol and the RNA was purified according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was carried out using SuperScript III RT (Invitrogen). Subsequently, the cDNA was used in semiquantitative PCR using the following primers (sequences from 5' to 3'): EBF1 forward CAACTTCTCCACTTCGTCTGG and reverse CACATTCTGGGTTCTGTCTTGG, EBF3 forward CGAGAAAACCAACAACGGCATC and reverse ATGATTACAGGGTCTGAGGGCG, and EBF4 forward CCAACTTCTCCACTTCGTCTGG and reverse CTTGTCTGCCCTCATAGATG. Amplification of glyceraldehyde-3-phosphate dehydrogenase was used as a control for RNA integrity for all samples. Following PCR, reaction products were resolved on 2% agarose gels and photographed using a Bio-Rad (Hercules, CA) gel documentation system.

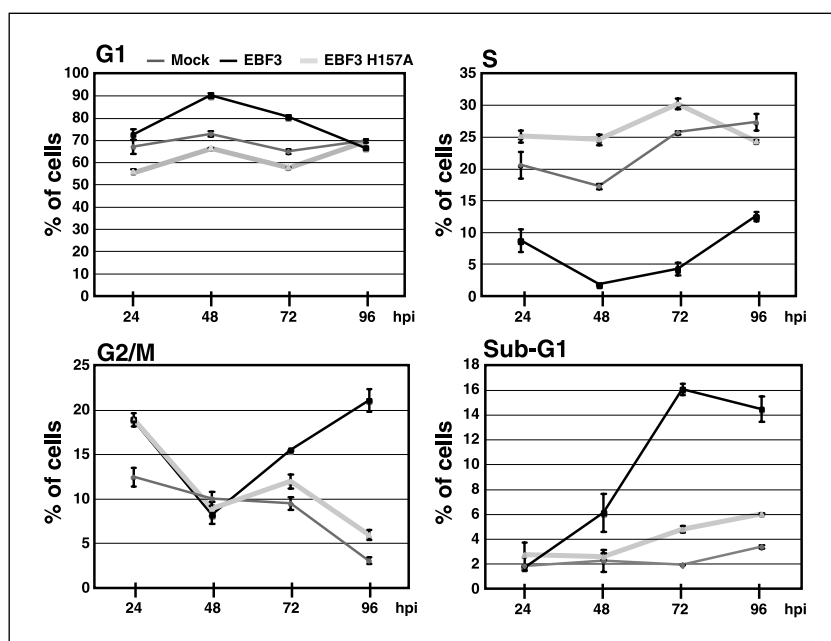
For reactivation experiments, cell lines were treated with 5  $\mu$ mol/L 5-aza-2'-deoxycytidine for 4 days (fresh drug was added every 24 hours) followed by a 24-hour treatment with 100 nmol/L trichostatin A. All chemicals were purchased from Sigma.

## Results

**Expression of EBF3 in tumor cells results in growth arrest and apoptosis.** To assess the effect of EBF3 expression on the growth and proliferation of tumor cells, we have transduced several tumor cell lines with recombinant adenovirus for wild-type EBF3 (Ad-EBF3) or the DNA-binding mutant H157A (Ad-EBF3 H157A). We found that EBF3 expression profoundly restricted the growth of several tumor cell lines, including glioblastoma U87 MG, osteosarcoma Saos2, and colon carcinoma HCT116. The growth of these tumor cells was almost completely suppressed within 4 days of infection with Ad-EBF3 but not with Ad-EBF3 H157A (see Fig. 1; data not shown). Saos2 cells continued to grow in mock and Ad-EBF3 H157A-treated cells. By contrast, the Ad-EBF3-infected cells started to detach from the culture plate by 48 hours postinfection, and most of the cells were detached with very few attached cells by 96 hours postinfection (Fig. 1A). Interestingly, EBF3-mediated killing of tumor cells seems to be selective, as several prostate cancer cell lines were completely resistant to Ad-EBF3 infection (data not shown).

EBF3-mediated killing of Saos2 cells and inactivation of EBF3 in brain tumors (18) suggest that EBF3 might have broad tumor suppression functions in diverse tumor cells. To further substantiate this possibility, we assayed effects of EBF3 expression on the growth of colon carcinoma cell HCT116. Empty vector, or that carrying cDNA for EBF3 or H157A mutant, was cotransfected with a plasmid carrying the puromycin-resistant gene. As shown in Fig. 1B, transfection of wild-type EBF3, but not the H157A mutant cDNA, drastically reduced the number of puromycin-resistant colonies. Thus, EBF3 suppresses the growth of colon cancer cells.

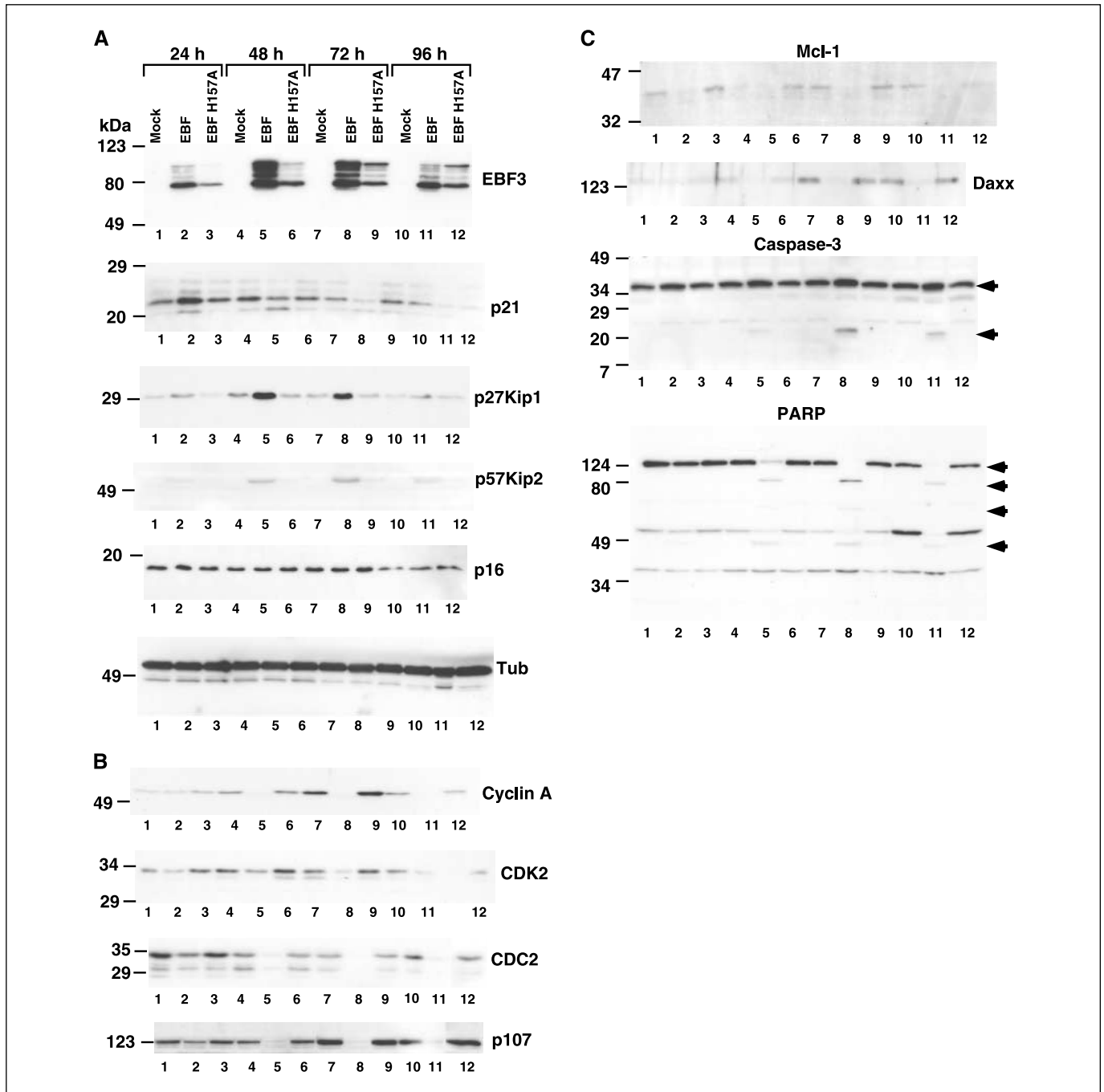
Flow cytometry analysis of Ad-EBF3-transduced Saos2 cells revealed that G<sub>1</sub> arrest was evident within 24 hours and peaked 48 hours postinfection with 90% of cells in the G<sub>1</sub> phase of the cell cycle (Fig. 2). EBF3 expression also resulted in inhibition of DNA replication, as the percentage of cells in the S phase was markedly reduced in cells with EBF3 expression as opposed to mock-infected or Ad-EBF3 H157A-infected cells. At a late time after infection with



**Figure 2.** EBF3 promotes cell cycle arrest and apoptosis. Saos2 cells were either not treated (*Mock*) or infected with Ad-EBF3 or Ad-EBF3 H157A mutant. Cells were harvested for flow cytometry analyses at different time points. Percentage of cells in each phase of the cell cycle as well as that of apoptotic cells with sub-G<sub>1</sub> DNA content under different treatments is separately plotted. *hpi*, hours postinfection. *Points*, results of two independent experiments; *bars*, SD. *Dark gray line*, mock; *black line*, EBF3; *thick light gray line*, EBF3 H157A.

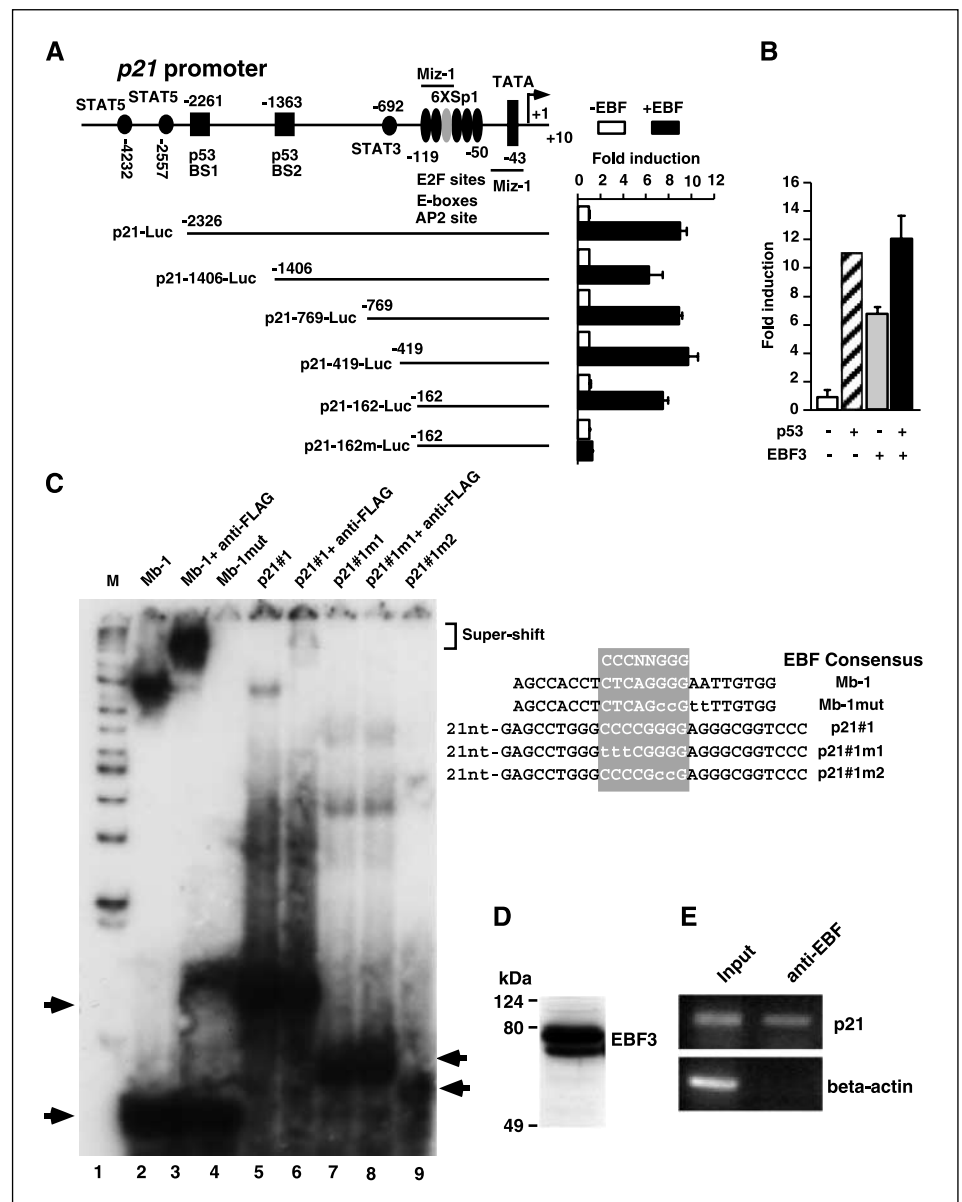
Ad-EBF3 (72 and 96 hours postinfection), cell cycle arrest at the G<sub>2</sub>-M phase was also evident (Fig. 2). Finally, Saos2 cells underwent apoptosis on EBF3 expression as cells with sub-G<sub>1</sub> DNA content were accumulating starting at 48 hours after Ad-EBF3 infection (Fig. 2). Importantly, the DNA-binding mutant H157A had no obvious effects on cell cycle progression and apoptosis (Fig. 2). Therefore, it is likely that EBF3 suppresses tumor cell growth and proliferation through cell cycle arrest and apoptosis.

**EBF3 activates genes involved in growth inhibition but represses genes required for cell growth and survival.** To understand the molecular mechanisms underlying EBF3-imposed restriction on cell proliferation, we analyzed the expression of some important players involved in the regulation of cell cycle progression and apoptosis. Strikingly, the expression of CDK inhibitors (CDKI) p21, p27, and p57 was markedly elevated 24 hours after Ad-EBF3 infection (Fig. 3A). The increased



**Figure 3.** Gene expression of the EBF3-mediated transcriptional program. Saos2 cells were either not treated (*Mock*) or infected with Ad-EBF3 or Ad-EBF3 H157A mutant. Cells were harvested for Western blot analyses at different time points. Antibodies for each blot are denoted. Protein concentration of cell extracts was measured with the Bradford method. Equal amount of total cellular proteins was loaded in each lane as shown in the  $\alpha$ -tubulin (*Tub*) blot. *Arrows*, intact and cleaved proteins in caspase-3 and PARP blots. *A*, EBF3 regulates the expression of genes for CDKIs. *B*, EBF3 represses the genes involved in cell proliferation and survival. *C*, EBF3 inhibits the expression of antiapoptotic genes.

**Figure 4.** EBF3 binds to and activates the p21 promoter. **A**, EBF3 activates the proximal p21 promoter. *Top*, schematic representation of the human p21 promoter. Some known *cis*-elements along with their positions are indicated. p53 BS, p53 binding site. The p21 promoter was deleted progressively from the distal 5' end and the 5' position of each deletion mutant is indicated. A putative EBF-binding site was mutated in p21-162m-Luc. The mutations were the same as in p21#1m1 in (C). The luciferase reporter with the specified fragment of the p21 promoter was transfected alone or together with a mouse EBF3 expression plasmid into Saos2 cells. Dual luciferase assays were done with the extracts of transfected cells 24 hours after transfection. **B**, effects of coexpression of p53 and EBF3 on the p21 promoter. The firefly luciferase reporter driven by the 2.4-kb p21 promoter (p21-Luc) was transfected into Saos2 cells alone or together with p53, EBF3 expression vector, or both. Dual luciferase reporter assays were done 24 hours after transfection. **C**, EBF3 binds to the p21 promoter. *Top*, DNA probe for each protein-DNA interaction assay. In some reactions, anti-FLAG antibody was included. *M*, 1-kb DNA size markers. *Arrows*, positions of free DNA probes. The DNA sequences of the used probes for EMSA are shown. The EBF3-binding site is indicated in white with gray background; mutated nucleotides are denoted with lowercase letter. *D*, purified FLAG-EBF3. The protein was purified using anti-FLAG affinity gel and eluted with FLAG peptide. The eluate was examined using Western blotting with anti-FLAG antibody. **E**, chromatin immunoprecipitation analysis of EBF3-p21 promoter interaction. Saos2 cells infected with Ad-EBF3 were subjected to chromatin immunoprecipitation with anti-EBF antibody and the immunoprecipitates were PCR amplified using primers specific to the proximal region of the p21 promoter or to the  $\beta$ -actin promoter. The amplified PCR fragment from the p21 promoter lies 95 bp 5' to the putative EBF-binding site.



expression of p27 and p57 persisted throughout the course of infection with peak expression at 48 and 72 hours postinfection for p27. The expression pattern of p27 correlated strictly with the G<sub>1</sub> arrest induced by EBF3. Thus, at 48 hours postinfection, 90% of the cells were arrested at the G<sub>1</sub> phase (Fig. 2), when the expression of p27 was also highest (Fig. 3A). Interestingly, p21 expression peaked at 24 hours postinfection but was then repressed at later times after Ad-EBF3 infection. By contrast, EBF3 did not influence the expression of p16<sup>INK4A</sup>, an inhibitor of cyclin D-CDK4/6 holoenzymes (Fig. 3A). Collectively, our findings suggest that EBF3 specifically activates the expression of p21, p27, and p57, all of which belong to the Cip/Kip family of CDKIs and specifically inhibit cyclin A-CDK2 or cyclin E-CDK2 holoenzymes. Of note, the levels of the wild-type EBF3 protein seemed to be higher than that of the H157A mutant (Fig. 3A). Nonetheless, differential expression of the two constructs did not influence these observations, as EBF3 were able to induce specific gene expression at a much lower dose (10-fold lower than that used for Fig. 3), whereas the H157A mutant

did not affect gene expression and cell growth at any dose tested (data not shown). EBF3-mediated activation of p21 and p27 is likely direct, as EBF3 can stimulate the promoters of both p21 and p27 in reporter gene assays (see below) and EBF3 binds to p21 promoter in gel electrophoresis mobility shift assay (EMSA) and chromatin immunoprecipitation experiments (see below).

We then analyzed the expression patterns of genes involved in cell growth and proliferation. As shown in Fig. 3B, EBF3 inhibited the expression of several key proteins involved in cell cycle progression, including cyclin A, CDC2, and CDK2 (Fig. 3B) as well as cyclin B (data not shown). Down-regulation of CDC2 and CDK2 was observed as early as 24 hours after Ad-EBF3 infection, and at later time points, the levels of these proteins were drastically reduced. In contrast, cyclin D1 expression was not affected (data not shown).

Saos2 cells do not contain functional retinoblastoma protein (Rb). Because Rb family proteins can inhibit both proliferation and apoptosis (21), we wondered whether EBF3 could regulate the

expression of Rb family proteins. Interestingly, EBF3 expression resulted in specific repression of p107, a member of Rb family (Fig. 3B). Down-regulation of p107 was seen as early as 24 hours postinfection with Ad-EBF3, and more profound repression was evident at later times during EBF3 expression (Fig. 3B). AKT expression was moderately down-regulated at 72 and 96 hours after Ad-EBF3 infection and the expression of mitogen-activated kinase p38 (MAPK14), ERK1/2, and JAK1 was not at all affected (data not shown).

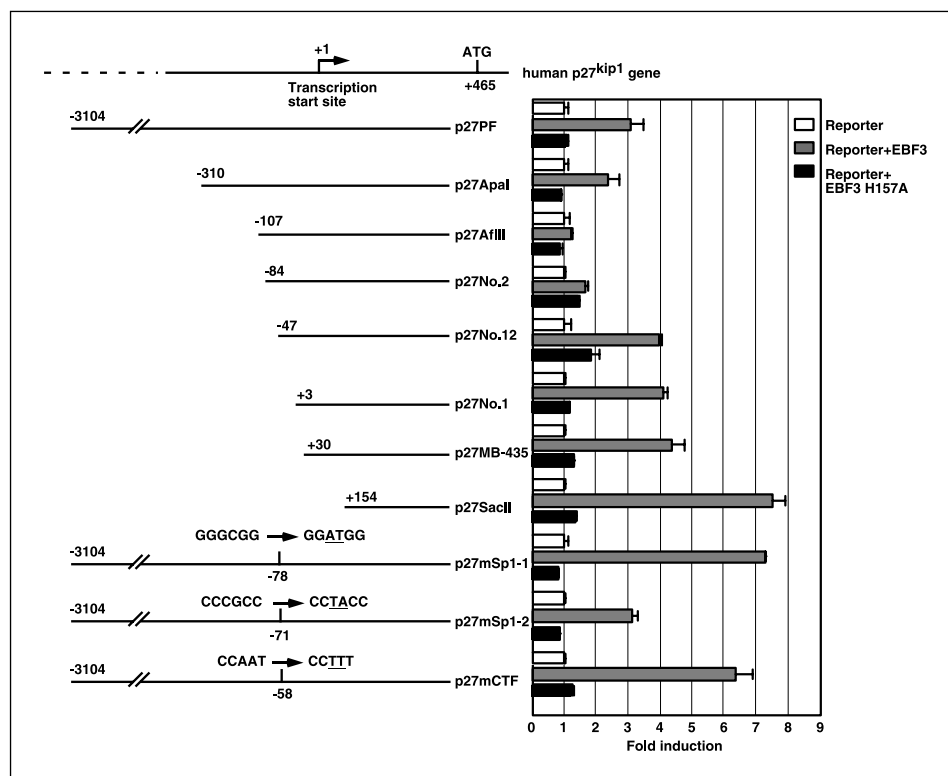
If EBF3 expression indeed triggers apoptosis, we would expect the appearance of apoptotic markers. We thus have examined the expression of various proapoptotic and antiapoptotic proteins. Whereas EBF3 did not affect the expression of either antiapoptotic Bcl-2 family protein Bcl-xL or the proapoptotic Bax (data not shown), it markedly repressed the expression of Mcl-1, an antiapoptotic Bcl-2 family protein (Fig. 3C). Interestingly, strong repression of Daxx expression by EBF3 was also observed, consistent with an antiapoptotic role for Daxx in this situation as we reported previously (19). Strikingly, activation of caspase-3 and cleavage of PARP, two hallmark events during apoptosis, occurred 48 hours postinfection of Ad-EBF3 (Fig. 3C). Thus, EBF3 expression resulted in apoptotic cell death.

**EBF3 regulates p21 and p27 promoters.** The results described above indicate that EBF3 regulates the expression of inhibitors of CDKs p21 and p27 (Fig. 3). To assess whether EBF3 directly mediates the transcription of both genes, we first did luciferase reporter gene assays. Data presented in Fig. 4A revealed that EBF3 expression markedly elevated the reporter activities driven by a set of constructs of the p21 promoter. The data also indicated that only the proximal region of the p21 promoter is required. Inspection of the DNA sequence of this region allowed us to identify a single putative EBF-binding site (-121 to -128) that resembles the consensus EBF-binding sequence (see Fig. 3C). We

mutated this site in the p21 reporter and found that this mutated reporter (p21-162m-Luc) was no longer responsive to EBF3 expression (Fig. 4A).

Whereas p21 is a prototypical p53 target gene, our data indicated that p53 probably does not influence EBF3-mediated regulation of p21. First, EBF3 could activate p21 expression in Saos2 cells that are deficient of p53 (Figs. 3 and 4A). Second, the p21 promoter constructs lacking the p53-binding sites (p21-769-Luc, p21-419-Luc, and p21-162-Luc in Fig. 4A) could still be activated by EBF3. Furthermore, we coexpressed p53 and EBF3 in reporter assays. Data presented in Fig. 4B indicate that these two factors showed no synergy in regulating the p21 promoter.

To verify whether EBF3 indeed binds to the putative EBF-binding site in the p21 promoter, we have done EMSA. Purified FLAG-tagged EBF3 from transfected Saos2 cells was incubated with a radioactive double-stranded oligonucleotide containing the EBF-binding site (p21#1, Fig. 4C). The mobility of this DNA probe was retarded (Fig. 4C, lane 5) and addition of anti-FLAG antibody resulted in supershift of the retarded band (Fig. 4C, lane 6). Mutations within the EBF-binding site abolished EBF3-binding to the DNA (p21#1m1 and p21#1m2, Fig. 4C, lanes 7-9; note that two shifted bands in lanes 7 and 8 were nonspecific because addition of anti-FLAG antibody did not result in supershift). We have also tested the interaction between EBF3 and several other oligonucleotide probes that together span the entire proximal region of the p21 promoter from -162 to +10. EBF3 did not bind to any DNA probe without the putative EBF-binding site (data not shown). As a positive control, we assayed the interaction of the purified FLAG-EBF3 with human Mb-1 promoter, a known target gene of EBF1 (1). FLAG-EBF3 could bind to the DNA probe containing the wild-type but not the mutated EBF-binding site from the human Mb-1 promoter (compare lanes 2 and 4 in Fig. 4C). Furthermore, addition of anti-FLAG antibody also resulted in supershift of the



**Figure 5.** EBF activates the p27 promoter. A schematic representation of the human p27 promoter. The transcription start site is designated as +1. The position of the translational initiation codon (ATG) is also indicated. The p27 promoter was deleted progressively from the distal 5' end and the 5' position of each deletion construct is indicated relatively to the +1 site. Each reporter construct is specifically named as described by Inoue et al. (20). Base substitutions were made at two putative Sp1 sites and a CTF (NF-Y) site. The luciferase reporter with a specified fragment of the p27 promoter was transfected alone or together with either vector for mouse EBF3 or the H157A mutant into Saos2 cells. Dual luciferase assays were done with the extracts of transfected cells 24 hours after transfection.

EBF3-DNA complex (Fig. 4C, lane 3). Therefore, EBF3 specifically binds to the p21 promoter.

To further assess the interaction of EBF3 with the p21 promoter, we have carried out chromatin immunoprecipitation assay. We found that in Saos2 cells infected by Ad-EBF3, the proximal region of the p21 promoter near the EBF-binding site was significantly enriched by the anti-EBF antibody, whereas  $\beta$ -actin promoter was not (Fig. 4E). Similar results were obtained from glioblastoma LN-229 cells that express endogenous EBF3 (data not shown). Therefore, EBF3 associates with the p21 promoter at the chromatin levels.

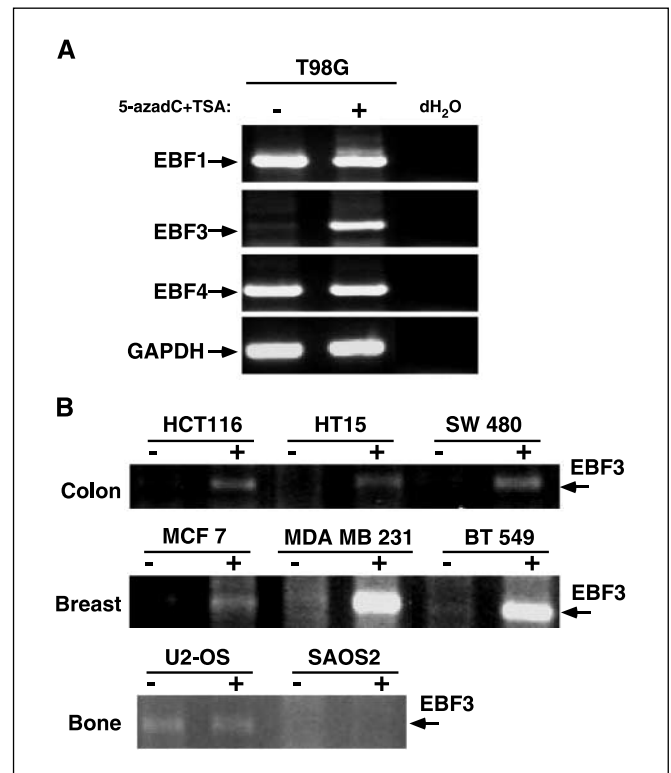
We also assayed whether EBF3 could activate the p27 promoter. Data shown in Fig. 5 indicate that EBF3, but not EBF3 H157A mutant, activated the p27 promoter using luciferase reporter gene assay. Interestingly, EBF3 had very little effects on constructs p27AflIII and p27No.2, but luciferase activity was restored or even increased on further deletion from the 5' end of the p27 promoter. A simple explanation for these observations is that one or more *cis*-acting elements locating around  $-107$  to  $-84$  might confer inhibition to EBF3-mediated transcription from the p27 promoter. This inhibition can be overcome in the presence of upstream DNA sequence. Consistent with this interpretation, mutation of a putative Sp1 site at  $-78$  (p27mSp1-1) or a putative CTF (p27mCTF) at  $-58$  resulted in increased reporter activities (Fig. 5). Collectively, our results suggest that EBF3 directly activates the expression of the p27 gene.

**Epigenetic inactivation of EBF3 in tumors.** We have examined a panel of brain tumor cell lines for the expression of EBF family of transcription factors using RT-PCR. Consistent with previous findings regarding inactivation of EBF3 in brain tumors (18), EBF3 expression was undetectable or very low in four of six brain tumor cell lines, whereas the levels of EBF1 expression were markedly higher and detectable in all six lines (see Fig. 6A; data not shown). In glioblastoma cell line T98G, treatment with inhibitors of DNA methyltransferase (5-aza-2'-deoxycytidine) and histone deacetylases (trichostatin A) resulted in strong reactivation of EBF3. By contrast, the same treatment did not cause increased expression of EBF1 or EBF4 (Fig. 6A). Thus, our data suggest that EBF3 is selectively silenced in brain tumors and its expression could be reactivated with 5-aza-2'-deoxycytidine and trichostatin A.

We have also examined EBF3 expression in tumor cell lines of various tissue origins and found that EBF3 is silenced in 5 of 8 (63%) colorectal tumor cell lines and 7 of 7 (100%) breast tumor cell lines and 1 of 2 bone tumor cell lines (Fig. 6B; data not shown) as well as 6 of 8 liver cancer lines (data not shown). With the exception of bone tumors, EBF3 can be reactivated with 5-aza-2'-deoxycytidine and trichostatin A in most of these examined tumor cells (Fig. 6B; data not shown). Taken together, our findings indicate that epigenetic silencing of EBF3 is a widely occurring phenomenon in human cancers; remarkably, EBF3 silencing can be reversed after treatment with anticancer drugs.

## Discussion

A previous study showed that EBF3 locus is inactivated in brain tumors (18). Here, we found that EBF3 gene is silenced in tumor cell lines of diverse tissue origins, such as breast, bone, and colorectal cancers. Importantly, our results provided a plausible explanation for EBF3-mediated tumor suppression. EBF3 expression in tumor cells results in growth suppression and apoptosis. It regulates a gene expression program in which genes involved in cell



**Figure 6.** Epigenetic silencing of EBF3 locus in tumor cells. **A**, expression of EBF family of transcription factors in brain tumor. Glioblastoma T98G cells were either untreated (–) or treated (+) with 5-aza-2'-deoxycytidine (5-azadC; 5  $\mu$ mol/L) + trichostatin A (TSA; 0.1  $\mu$ mol/L). RT-PCR was done using RNA extracted from the cells and specific primers to EBF1, EBF3, or EBF4. **B**, epigenetic silencing of EBF3 locus in colon, breast, and bone tumors. Tumor cell lines were either untreated or treated with 5-aza-2'-deoxycytidine (5  $\mu$ mol/L) + trichostatin A (0.1  $\mu$ mol/L). RT-PCR was done using RNA extracted from the cells and specific primers to EBF3.

cycle arrest, such as the Cip/Kip family of CDKs are selectively up-regulated, and in the same time, genes involved in cell proliferation (e.g., cyclins and CDKs) and survival (Daxx and Mcl-1) are repressed. Apoptosis was induced on EBF3 expression as caspase-3 activation and cleavage of PARP were observed.

For many of the genes whose expression was affected on EBF3 expression, EBF3 may directly mediate their transcriptional activation or repression through interacting with specific binding sites in the promoters of these genes. Indeed, EBF3 binds to the p21 promoter and regulates the expression of p21 and p27 in reporter gene assays. In support of this interpretation, the EBF3 H157A, a DNA-binding mutant, failed to elicit specific gene expression and killing of tumor cells. Notably, EBF3 constitutively activated the expression of p27 and p57 (Fig. 3A). Therefore, EBF3 regulates the expression of all three members of Cip/Kip family of CDKs, which specifically inhibit cyclin A-CDK2 or cyclin E-CDK2 holoenzymes. Additionally, EBF3 also represses the expression of CDK2 and cyclin A (Fig. 3B). In conjunction with our finding that it does not affect the expression of p16<sup>INK4A</sup>, our data suggest that EBF3 specifically and profoundly suppresses the cyclin A-CDK2 activities at multiple levels, which may account for EBF3-mediated cell cycle arrest.

Interestingly, for the p21 gene, we have observed a striking switch from activation at early time point of EBF3 expression to repression at a later time point (see Fig. 3A). This switch seems to



coincide with the onset of apoptosis as activation of caspase-3 and cleavage of PARP started around 48 hours after EBF3 expression. This observation suggests that down-regulation of p21 may be necessary for the activation of apoptosis. It has been documented that cytoplasmic localization of overexpressed p21 correlates with inhibition of apoptosis, cancer cell survival, and poor prognosis of cancer patients (22). How this activation-repression switch of p21 expression is achieved is unknown. One scenario could be an ordered exchange of activators and repressors on the p21 promoter in a situation similar to signal-dependent activation of c-Jun transcription factor although in a reversed order of events (23). Further studies on the mechanisms underlying EBF3-regulated p21 expression might shed important insight into how cells coordinate transcription and apoptosis.

We were able to detect EBF3 expression in several tumor cell lines, such as colon tumor lines SW48 and LoVo and glioblastoma cell line LN-229 (data not shown) as well as osteosarcoma U2-OS (Fig. 6B). Thus, EBF3 is not universally silenced. In conjunction with the findings that EBF3 is expressed in normal brain cells (18), our data suggest that EBF3 is probably expressed in differentiated cells of different organs and epigenetic silencing of EBF3 occurs specifically in tumor cells. On treatment of tumor cells with 5-aza-2'-deoxycytidine and trichostatin A, EBF3 was reactivated in most of the

studied cell lines. However, in a small portion of these cell lines, such as colon cancer cell lines HT29 and T84, breast tumor cell line SKBR3 (data not shown) and bone tumor Saos2 (Fig. 6B), the same drug treatment was unable to promote EBF3 expression. Although it is unknown what the cause for the failure to reactivate EBF3 in these tumor cells was, one possibility is that the EBF3 locus is completely deleted in these tumor cell lines, as deletion of EBF3 locus has been found in glioblastoma (18). Additionally, small-scale inactivating mutations of the EBF3 coding sequence, such as point mutations, small insertions, and deletions, could also occur in tumors, especially in tumor cells with detectable expression of EBF3. Further studies will be required to determine whether genetic mutations occur within the coding sequence of the EBF3 gene.

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