ZBP-89 Promotes Growth Arrest through Stabilization of p53

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Transcription factor p53 can induce growth arrest and/or apoptosis in cells through activation or repression of downstream target genes. Recently, we reported that ZBP-89 cooperates with histone acetyltransferase coactivator p300 in the regulation of p21^{waf1}, a cyclin-dependent kinase inhibitor whose associated gene is a target gene of p53. Therefore, we examined whether ZBP-89 might also inhibit cell growth by activating p53. In the present study, we demonstrate that elevated levels of ZBP-89 induce growth arrest and apoptosis in human gastrointestinal cell lines. The ZBP-89 protein accumulated within 4 h, and the p53 protein accumulated within 16 h, of serum starvation without changes in p14ARF levels, demonstrating a physiological increase in the cellular levels of these two proteins. Overexpression of ZBP-89 stabilized the p53 protein and enhanced its transcriptional activity through direct protein-protein interactions. The DNA binding and C-terminal domains of p53 and the zinc finger domain of ZBP-89 mediated the interaction. A point mutation in the p53 DNA binding domain, R273H, greatly reduced ZBP-89-mediated stabilization but not their physical interaction. Furthermore, ZBP-89 formed a complex with p53 and MDM2 and therefore did not prevent the MDM2-p53 interaction. However, heterokaryon assays demonstrated that ZBP-89 retained p53 in the nucleus. Collectively, these data indicate that ZBP-89 regulates cell proliferation in part through its ability to directly bind the p53 protein and retard its nuclear export. Our findings further our understanding of how ZBP-89 modulates cell proliferation and reveals a novel mechanism by which the p53 protein is stabilized.

The tumor suppressor p53 is one of the most important regulators of cell proliferation, and its gene is frequently mutated in human cancers (21). The p53 protein is a potent transcription factor that can activate target genes and initiate growth arrest, DNA repair, and apoptosis in response to cellular genotoxic stress, e.g., DNA damage, oncogene activation, and hypoxia (15, 26). One of the gene products induced by p53 is p21^{waf1}, an inhibitor of cyclin-dependent kinases, which can initiate cell cycle arrest (12, 17). Other targets include GADD45, MDM2, cyclin G, and Bax genes, whose gene products function as regulators of several aspects of cell growth (27, 31, 49).

p53 is tightly regulated, and its protein level in normal cells is very low. The p53 protein is regulated largely at the post-translational level through its interaction with MDM2. The MDM2 protein restricts p53 transactivation function by binding to the N-terminal domain of p53, mediating ubiquitination and rapid degradation of p53 by the proteasome (20, 28, 50, 51). Since p53 stimulates the production of its inhibitor, MDM2 is an important negative-feedback regulator of p53. In cancer cells, mutant p53 loses its transactivation function and does not induce MDM2 gene expression. Therefore mutant p53 is not degraded and its half-life in cells is prolonged (7). While many have attributed p53 overexpression to the presence of a mutated protein (54), the presence of MDM2 does not explain why elevated levels of wild-type p53 can be sustained in cancers. Thus, detection of p53 in colon cancer does not always correlate with the presence of p53 gene mutations

(13). Although viral proteins can also bind and stabilize mutant p53, few cellular proteins other than MDM2 and p14ARF have been reported to regulate p53 levels (49). This suggests that there may be other mechanisms recruited to increase wild-type p53. p53 mutant status is clinically relevant since those cancers expressing wild-type p53 appear to be more sensitive to chemotherapeutic agents (33).

ZBP-89 (BFCOL1, BERF1, ZNF 148) is a zinc finger transcription factor that is universally expressed (34). It has been shown that ZBP-89 binds to GC-rich DNA elements in promoters involved in cell growth regulation, e.g., promoters for gastrin, ornithine decarboxylase, and the cyclin-dependent kinase inhibitor p21^{waf1} (5, 18, 30, 34). However, its ability to regulate cell growth has not been extensively demonstrated. For the rat pituitary adenoma cell line GH4, we showed that elevated expression of ZBP-89 inhibits cell proliferation (39). ZBP-89 expression is significantly induced by *trans*-retinoic acid or butyrate, which also induces terminal differentiation of a colon cancer cell line (5, 9). Moreover, we recently found that ZBP-89 cooperates with p300 to potentiate the butyrate-induced activation of p21^{waf1} (5). Studies by Hasegawa et al. have shown that BFCOL1, the mouse homologue of ZBP-89, interacts with a GADD34-like protein (19). The GADD34 gene is a growth arrest-associated gene that can be induced by DNA damage (55).

Because p53 and ZBP-89 are both implicated in the regulation of p21^{waf1} expression and control of cell growth, we investigated the possibility of a functional interaction between p53 and ZBP-89. We demonstrate here that elevated levels of ZBP-89 induce growth arrest and apoptosis in human gastrointestinal cell lines. Furthermore, we show that ZBP-89 stabilizes p53 through direct protein contact and retention in the nucleus, which subsequently potentiates the transcriptional ac-

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tivity of p53. In addition, the p53R273H mutant, which is common in human colon and stomach tumors, is resistant to ZBP-89-mediated stabilization. These results strongly suggest that ZBP-89 regulates cell growth through stabilization of the p53 protein.

MATERIALS AND METHODS

Plasmids and constructs. The pcDNA3-Flag-ZBP-89 gene encoding the full-length Flag-tagged rat ZBP-89 (amino acids 1 to 794) has been described previously (5). To generate various glutathione *S*-transferase (GST) fusion proteins, the partial ZBP-89 cDNA fragments encoding the N-terminal domain (amino acids 1 to 154), zinc finger DNA binding domain (amino acids 154 to 300), N-terminal and zinc finger domains (amino acids 1 to 300), and C-terminal domain (amino acids 300 to 794) were amplified by PCR and cloned into pGEX 5X-1 (Pharmacia). The PCR primers used were as follows: to construct the N-terminal portion of the ZBP-89 fusion protein, forward primer 5'-TACGAA TTCAACATTGACGACAACTGGAAG-3' and backward primer 5'-ATTGCGCCGCGATTGTCAGAGAGCGTTG-3'; to construct the zinc finger DNA binding domain, forward primer 5'-ATCGAATTCCTTACAATAAATG AGGATGGATC-3' and backward primer 5'-TAAATATAGCGGCCGCTTAA TCTTCTCTGATGTCAGAAG-3'; to construct the N-terminal and zinc finger domains, forward primer 5'-TACGAATTCACATTGACGACAACTGGAA G-3' and backward primer 5'-TAAATATAGCGGCCGCTTAACTTCTCTCT GATGTCAGAAG-3'; and to construct the C-terminal fusion protein, forward primer 5'-ATCGAATTCGATTCTGGCTTTTCTACGTCACC-3' and backward primer 5'-TAAATATAGCGGCCGCTTACGCAAAAGTCTGGCCAG-3'. The plasmid encoding the full-length rat ZBP-89 GST fusion protein has been described previously (34). The pCMV- β -gal reporter was obtained from Clontech. PG13 (contains 13 copies of p53 DNA binding sites), MG15 (contains 15 copies of mutant p53 DNA binding sites), and p21^{waf1}/2300-Luc (contains 2.3 kb of the p21^{waf1} promoter) reporter constructs (12) and pBS-p53, pCMV-p53, and pCMV-p53 (R273H) expression vectors were all gifts from Bert Vogelstein (Johns Hopkins University). Plasmids encoding various GST-p53 fusion proteins [pGThp53, pGThp53C-(160-393), pGThp53C1-(160-318), pGThp53C2-(318-393), and pGThp53N-(1-160)] were kindly provided by Thomas Shenk (Princeton University) (22). GST-p53N (1-90), GST-p53D (90-300), and GST-p53C (300-393) were provided by Ken-ichi Yamamoto (Kanazawa University, Kanazawa, Japan) (25). hp53-Luc, which contains 2.4 kb of the human p53 promoter region, was kindly provided by Moshe Oren (Weizmann Institute of Science, Rehovot, Israel).

Cell culture and transfections. AGS gastric carcinoma and p53-deficient mouse embryonic fibroblast (MEF) cells (provided by Larry Donehower, Baylor College of Medicine) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). HCT116 p53^{+/+} and p53^{-/-} cells were gifts from Bert Vogelstein and were cultured in McCoy's 5A medium with 10% FBS. HT-29, a colon cancer cell line, was grown in McCoy's 5A medium with 10% FBS. Cells were grown on 12-well plates and transfected using FUGENE 6 (Roche) according to the manufacturer's protocol. Luciferase and β -galactosidase assays were performed 48 h after transfection using a Berthold AutoLumat luminometer (LB953; EG&G, Gaithersburg, Md.). To correct for the transfection efficiency, pCMV- β -gal was cotransfected to normalize the luciferase values to β -galactosidase activity.

Serum starvation of cells. AGS cells were seeded on 60-mm-diameter plates in DMEM with 10% FBS. Sixteen hours later, the medium was changed to F-12 medium (Life Technologies) without FBS. Whole-cell or nuclear extracts were used for immunoblot analysis or immunoprecipitation.

Adenovirus infection of cells. Replication-deficient recombinant Ad5-ZBP-89, which contains the rat full-length Flag-tagged ZBP-89 cDNA, has been previously described (5). The control recombinant adenoviruses, Ad5-vector containing the cytomegalovirus (CMV) promoter alone and a poly(A) signal sequence, and Ad5- β -gal expressing β -galactosidase from the CMV promoter were obtained from the University of Michigan Cancer Center Vector Core. Cells were grown in 10-cm-diameter cell culture dishes until 60% confluent and then infected with replication-deficient recombinant adenoviruses for 6 h. The amount of virus (in terms of multiplicity of infection [MOI]) which resulted in at least 70% infection efficiency was determined empirically. The lowest MOI which resulted in infection of more than 70% of the cells was used for all experiments.

Flow cytometry. Cells were seeded in 10-cm-diameter cell culture dishes and infected with recombinant adenoviral vectors as described above. Forty-eight hours later, the cells were collected and fixed with 70% ethanol for 2 h. The cells were collected, washed with phosphate-buffered saline (PBS), resuspended in

FIG. 1. Recombinant replication-deficient adenovirus delivery of ZBP-89. AGS cells were mock infected or infected with the control Ad5-vector (Ad-vector) or Ad5-ZBP-89 (Ad-ZBP-89) at MOIs of 2 and 10. Forty-eight hours later, cells were collected, lysed, and separated by SDS-PAGE. The monoclonal Flag M2 and polyclonal ZBP-89 antibodies were used to detect Flag-tagged and endogenous ZBP-89 proteins. A monoclonal actin antibody was used to control for the amount of protein loaded.

PBS containing 25 μ g of propidium iodide/ml and 0.1% RNase A, and then incubated at 37°C for 1 h. The DNA content was measured using a FACSCaliber (Becton Dickinson). The data were plotted using Cell Quest software (Becton Dickinson). At least 10,000 events were analyzed for each sample.

TUNEL assay. Cells were grown on glass coverslips and infected with recombinant adenoviruses at an MOI of 10. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assays were performed using an in vitro cell death detection kit (Roche) according to the manufacturer's instructions.

Immunoblot analyses. Rabbit anti-Mdm2, -p53, -p21^{waf1}, -p14ARF, and -actin, mouse anti-p53 (DO-1); and mouse immunoglobulin G (IgG) were purchased from Santa Cruz Biotechnology. The rabbit ZBP-89 antibody has been previously described (47). The mouse monoclonal Flag M2 antibody was purchased from Sigma. Mouse monoclonal anti- β -galactosidase and rabbit anti-caspase 3 antibodies were obtained from Oncogene Science. Whole-cell extracts were prepared in lysis buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 0.1% Nonidet P-40, 0.5 mM EDTA, 1 mM dithiothreitol [DTT]) and analyzed by immunoblotting as described previously (5). Coprecipitation of p53 or ZBP-89 was carried out using whole-cell extracts and specific antibodies as described previously (5).

Immunofluorescence. Cells were grown on coverslips transfected with various plasmids or infected with recombinant adenoviruses at an MOI of 10. Forty hours later, the cells were washed twice with PBS, fixed in 4% paraformaldehyde in PBS for 20 min, and then permeabilized with 0.1% Nonidet P-40 in PBS for 10 min. The cells were washed again with PBS and blocked with 10% fetal calf serum (FCS) at 37°C for 30 min. The primary rabbit anti-ZBP-89 IgGs (1:500 dilution) or mouse anti-p53 (1:200 dilution) antibodies were added to the coverslips in PBS with 5% FCS at room temperature for 1 h. After being washed with PBS, the coverslips were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (1:200 dilution) or Texas red-conjugated anti-rabbit IgG (1:500) at room temperature for 1 h. After being mounted, the cells were visualized with an Olympus BX60 fluorescence microscope and photographed with the digital SPOT camera (Diagnostic Inst.).

For 5-bromo-2'-deoxyuridine (BrdU) labeling, 24 h after transfection, the cells were cultured in DMEM with 10% FBS and 100 μ M BrdU (Sigma) for an additional 24 h. The cells were washed with PBS, fixed with 4% paraformaldehyde in PBS, permeabilized with 0.25% Triton X-100 in PBS for 10 min, and then washed with PBS followed by distilled water. The cells were treated with 2 N HCl at room temperature for 30 min to denature the DNA, neutralized with 0.1 M sodium borate at room temperature for 5 min, and then washed with PBS. A mouse monoclonal antibody against BrdU (1:50 dilution) was added to the

FIG. 2. ZBP-89 induces growth arrest and apoptosis in AGS cells. (A) AGS cells were mock infected or infected with the indicated recombinant adenovirus (vector alone [Ad-vector] or ZBP-89 [Ad-ZBP-89]) at an MOI of 10. Two days later, cells were collected, fixed, and stained with propidium iodide. The DNA content was quantified using a FACSCaliber. Shown are the means of three experiments performed in triplicate $\pm$ standard errors of the means (SEM). (B) AGS cells were transiently transfected with the pcDNA3 vector or pcDNA3-Flag-ZBP-89 and then labeled with BrdU as described in Materials and Methods. DAPI was used to stain the nuclei (blue). Rabbit anti-ZBP-89 (followed by Texas red-conjugated anti-rabbit IgG) and FITC-conjugated anti-BrdU antibodies were used to stain ZBP-89 (red) and BrdU (green), respectively. The

coverslips for 2 h at 37°C. After the cells were washed with PBS, rabbit anti-ZBP-89 IgG (1:500 dilution) was added to the coverslips at room temperature for 1 h, after which the cells were washed with PBS. Coverslips were subsequently incubated with a mixture of FITC-conjugated anti-mouse IgG (1:200) and Texas red-conjugated anti-rabbit IgG (1:500) at room temperature for 1 h, and the cells were mounted and visualized with a fluorescence microscope using single and double filters as described above.

Human-mouse heterokaryon assay. AGS cells were cultured in six-well plates and transfected with 2 µg of the pcDNA3-Flag-ZBP-89 expression vector or the pcDNA3 empty vector. Twenty-four hours after transfection, the cells were trypsinized, mixed with p53-deficient MEF cells at a ratio of 1:1, and seeded on glass coverslips. Sixteen hours later, the cells were treated with 100 µg of cycloheximide/ml for 25 min at 37°C, and then cell fusion was induced by 50% (wt/vol) polyethylene glycol 8000 (Sigma) in DMEM for 2 min. The cells were then incubated at 37°C for 1 h in the presence of 100 µg of cycloheximide/ml. Subsequently, the cells were fixed with 4% paraformaldehyde in PBS for 15 min at 4°C and permeabilized with 0.2% Triton X-100 in PBS for 5 min at 4°C. After being washed three times with PBS containing 0.5% bovine serum albumin (BSA), the cells were blocked with PBS containing 0.5% BSA and 10% normal goat serum for 1 h at room temperature. The cells were first incubated with mouse anti-p53 (DO-1; Santa Cruz Biotechnology; 1:400 dilution) and rabbit anti-ZBP-89 IgG (0.4 µg/ml) for 1 h at room temperature and rinsed. Then they were incubated with Texas red-conjugated goat anti-rabbit IgG (Becton Dickinson; 1:500)-FITC-conjugated goat anti-mouse IgG (Becton Dickinson; 1:200)-4',6-diamidino-2-phenylindole (DAPI) (Sigma, 5 ng/ml) for an additional hour at room temperature.

Measurement of p53 protein stability. The cells were infected with control Ad5-β-gal or Ad5-ZBP-89 adenoviruses at an MOI of 10. Eighteen hours later, the infected cells were prestarved by replacing the culture media with DMEM without L-methionine for 30 min at 37°C. The cells, at a concentration of 6×10^5 cells/ml, were labeled in vivo with 100 µCi of [³⁵S]methionine/ml (Amersham) for 30 min. After being labeled, the cells were immediately washed one time with DMEM containing 5 mM L-methionine and 5% FBS and then incubated in the same medium for various chase times. The cells were harvested and labeled, and p53 proteins were immunoprecipitated as described above. Labeled p53 was visualized by autoradiography and quantified using a PhosphorImager (Molecular Dynamics). Background was calculated from the same area in each lane and subtracted from the value for labeled p53 in that lane. At time zero, the p53 protein amount was set at 100%. The data were plotted on a semilog scale and calculated using nonlinear regression with the Prism program (GraphPad Software, San Diego, Calif.).

In vitro transcription and translation and protein binding assays. [³⁵S]methionine-labeled p53 and ZBP-89 were synthesized by the TNT quick-coupled transcription/translation system (Promega) using pBS-p53 or pcDNA3-Flag-ZBP-89 as the template. To determine the binding of radioactive proteins to recombinant GST fusion proteins, [³⁵S]methionine-labeled p53 and ZBP-89 were produced by in vitro transcription and translation as described above. The translation products were then incubated with various GST fusion proteins immobilized on beads in the binding buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 5 mM EDTA, 0.2% Nonidet P-40, 1 mM DTT, 2 mg of BSA/ml) for 1 h at 4°C. The pellets were washed with binding buffer five times. Coprecipitated proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie blue staining and autoradiography.

Alternatively, in vitro-translated proteins were mixed with the products of parallel in vitro translation reactions carried out in the presence of nonradioactive methionine only. Briefly, 2 µl of each of the translation product was mixed in a final volume of 250 µl containing 20 mM HEPES, pH 7.5, 40 mM KCl, 3 mM MgCl₂, 1 mM DTT, and 5% glycerol at 4°C for 1 h. At the completion of the incubation, 2 µg of affinity-purified rabbit anti-ZBP-89 IgG, p53 polyclonal antibody, or preimmune serum was added to the reaction mixture, which was gently rotated overnight at 4°C. Twenty microliters of packed protein A and G-Sepharose beads (Santa Cruz Biotechnology) was then added to each reaction mixture, and the incubation was continued for 1 h at 4°C. The beads were washed

three times with the incubation buffer and resuspended in 2× SDS sample buffer before electrophoresis. Coprecipitated radioactive proteins were analyzed by SDS-PAGE followed by autoradiography as described above. Two microliters of each translated product was diluted in 100 µl of binding buffer, and 20 µl of the dilution was loaded as the input.

RNA isolation and Northern blotting. Total RNAs were isolated from AGS and HCT 116 p53^{+/+} cells using TRIZOL reagent (Life Technologies). Twenty micrograms of total RNA was size-fractionated on 1.2% agarose gels containing 2.4 M formaldehyde and transferred to nylon membranes (Hybond N⁺; Amersham). Hybridizations and washings were performed under high-stringency conditions using ExpressHyb (Clontech). A ³²P-labeled p53 riboprobe was prepared from the T3 promoter after linearizing the pBS-p53 plasmid with *Nco*I using the MAXIScript kit (Ambion). To control for the loading of RNA samples, the blot was stripped and reprobed with a radiolabeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobe transcribed from pTRI-hGAPDH (Ambion).

RESULTS

ZBP-89 induces growth arrest and apoptosis. To examine the effect of ZBP-89 overexpression on cell growth, AGS cells were infected with two concentrations of adenovirus vectors (Fig. 1). Immunoblots were performed with the Flag antibody to detect transfected amounts of ZBP-89 and with the ZBP-89 antibody to detect the presence of endogenous ZBP-89. In mock-infected cells or those cells infected with control adenovirus, ZBP-89 was not detected by immunoblotting although very small amounts of endogenous hZBP-89 protein were detected upon overexposure of the blot (Fig. 1). Increasing amounts of adenovirus were accompanied by an increase in ZBP-89 expression. The percentage of cells infected was determined by fluorescent-cell sorting. At an MOI of 10, more than 70% of all cells analyzed expressed ZBP-89. This MOI was used in subsequent experiments.

Next, we used flow cytometry to determine if overexpression of ZBP-89 regulated cell growth. AGS cells infected with control recombinant virus, Ad5-vector, or Ad5-β-gal had no effect on the cell cycle and were the same as mock-infected cells (Fig. 2A). However, cell proliferation was inhibited after infection with Ad5-ZBP-89. The percentages of G₀/G₁- and G₂/M-phase cells were increased from 50 to 62% and 22 to 30%, respectively, whereas the S-phase population sharply decreased from 27% in the controls to 7% in the ZBP-89-expressing cells. Consistent with the flow cytometry, immunofluorescence revealed reduced or absent DNA synthesis, as indicated by decreased BrdU incorporation in cells expressing ZBP-89 (Fig. 2B). Collectively, these data show that overexpression of ZBP-89 induces growth arrest in AGS cells primarily by suppressing the S phase of the cell cycle.

Twenty-four to 48 h after infection with Ad5-ZBP-89, AGS cells showed condensed nuclei, nuclear blebbing, and floating dead cells, which were not evident in cells infected with the control virus, Ad5-vector, or Ad5-β-gal at the same MOI. To determine whether these morphological changes correlated with apoptosis, infected cells were cultured for 48 h and then analyzed for the presence of DNA fragmentation using the

results shown are representative of three different experiments. (C) The presence of apoptotic cells in AGS cells was determined by the TUNEL assay. DAPI was used to stain the nuclei (blue). The immunolocalization of ZBP-89 was as described for panel B. FITC-conjugated dUTP labeled apoptotic cells green (TUNEL). Yellow indicates the colocalization of the ZBP-89 protein with apoptotic events in the same cell photographed with a dual filter on an Olympus BX60. Results shown are representative of three experiments. (D) The populations of sub-G₁ cells from mock infection and each recombinant adenovirus-infected cell population were determined by flow cytometry. Shown are the means of three experiments ± SEM.

FIG. 3. ZBP-89 induces wild-type p53 protein accumulation. (A) AGS cells were mock infected or infected with the indicated recombinant adenoviruses. The protein expression levels of Flag-tagged ZBP-89, wild-type p53, procaspase-3, and p21^{waf1} were measured by immunoblotting. Lane 1, mock infection; lane 2, Ad5-vector; lane 3, Ad5-ZBP-89. (B) ZBP-89 and induced p53 expression colocalizes to the nucleus. The AGS cells were infected with control Ad5-β-gal or Ad5-ZBP-89 at an MOI of 10. Forty-eight hours after infection, the cells were immunostained with mouse anti-p53 (green) and rabbit anti-ZBP-89 (red) antibodies. Left, Ad5-β-gal-infected cells; right, Ad5-ZBP-89-infected cells. DAPI was used to stain the nuclei (blue). Yellow indicates colocalization of ZBP-89 and p53, which were photographed with a dual filter. (C) Flow cytometry analysis of HCT 116 wild-type and p53 null cells. Mock-infected, Ad5-vector, or Ad5-ZBP-89 recombinant adenoviruses were used infect HCT 116 wild-type and p53 null cells at an MOI of 40 as described above prior to flow analysis. (D) The sub-G₁ population was determined by flow cytometry as described above. Shown are the means $\pm$ standard errors of the means for three experiments.

FIG. 4. Serum starvation induces ZBP-89 and p53 expression. (A) AGS cells were cultured in serum-free F-12 medium for the indicated times, and immunoblots were used to detect the expression profiles of ZBP-89, p53, and p14ARF. (B) AGS cells were grown in serum-free medium for 24 h, and 200 μ g of nuclear extracts was used for immunoprecipitation (IP). Lanes 1 and 2, immunoprecipitation with mouse anti-p53 antibody (DO-1) at 0 and 24 h after serum starvation; lane 3, immunoprecipitation with control mouse IgG at 24 h. Blotting was with rabbit anti-ZBP-89 or anti-p53 antibodies.

TUNEL assay and flow cytometry (Fig. 2C and D). Quantitative analysis of apoptosis by flow cytometry revealed a fourfold increase in the sub-G₁ cell populations with ZBP-89 overexpression (Fig. 2D). Cells undergoing apoptosis activate a family of cysteine proteases called caspases, which play a central role in the execution of apoptosis (11). Caspases are usually synthesized as inactive proenzymes (zymogens) and then are proteolytically cleaved to release the active forms. The 32-kDa procaspase 3 enzyme is cleaved into the active 17- and 11-kDa subunits at multiple aspartic acid residues. Activated caspase 3 participates in the proteolysis of several important effector molecules, such as PARP, the poly(ADP-ribose) polymerase that takes part mainly in regulation of DNA repair in the nucleus, and Bcl-2 (11). Therefore, we performed immunoblot analysis to assess whether protein levels of procaspase 3 are altered with ZBP-89 overexpression (Fig. 3A). Indeed, procaspase 3 levels decreased with ZBP-89 overexpression, consistent with the TUNEL assay and flow-cytometric indicators of apoptosis.

FIG. 5. ZBP-89 stabilizes the p53 protein. (A) AGS and HCT 116 cells were infected with control and ZBP-89-expressing adenovirus vectors at an MOI of 10 for 2 days. Total RNA was prepared for Northern blot analysis. The blot was first probed with a p53 riboprobe (top) and then stripped and reprobed for GAPDH (bottom). Lanes 1 and 4, mock infection; lanes 2 and 5, Ad5- β -gal; lanes 3 and 6, Ad5-ZBP-89. (B) AGS cells were infected with the control Ad5- β -gal or Ad5-ZBP-89 for 16 h. The half-life of p53 was then determined by [³⁵S]methionine pulse-chase analysis. The p53 protein was immunoprecipitated with mouse anti-p53 antibody (DO-1) and then resolved on a NOVEX 4 to 12% gradient gel. (C) The p53 band intensity was quantified by PhosphorImager analysis. Band intensities were expressed as percentages of the control signal (pulse only, no chase). Results shown are representative of three experiments.

ZBP-89 increases wild-type p53 protein levels. Since p53 mediates cell cycle arrest and apoptosis and since both p53 and ZBP-89 stimulate the p21^{waf1} promoter, we examined whether ZBP-89 might regulate p53. Expression of both the p53 and

A **B** **C** **D** **E** **F**

p21^{waf1} proteins increased 48 h after infection with Ad5-ZBP-89 (Fig. 3A). As observed for ZBP-89, p53 arrests the cell cycle at both the G₁/S and G₂/M interfaces (8). Similarly, their common target, p21^{waf1}, also inhibits cell proliferation at both the G₁/S and the G₂/M transitions (49). Colocalization of ZBP-89 and p53 expression by immunofluorescence demonstrated that p53 and ZBP-89 occupy the nuclear compartment (Fig. 3B). Thus, we concluded from these studies that p53 may also be a downstream target of ZBP-89.

These results raised the question of whether ZBP-89 stimulates growth arrest and apoptosis in a p53-dependent manner. To examine this question, Ad5-ZBP-89 was used to infect HCT 116 p53 wild-type and null cells (Fig. 3C and D). Flow cytometry was performed to analyze the percentages of cells in different phases of the cell cycle and the sub-G₁ phase. ZBP-89 expression in the HCT 116 wild-type cells resulted in a 40% reduction of the cells in S phase, as observed with the AGS cells. However, in the p53 null cells expressing ZBP-89, there was no change in the percentage of the cells in S phase. Therefore we concluded that the S phase inhibition mediated by ZBP-89 is p53 dependent. There was no change in the number of cells entering the sub-G₁ phase, demonstrating that ZBP-89-induced apoptosis is p53 independent (Fig. 3D).

Overexpression of ZBP-89 inhibits cell growth primarily by reducing the number of cells in S phase. Since this process is p53 dependent, we examined whether physiological arrest of cell growth by serum starvation stimulates ZBP-89 expression. AGS cells were placed in serum-free media for up to 24 h. Whole-cell extracts were prepared for immunoblot analysis at 0, 4, 8, 16, and 24 h (Fig. 4A). The results show that an increase in endogenous ZBP-89 protein occurs within 4 h of serum removal. This increase occurred before the rise in p53 protein levels, which was evident by 16 h. It is known that p53 protein levels may stabilize due to an increase in p14ARF activity (37, 46); however there was no change in p14ARF protein levels during the first 24 h of serum starvation. Since changes in p53 protein levels frequently occur as a result of protein-protein interactions (15), we examined whether ZBP-89 induced during serum starvation formed a complex with p53. The results indicate that ZBP-89 coprecipitates with p53 (Fig. 4B). Therefore both physiological induction and overexpression of ZBP-89 increase p53 protein levels.

ZBP-89 stabilizes the wild-type p53 protein. To examine further whether ZBP-89 increases the p53 protein through a

transcriptional or translational mechanism, we determined the level of p53 mRNA by Northern blot analysis with the AGS gastric cancer cell line and the HCT 116 colon cancer cell line (Fig. 5A). Both cell lines express low levels of wild-type p53 protein. There was no increase in the amount of p53 mRNA observed in either cell line with ZBP-89 overexpression. In addition, cotransfection of a p53 reporter and ZBP-89 expression vector did not result in an increase in p53 promoter activity (data not shown). Thus, we concluded that ZBP-89 did not increase p53 by transcriptional or posttranscriptional mechanisms. It has been reported that hypoxia-inducible factor 1 α (1) and BRCA1 (45) increase p53 protein levels through protein stabilization. Therefore, to examine whether ZBP-89 increases p53 protein levels by a similar mechanism, we performed pulse-chase analysis. In mock- and control adenovirus-infected cells, the half-life of wild-type p53 was ~30 min as previously reported (1, 3), whereas the p53 protein from Ad5-ZBP-89-infected cells had a half-life of ~85 min (Fig. 5B and C). Similar results were also obtained with HCT 116 cells (data not shown). These data indicate that ZBP-89 stabilizes the wild-type p53 protein.

Direct interaction between ZBP-89 and p53. Since ZBP-89 stabilized the p53 protein, we queried whether this occurs by direct contact. The p53 protein participates in multiple protein-protein interactions that modulate its activity in a variety of ways, including protein stabilization (1, 38). In an effort to determine whether ZBP-89 stabilizes p53 through direct contact, protein binding assays were performed. AGS cells were mock infected or infected with ZBP-89-containing recombinant adenovirus. Cell extracts were subjected to immunoprecipitation with a rabbit polyclonal p53 antibody followed by immunoblotting with mouse anti-Flag M2 to detect transfected ZBP-89, with rabbit anti-ZBP-89 to detect endogenous and transfected proteins, or with the mouse anti-p53 antibody to detect the p53 protein. Figure 6A shows that ZBP-89 coprecipitated with p53. Moreover, coprecipitation was also performed using unlabeled in vitro-translated or [³⁵S]methionine-labeled ZBP-89 or p53 proteins. ZBP-89 coprecipitated with the p53 antibody, and p53 coprecipitated with the ZBP-89 antibody. Neither protein was immunoprecipitated with pre-immune serum (Fig. 5B).

To identify the domains that mediated the interaction between ZBP-89 and p53, we carried out GST pull-down experiments. Various recombinant ZBP-89 deletion mutants were

FIG. 6. ZBP-89 interacts with p53 in vivo and in vitro. (A) Coimmunoprecipitation of p53 and ZBP-89 from mock-infected AGS cells (lane 1) or from cells infected with Ad5- β -gal (lane 2) or Ad5-ZBP-89 (lane 3). After cell lysis, immunoprecipitation (IP) was performed with the polyclonal p53 antibody followed by immunoblotting with p53 and Flag M2 antibodies. (B) Immunoprecipitation of ZBP-89 and p53 was performed with in vitro-transcribed and -translated ZBP-89 and p53 proteins. Lane 1, input of [³⁵S]methionine-labeled ZBP-89; lane 2, [³⁵S]methionine-labeled p53; lane 3, unlabeled ZBP-89 incubated with radiolabeled p53 and immunoprecipitated with control rabbit serum; lane 4, unlabeled ZBP-89 incubated with radiolabeled p53 and immunoprecipitated with rabbit ZBP-89 antibody; lane 5, unlabeled p53 incubated with radiolabeled ZBP-89 and immunoprecipitated with control rabbit serum; lane 6, unlabeled p53 incubated with radiolabeled ZBP-89 and immunoprecipitated with rabbit anti-p53 antibody. PI, preimmune serum. (C) Mapping of the p53-interacting domain of ZBP-89. The various ZBP-89 constructs used in GST pull-down assays are shown. The ZBP-89 GST fusion proteins were incubated with [³⁵S]methionine-labeled, in vitro-translated p53, pelleted, and then analyzed by SDS-PAGE. Lane 1, input of p53; lane 2, GST alone. (D) Mapping of the ZBP-89-interacting domain of p53. The various p53 constructs used in GST pull-down assays are shown. These proteins were incubated with [³⁵S]methionine-labeled, in vitro-translated ZBP-89 and analyzed as described above for p53. Lane 1, input of labeled ZBP-89; lane 2, GST alone. (E) GST pull-down was performed in the presence of 10 μ g of ethidium bromide/ml, and blotting was performed with the M2 anti-Flag antibody. (F) Schematic representation of the ZBP-89 and p53 interaction domains. ZBP-89 interacts with amino acids 160 to 393 of p53. p53 contains an activation domain (AD), DNA binding domain (DNA BD), tetramerization domain (TD), and regulatory domain (RD). p53 binds to the zinc finger DNA binding domain of ZBP-89 (amino acids 154 to 300). ZBP-89 contains an acidic domain (AD), zinc finger DNA binding domain, basic domain (BD), and C-terminal domain (C-TER).

FIG. 7. ZBP-89 enhances p53 transcriptional activation. (A) HCT 116 p53^{-/-} cells were cotransfected with 200 ng of PG13 or MG15, 5 ng of pCMV-p53, and/or 100 ng of pcDNA3-Flag-ZBP-89. Relative luciferase activities were obtained by normalizing the luciferase activity with β -galactosidase activity. Values are the means $\pm$ standard errors of the means (SEM) for three independent experiments performed in triplicate. (B) HCT 116 p53^{-/-} cells were cotransfected with 200 ng of p21^{waf1}-Luc, 5 ng of pCMV-p53, and/or 100 ng of pcDNA3-Flag-ZBP-89. Relative luciferase activities were obtained by normalizing the luciferase activity with β -galactosidase activity. Shown are the means $\pm$ SEM for three experiments performed in triplicate. (C) Immunoblot analysis of transiently transfected HCT 116 p53^{-/-} cells. Eighty micrograms of whole-cell extracts was resolved by SDS-PAGE. The anti- β -galactosidase, anti-p53, and anti-Flag M2 monoclonal antibodies were used. Results shown are representative of three independent experiments.

cloned as GST fusion proteins. The p53 protein was labeled with [³⁵S]methionine by in vitro translation and then incubated with the various immobilized GST-ZBP-89 fusion proteins, eluted, and analyzed by SDS-PAGE. The p53 protein interacted with both full-length ZBP-89 and with ZBP-89 mutants containing the zinc finger DNA-binding domain but not the N-terminal or C-terminal domain (Fig. 6C). This result demonstrated that the ZBP-89-p53 interaction occurs exclusively through the zinc fingers of ZBP-89. Note that the 53- and 40-kDa forms (translated from an internal translation start site) both bound to ZBP-89. The presence of the 40-kDa form

suggested that the N-terminal domain of p53 is not required for the ZBP-89 interaction.

To directly identify the p53 domain that is required for the interaction with ZBP-89, [³⁵S]methionine-labeled, in vitro-translated ZBP-89 was incubated with various immobilized GST-p53 fusion proteins and analyzed as described above (Fig. 6D). Mutants comprising the DNA binding domain or C-terminal domain alone or together were able to interact with ZBP-89 (Fig. 6D). This result is somewhat consistent with the site of interaction for BRCA1, which stabilizes p53 by binding to its C terminus and enhancing its transcriptional activation

FIG. 8. The p53R273H mutation prevents ZBP-89-mediated stabilization. (A) HCT 116 (wild-type p53 and null) and HT-29 colon cell lines were treated as described in the legend for Fig. 1. The expression of ZBP-89 and p53 was determined by immunoblot analysis. Lanes 1, 4, and 7, mock infection; lanes 2, 5, and 8, Ad5-vector; lanes 3, 6, and 9, Ad5-ZBP-89. (B) Two hundred micrograms of HT-29 whole-cell extracts was incubated with 20 μ l of GST or GST-ZBP-89 beads at 4°C for 1 h. The pellets were washed; proteins were eluted from beads and resolved by SDS-PAGE for immunoblot analysis. Twenty micrograms of whole-cell extracts was loaded as the input. (C) HCT 116 p53^{-/-} cells were cotransfected with 200 ng of PG13 or p21^{waf1}-Luc, 5 ng of pCMV-p53R273H, and/or 100 ng of pcDNA3-Flag-ZBP-89. Relative luciferase activities were obtained by normalizing the luciferase activity with β -galactosidase activity. Values are means $\pm$ standard errors of the means from three independent experiments performed in triplicate. (D) Western blot analysis of transiently transfected HCT 116 p53^{-/-} cells. Eighty micrograms of whole-cell extracts was separated by SDS-PAGE for immunoblot analysis.

(45, 56). To exclude the possibility that the *in vitro* protein-protein interaction was due to binding to nucleic acids, the pull-down assay was performed in the presence of ethidium bromide and revealed no difference in the protein-protein interaction (Fig. 6E). Thus, the zinc finger domain of ZBP-89 physically interacts with the DNA binding and C-terminal domains of p53 to stabilize the protein (Fig. 6F).

ZBP-89 enhances p53 transcriptional activation. Since ZBP-89 stabilizes p53 through direct protein interaction, we examined whether this translated into an increase in p53 transcriptional activation. An important function of p53 is to trans-

activate downstream target genes through direct DNA binding. To determine whether the elevated protein levels of p53 induced by ZBP-89 resulted in higher p53-specific transcriptional activity, HCT 116 p53 null cells were cotransfected with p53 and/or ZBP-89 expression vectors and p53 reporter constructs. ZBP-89 alone had no effect on the PG13 reporter but significantly potentiated p53 transcriptional activity (Fig. 7A). This result was consistent with the inability of ZBP-89 overexpression to stimulate p53 gene expression and p53 reporter constructs. p53 alone or with ZBP-89 had no effect on the MG15 reporter, which contains 15 copies of the mutant p53 binding

FIG. 9. ZBP-89 stabilizes p53 without interrupting the p53-MDM2 interaction. (A) AGS cells were infected, and an immunoblot was performed, as described in the legend for Fig. 1. The polyclonal p53, ZBP-89, actin, and monoclonal Flag M2 and MDM2 antibodies were used. Lane 1, mock infection; lane 2, Ad5- β -gal; lane 3, Ad5-ZBP-89. (B) AGS whole-cell extracts were immunoprecipitated with the mouse p53 antibody (DO-1). The pellets were resolved on a NOVEX 4 to 12% gel followed by immunoblotting as described in the legend for Fig. 7. Lane 1, mock infection; lane 2, Ad5- β -gal; lane 3, Ad5-ZBP-89.

sites. To assess whether ZBP-89 potentiated the effect of p53 on an endogenous promoter, transfection experiments were performed with HCT 116 p53 null cells with the p21^{waf1}/2300-Luc reporter, which contains two endogenous p53 binding sites (12) (Fig. 7B). The results show that ZBP-89 potentiated p53 activation of the p21^{waf1} promoter. In the absence of wild-type p53, ZBP-89 alone did not activate the p21^{waf1} promoter, as we have shown previously for the colon cancer cell line HT-29 expressing mutant p53 (5). An immunoblot confirmed (Fig.

7C) that there was a substantial accumulation of wild-type p53 protein in HCT 116^{-/-} cells cotransfected with both ZBP-89 and p53 expression vectors compared to that in cells transfected with p53 alone.

ZBP-89 induced accumulation of the p53 protein in both gastric (AGS) and colon (HCT 116) cell lines that express small amounts of wild-type p53 (Fig. 8A). By contrast, ZBP-89 did not stabilize p53 in the HT-29 colon cancer cell line that expresses mutant p53 (Fig. 8A). The p53 mutation in this cell line is R273H, which is a common mutation in both colon and gastric cancers (40, 42). The p53R273H mutant did not prevent ZBP-89 binding as shown in the GST pull-down assay (Fig. 8B). The R273H mutation abolishes the transactivation function of p53 as previously reported (42), and cotransfection with ZBP-89 did not overcome the transcriptional inhibition (Fig. 8C). To ensure that the lack of stabilization in HT-29 cells was due to the mutation and not to other genetic abnormalities, we cotransfected the p53R273H mutant expression vector into HCT 116 p53 null cells. As observed for the HT-29 cell line, ZBP-89 was not able to stabilize the mutant p53 protein (compare Fig. 8D and 7C). Together, these data indicate that ZBP-89 enhances the transcriptional activity of wild-type p53 by stabilizing protein levels whereas mutations prevent the accumulation of the p53 protein. Therefore, ZBP-89 does not appear to contribute to the accumulation of at least some mutant forms of p53.

ZBP-89 does not prevent the interaction between MDM2 and p53. Wild-type p53 usually has a very short half-life in normal cells. This is largely due to MDM2-directed degradation. MDM2 binds to p53 and promotes its ubiquitination and subsequent degradation by the proteasome (20, 28). Inhibition of this degradation is the primary mechanism that results in stabilization of wild-type and mutant p53 proteins (43) and may be accomplished by inhibiting MDM2 activity (3). p14ARF forms a complex with MDM2 and p53 and inhibits MDM2-directed degradation (48). However, p14ARF protein levels remained unchanged during serum starvation and the subsequent rise in ZBP-89 and p53 protein levels, suggesting that p14ARF was not required (Fig. 4). Therefore, we examined whether MDM2 levels are altered or whether the MDM2-p53 interaction is disrupted. We found that enhanced expression of ZBP-89 had no effect on total cell MDM2 protein levels (Fig. 9A). In addition, overexpression of ZBP-89 did not prevent MDM2 from forming a complex with p53 (Fig. 9B). These results demonstrate that ZBP-89 stabilizes the p53 protein independently of MDM2.

ZBP-89 retains p53 in the nucleus. Since ZBP-89-mediated stabilization of p53 was independent of p14ARF and MDM2, we used heterokaryon assays to examine whether ZBP-89 overexpression retained p53 in the nucleus. The results show that endogenous p53 in human AGS cells translocates to the mouse MEF p53 null cell nucleus in the absence of ZBP-89 overexpression (Fig. 10). However, in AGS cells overexpressing ZBP-89 that are fused to mouse p53 null cells, p53 is retained in the AGS cell nucleus. These results demonstrate that ZBP-89 prevents p53 nuclear export (Fig. 10).

DISCUSSION

The results in this study establish a fundamental role for ZBP-89 in the regulation of cell proliferation. Elevated expres-

FIG. 10. ZBP-89 prevents the nuclear export of p53. AGS cells were transfected with the pcDNA3 empty vector or the pcDNA3-Flag-ZBP-89 expression vector. Forty hours after transfection, the heterokaryon assay was performed as described in Materials and Methods. Phase-contrast optics were used to confirm the fusion of AGS (arrows) with MEF p53 null cells. The nucleus was stained with DAPI and visualized using a UV filter (blue). p53 was stained with a FITC-labeled anti-mouse IgG (green), and ZBP-89 was stained with a Texas red-conjugated anti-rabbit IgG (red).

sion of ZBP-89 induced growth arrest and apoptosis. However, the S-phase inhibition observed with ZBP-89 overexpression was abolished in a p53 null cell line. This result confirmed that the growth arrest mediated by ZBP-89 was p53 dependent whereas ZBP-89-mediated apoptosis was p53 independent. Elevated levels of ZBP-89 stabilize the p53 protein through di-

rect physical contact, which potentiates the transcriptional activation of both a synthetic p53-responsive reporter and its endogenous target, the p21^{waf1} gene. The potentiation was p53 dependent since ZBP-89 could not activate the promoter in the absence of the p53 protein. Similarly, our prior study showed that ZBP-89 activation of the p21^{waf1} promoter is butyrate

dependent in a p53-deficient cell line and that ZBP-89 exerts no transcriptional regulation on the p21^{waf1} promoter alone (5). ZBP-89 binds directly to the p21^{waf1} promoter (5, 19), but, in the absence of p53, ZBP-89 requires recruitment of a histone acetylase and inhibition of deacetylase activity (5). Thus both p53 and p21^{waf1} appear to be downstream targets of ZBP-89. Accumulation of p53 is normally transient due to the induction of the p53 inhibitor MDM2 or decreased p14ARF activity. This raised the possibility that ZBP-89 might affect p53 protein levels by modulating p14ARF or MDM2 expression. However, both the MDM2-p53 interaction and p14ARF levels remained unperturbed despite elevated levels of ZBP-89. Thus ZBP-89 was able to overcome the tendency for p53 to be rapidly degraded by binding to a site distal to the MDM2 binding domain and formation of the p14ARF-MDM1-p53 trimeric complex, thereby preventing p53 nuclear export.

In transiently cotransfected HCT 116 p53 null cells, ZBP-89 caused an approximately five- to eightfold increase in cotransfected p53 protein, whereas the transcriptional activity of transfected p53 alone was potentiated only approximately twofold. The discrepancy between protein levels and activity is consistent with another report showing that stabilized p53 is not able to totally regain its transcriptional activation due to the formation of a trimeric complex containing MDM2, p53, and the retinoblastoma protein (23). Further, the accumulation of p53 mediated by ZBP-89 may not directly translate to transcriptional activity due to the inhibitory effects of MDM2 suppressing the overall level of transcription.

A major mechanism that results in p53 stabilization involves activation of tumor suppressor protein p14ARF (mouse p19ARF) (37, 46). An increase in the activity of p14ARF promotes sequestration and degradation of MDM2, thereby reducing its activity (51). p14ARF binds directly to MDM2 in a region distinct from the p53 binding domain and therefore does not disrupt the interaction between p53 and MDM2. The tumor suppressor protein BRCA1 (45, 56) and oncogene products, such as c-Abl, c-Myc, Ras, and E1A (10, 36, 44, 57), stabilize p53 through activation of p14ARF. For BRCA1, overexpression induces p14ARF expression, which in turn inhibits MDM2 (45, 56). p14ARF is required for the BRCA1 effect since overexpression of this tumor suppressor protein in p14ARF-deficient cells failed to induce accumulation of wild-type p53 (45, 56). BRCA1 binds to the C terminus of wild-type p53 and stabilizes the protein through direct binding. While BRCA1 induces accumulation of wild-type p53, its overexpression failed to induce accumulation of mutant p53. These studies were carried out with a prostate cell line (DU-145) which has a double point mutation of p53 (14). Since BRCA1 and ZBP-89 both bind to the C terminus of p53 and preferentially stabilize wild-type over mutant forms of p53, we considered the possibility that ZBP-89 might also stabilize p53 in a p14ARF-dependent manner. However, the time course of serum starvation and lack of correlation with p14ARF protein expression diminished the likelihood that this tumor suppressor is required for the ZBP-89-induced stability of p53.

Most of the p53 mutations that occur in cancer are located in the DNA binding domain and effectively block its transactivating activity. p53 mutations also prevent MDM2 from targeting the protein for degradation, allowing mutant forms of p53 to accumulate in the cell (6). Elevated mutant forms of p53

are thought to have a deleterious effect on p53 function by dimerizing with the remaining normal p53 proteins in the cell (7, 42). We found that a single p53 mutation, R273H, prevented ZBP-89 from inducing accumulation of the mutant p53 protein. Thus it appears so far that the effect of ZBP-89 on the p53 protein is specific for the wild-type form. This result may have relevance in cancers that tend to accumulate wild-type rather than mutant p53 despite activation of oncogenes or other cell stresses (13). Cancer cells that accumulate wild-type p53 tend to undergo apoptosis and are more susceptible to radiotherapy and chemotherapy (33). Therefore, the studies described here may further our understanding of how wild-type p53 might accumulate in transformed cells.

p53 may accumulate in cells due to mechanisms that interfere with MDM2 binding or activity. This may be accomplished by phosphorylation of the p53 N-terminal domain, subsequently blocking MDM2 binding and activity (2). It is not clear how the ZBP-89-p53-MDM2 trimeric complex protects p53 from MDM2-mediated degradation. However, the heterokaryon assay clearly demonstrated that elevated levels of ZBP-89 prevent p53 nuclear export. The knowledge that ZBP-89 binds preferentially to the middle (DNA binding) and C-terminal domains of p53 and not to the N-terminal domain, recognized by the MDM2-p14ARF complex, suggests that an alternative mechanism is employed to stabilize p53. It has been shown recently that both the DNA binding domain and extreme C terminus of p53 are necessary for MDM2-mediated degradation (2, 29). Partial deletions or mutations of the p53 C terminus interrupt MDM2-directed degradation (29). A recent study shows that p53 C-terminal lysine residues are the main sites of MDM2-mediated ubiquitin ligation, which targets p53 for proteasome degradation (41). Modifications of the p53 C terminus, including phosphorylation (24, 52) and acetylation (4, 16, 32, 53), enhance the transcriptional activity of p53. Acetylation of p53 at these C-terminal lysines prevents nuclear ubiquitination (35). Further, histone acetylase coactivator p300 binds the N-terminal domain of ZBP-89 and the C-terminal domain of p53 (5, 32). Thus, ZBP-89 may protect p53 from MDM2-mediated degradation by sterically masking the sites on p53 that confer sensitivity to degradation or by recruiting p300 to modify p53 through increased acetylation. This hypothesis would explain why the cellular MDM2 protein levels are not directly affected by ZBP-89 overexpression. Collectively, the results reported here reveal a novel function of ZBP-89 that supports its physiological role in growth regulation through a p53-dependent mechanism.

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REFERENCES

- An, W. G., M. Kanekal, M. C. Simon, E. Maltepe, M. V. Blagosklonny, and L. M. Neckers. 1998. Stabilization of wild-type p53 by hypoxia-inducible factor 1 α . *Nature* **392**:405–408.
- Ashcroft, M., M. H. Kubbutat, and K. H. Vousden. 1999. Regulation of p53 function and stability by phosphorylation. *Mol. Cell. Biol.* **19**:1751–1758.
- Ashcroft, M., and K. H. Vousden. 1999. Regulation of p53 stability. *Oncogene* **18**:7637–7643.
- Avantaggiati, M. L., V. Ogryzko, K. Gardner, A. Giordano, A. S. Levine, and K. Kelly. 1997. Recruitment of p300/CBP in p53-dependent signal pathways. *Cell* **89**:1175–1184.
- Bai, L., and J. L. Merchant. 2000. Transcription factor ZBP-89 cooperates with histone acetyltransferase p300 during butyrate activation of p21waf1 transcription in human cells. *J. Biol. Chem.* **275**:30725–30733.
- Blagosklonny, M. V. 1997. Loss of function and p53 protein stabilization. *Oncogene* **15**:1889–1893.
- Blagosklonny, M. V. 2000. p53 from complexity to simplicity: mutant p53 stabilization, gain-of-function, and dominant-negative effect. *FASEB J.* **14**: 1901–1907.
- Burns, T. F., and W. S. El-Deiry. 1999. The p53 pathway and apoptosis. *J. Cell. Physiol.* **181**:231–239.
- Dawson, M. I., J. H. Park, G. Chen, W. Chao, L. Dousman, N. Waleh, P. D. Hobbs, L. Jong, L. Toll, X. Zhang, J. Gu, A. Agadir, J. L. Merchant, L. Bai, A. K. Verma, S. M. Thacher, R. A. Chandraratna, B. Shroot, and D. L. Hill. 2001. Retinoic acid (RA) receptor transcriptional activation correlates with inhibition of 12-O-tetradecanoylphorbol-13-acetate-induced ornithine decarboxylase (ODC) activity by retinoids: a potential role for trans-RA-induced ZBP-89 in ODC inhibition. *Int. J. Cancer* **91**:8–21.
- de Stanchina, E., M. E. McCurrach, F. Zindy, S. Y. Shieh, G. Ferbeyre, A. V. Samuelson, C. Prives, M. F. Roussel, C. J. Sherr, and S. W. Lowe. 1998. E1A signaling to p53 involves the p19(ARF) tumor suppressor. *Genes Dev.* **12**: 2434–2442.
- Earnshaw, W. C., L. M. Martins, and S. H. Kaufmann. 1999. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu. Rev. Biochem.* **68**:383–424.
- El-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, E. Mercer, K. W. Kinzler, and B. Vogelstein. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**:817–825.
- el-Mahdani, N., J. C. Vaillant, M. Guiguet, S. Prevot, V. Bertrand, C. Bernard, R. Parc, G. Berezat, and B. Hermelin. 1997. Overexpression of p53 mRNA in colorectal cancer and its relationship to p53 gene mutation. *Br. J. Cancer* **75**:528–536.
- Fan, S., J. A. Wang, R. Q. Yuan, Y. X. Ma, Q. Meng, M. R. Erdos, L. C. Brody, I. D. Goldberg, and E. M. Rosen. 1998. BRCA1 as a potential human prostate tumor suppressor: modulation of proliferation, damage responses and expression of cell regulatory proteins. *Oncogene* **16**:3069–3082.
- Giaccia, A. J., and M. B. Kastan. 1998. The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev.* **12**:2973–2983.
- Gu, W., and R. G. Roeder. 1997. Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal. *Cell* **90**:595–606.
- Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi, and S. J. Elledge. 1993. The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75**:805–816.
- Hasegawa, T., A. Takeuchi, O. Miyaishi, K.-I. Isobe, and B. D. Crombrughe. 1997. Cloning and characterization of a transcription factor that binds to the proximal promoters of the two mouse type I collagen genes. *J. Biol. Chem.* **272**:4915–4923.
- Hasegawa, T., H. Xiao, and K.-I. Isobe. 1999. Cloning of a GADD34-like gene that interacts with the zinc-finger transcription factor which binds to the p21^{WAF} promoter. *Biochem. Biophys. Res. Commun.* **256**:249–254.
- Haupt, Y., R. Maya, A. Kazaz, and M. Oren. 1997. Mdm2 promotes the rapid degradation of p53. *Nature* **387**:296–299.
- Hollstein, M., D. Sidransky, B. Vogelstein, and C. C. Harris. 1991. p53 mutations in human cancers. *Science* **253**:49–53.
- Horikoshi, N., A. Usheva, J. Chen, A. J. Levine, R. Weinmann, and T. Shenk. 1995. Two domains of p53 interact with the TATA-binding protein, and the adenovirus 13S E1A protein disrupts the association, relieving p53-mediated transcriptional repression. *Mol. Cell. Biol.* **15**:227–234.
- Hsieh, J. K., F. S. Chan, D. J. O'Connor, S. Mitnacht, S. Zhong, and X. Lu. 1999. RB regulates the stability and the apoptotic function of p53 via MDM2. *Mol. Cell* **3**:181–193.
- Hupp, T. R., A. Sparks, and D. P. Lane. 1995. Small peptides activate the latent sequence-specific DNA binding function of p53. *Cell* **83**:237–245.
- Ikeda, A., X. Sun, Y. Li, Y. Zhang, R. Eckner, T. S. Doi, T. Takahashi, Y. Obata, K. Yoshioka, and K. Yamamoto. 2000. p300/CBP-dependent and -independent transcriptional interference between NF- κ B RelA and p53. *Biochem. Biophys. Res. Commun.* **272**:375–379.
- Kern, S. E., K. W. Kinzler, A. Bruskin, D. Jarosz, P. Friedman, C. Prives, and B. Vogelstein. 1991. Identification of p53 as a sequence-specific DNA-binding protein. *Science* **252**:1708–1711.
- Ko, L. J., and C. Prives. 1996. p53: puzzle and paradigm. *Genes Dev.* **10**:1054–1072.
- Kubbutat, M. H., S. N. Jones, and K. H. Vousden. 1997. Regulation of p53 stability by Mdm2. *Nature* **387**:299–303.
- Kubbutat, M. H., R. L. Ludwig, M. Ashcroft, and K. H. Vousden. 1998. Regulation of Mdm2-directed degradation by the C terminus of p53. *Mol. Cell. Biol.* **18**:5690–5698.
- Law, G. L., H. Itoh, D. J. Law, G. J. Mize, J. L. Merchant, and D. R. Morris. 1998. Transcription factor ZBP-89 regulates the activity of the ornithine decarboxylase promoter. *J. Biol. Chem.* **273**:19955–19964.
- Levine, A. J. 1997. p53, the cellular gatekeeper for growth and division. *Cell* **88**:323–331.
- Lill, N. L., S. R. Grossman, D. Ginsberg, J. DeCaprio, and D. M. Livingston. 1997. Binding and modulation of p53 by p300/CBP coactivators. *Nature* **387**:823–827.
- Lowe, S. W., S. Bodis, A. McClatchey, L. Remington, H. E. Ruley, D. E. Fisher, D. E. Housman, and T. Jacks. 1994. p53 status and the efficacy of cancer therapy in vivo. *Science* **266**:807–810.
- Merchant, J. L., G. R. Iyer, B. R. Taylor, J. R. Kitchen, E. R. Mortensen, Z. Wang, R. J. Flintoft, J. Michel, and R. Bassel-Duby. 1996. ZBP-89, a Kruppel-type zinc finger protein, inhibits EGF induction of the gastrin promoter. *Mol. Cell. Biol.* **16**:6644–6653.
- Nakamura, S., J. A. Roth, and T. Mukhopadhyay. 2000. Multiple lysine mutations in the C-terminal domain of p53 interfere with MDM2-dependent protein degradation and ubiquitination. *Mol. Cell. Biol.* **20**:9391–9398.
- Palmero, I., C. Pantoja, and M. Serrano. 1998. p19ARF links the tumour suppressor p53 to Ras. *Nature* **395**:125–126.
- Pomerantz, J., N. Schreiber-Agus, N. J. Liegeois, A. Silverman, L. Alland, L. Chin, J. Potes, K. Chen, I. Orlow, H. W. Lee, C. Cordon-Cardo, and R. A. DePinho. 1998. The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell* **92**:713–723.
- Prives, C., and P. A. Hall. 1999. The p53 pathway. *J. Pathol.* **187**:112–126.
- Remington, M. C., S. A. Tarle, B. Simon, and J. L. Merchant. 1997. ZBP-89, a Kruppel-type zinc finger protein, inhibits cell proliferation. *Biochem. Biophys. Res. Commun.* **237**:230–234.
- Rodrigues, N. R., A. Rowan, M. E. Smith, I. B. Kerr, W. F. Bodmer, J. V. Gannon, and D. P. Lane. 1990. p53 mutations in colorectal cancer. *Proc. Natl. Acad. Sci. USA* **87**:7555–7559.
- Rodriguez, M. S., J. M. Desterro, S. Lain, D. P. Lane, and R. T. Hay. 2000. Multiple C-terminal lysine residues target p53 for ubiquitin-proteasome-mediated degradation. *Mol. Cell. Biol.* **20**:8458–8467.
- Roemer, K. 1999. Mutant p53: gain-of-function oncoproteins and wild-type p53 inactivators. *Biol. Chem.* **380**:879–887.
- Shieh, S. Y., M. Ikeda, Y. Taya, and C. Prives. 1997. DNA damage-induced phosphorylation of p53 alleviates inhibition by MDM2. *Cell* **91**:325–334.
- Sionov, R. V., E. Moallem, M. Berger, A. Kazaz, O. Gerlitz, Y. Ben-Neriah, M. Oren, and Y. Haupt. 1999. c-Abl neutralizes the inhibitory effect of Mdm2 on p53. *J. Biol. Chem.* **274**:8371–8374.
- Somasundaram, K., T. K. MacLachlan, T. F. Burns, M. Sgagias, K. H. Cowan, B. L. Weber, and W. S. el-Deiry. 1999. BRCA1 signals ARF-dependent stabilization and coactivation of p53. *Oncogene* **18**:6605–6614.
- Stott, F. J., S. Bates, M. C. James, B. B. McConnell, M. Starborg, S. Brookes, I. Palmero, K. Ryan, E. Hara, K. H. Vousden, and G. Peters. 1998. The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. *EMBO J.* **17**:5001–5014.
- Taniuchi, T., E. R. Mortensen, A. Ferguson, J. Greenson, and J. L. Merchant. 1997. Overexpression of ZBP-89, a zinc finger DNA binding protein, in gastric cancer. *Biochem. Biophys. Res. Commun.* **233**:154–160.
- Tao, W., and A. J. Levine. 1999. p19(ARF) stabilizes p53 by blocking nucleocytoplasmic shuttling of Mdm2. *Proc. Natl. Acad. Sci. USA* **96**:6937–6941.
- Vogelstein, B., D. Lane, and A. J. Levine. 2000. Surfing the p53 network. *Nature* **408**:307–310.
- Vousden, K. H. 2000. p53: death star. *Cell* **103**:691–694.
- Vousden, K. H., and G. F. Woude. 2000. The ins and outs of p53. *Nat. Cell Biol.* **2**:E178–E180.
- Wang, Y., and C. Prives. 1995. Increased and altered DNA binding of human p53 by S and G₂/M but not G₁ cyclin-dependent kinases. *Nature* **376**:88–91.
- Yang, X. J., V. V. Ogryzko, J. Nishikawa, B. H. Howard, and Y. Nakatani. 1996. A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* **382**:319–324.
- Zambetti, G. P., and A. J. Levine. 1993. A comparison of the biological activities of wild-type and mutant p53. *FASEB J.* **7**:855–865.
- Zhan, Q., K. A. Lord, I. Alamo, Jr., M. C. Hollander, F. Carrier, D. Ron, K. W. Kohn, B. Hoffman, D. A. Liebermann, and A. J. Fornace, Jr. 1994. The *gadd* and *MyD* genes define a novel set of mammalian genes encoding acidic proteins that synergistically suppress cell growth. *Mol. Cell. Biol.* **14**:2361–2371.
- Zhang, H., K. Somasundaram, Y. Peng, H. Tian, D. Bi, B. L. Weber, and W. S. El-Deiry. 1998. BRCA1 physically associates with p53 and stimulates its transcriptional activity. *Oncogene* **16**:1713–1721.
- Zindy, F., C. M. Eischen, D. H. Randle, T. Kamijo, J. L. Cleveland, C. J. Sherr, and M. F. Roussel. 1998. Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev.* **12**:2424–2433.