[CANCER RESEARCH 64, 8318–8327, November 15, 2004]

Transactivation of the *EGR1* Gene Contributes to Mutant p53 Gain of Function

Lilach Weisz, Amir Zalcenstein, Perry Stambolsky, Yehudit Cohen, Naomi Goldfinger, Moshe Oren, and Varda Rotter

Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot, Israel

ABSTRACT

Tumor-associated mutants of the p53 tumor suppressor protein exert biological activities compatible with an oncogenic gain of function. To explore the underlying molecular mechanism, we performed microarray analysis, comparing p53-null cells to mutant p53-expressing cells. One of the genes up-regulated in the presence of mutant p53 was *EGR1*, a transcription factor implicated in growth control, apoptosis, and cancer. EGR1 induction by various types of stress is markedly augmented in cells expressing mutant p53. Moreover, chromatin immunoprecipitation analysis indicates that mutant p53 is physically associated with the *EGR1* promoter. Functional assays indicate that induction of EGR1 by mutant p53 contributes to enhanced transformed properties and resistance to apoptosis. We propose that EGR1 is a significant contributor to mutant p53 gain of function.

INTRODUCTION

It is well accepted that inactivation of the *p53* tumor suppressor gene is an important event in the process of tumor development (1). Elimination of the normal functions of p53, one of the key players in the maintenance of genome stability, leads to accumulation of genetic aberrations that eventually cause malignant transformation of normal cells (2). A high percentage of human tumors maintain overexpression of various mutant forms of p53 (3). Furthermore, a growing number of epidemiologic surveys suggest a significant correlation between overexpression of specific mutant p53 forms and tumor prognosis (4). However, it remains unclear whether different p53 mutants initially act via a negative dominant mechanism, in which the protein expressed from the residual wild-type p53 (wtp53) allele is inactivated by the concomitantly expressed mutant p53, or whether mutant p53 acts primarily by a gain of function mechanism (5). The idea that mutant p53 functions by gain of function is supported by the observations that overexpression of mutant p53 forms in p53-null cells accentuated their transformed phenotype (6). Several p53 mutants were reported to increase tumorigenicity in mice (7, 8), mutation frequency (9), and metastatic potential (8). Interestingly, it was found that mutant p53 interferes with stress-induced apoptosis and overexpression of different p53 mutants in p53-null cells conferred increased resistance to chemotherapeutic drugs (10–12). However, anti-apoptotic activity mediated by p53 "core" mutants was relieved when these p53 mutants were also genetically modified at their NH_2-terminal domain (13), corresponding to the transactivation domain of the wtp53 protein (14).

The fact that mutant p53 facilitates transcription of transformation-related genes was shown in several studies. Different viral and cellular promoters were shown to be transactivated by mutant p53, including the MDR-1 (7, 15), c-myc (16), interleukin-6 (17), heat shock protein 70 (18), human epidermal growth factor receptor (19), and HIV-1 LTR promoters (20). Furthermore, transcription inhibitors were shown to counter the mutant p53 anti-apoptotic gain of function effect (12). It is presently unclear whether mutant p53 regulates transcription of particular genes by binding directly to specific regulatory DNA sequences, through interaction with other DNA-binding proteins, or both. Of note, chromatin immunoprecipitation analysis indicates that different p53 mutants bind different DNA sequences (21). In search of signature genes regulated by mutant p53, we used Affymetrix DNA microarrays to compare gene expression patterns of p53-null cells and their mutant p53-expressing derivatives. Induction of Early Growth Response 1 (*EGR1*) gene expression was one of the significant changes observed upon overexpression of mutant p53 in human prostate cancer– and lung cancer–derived cells. The EGR1 protein is a M_r 59,000 transcription factor involved in various biological functions including regulation of proliferation, growth, apoptosis, and angiogenesis (22, 23). The fact that this protein plays an important role in pathways that pertain directly to cancer progression, prompted us to focus on the analysis of the relationship between mutant p53 expression and the induction of EGR1. We found that mutant p53 protein can induce the transcription of *EGR1* and elevate the expression of EGR1 downstream genes such as vascular endothelial growth factor (*VEGF*). Furthermore, mutant p53 can associate with *EGR1* gene promoter DNA sequences. Knockdown of EGR1 in mutant p53-overproducing cells attenuates colony formation under stress conditions and renders these cells more vulnerable to genotoxic stress. Together, these findings suggest that EGR1 plays an important role in a mutant p53-regulated oncogenic pathway.

MATERIALS AND METHODS

Cells, Plasmids, and Reagents. H1299 cells were obtained from American Type Culture Collection (Manassas, VA). H1299 cells were maintained in RPMI (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (Sigma). PC3 and HeLa cells were maintained in Dulbecco's modified Eagle's medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum. SKBR3 cells were maintained in McCoy's medium (Sigma) supplemented with 10% fetal calf serum.

Plasmids, pCMV-neo-Bam-p53R175H carrying either Arg or Pro at position 72, were obtained from B. Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MD) and W. Kaelin (Dana-Farber Cancer Institute, Boston, MA), respectively. A pCDNA3-based expression plasmid for p53R175H and p53R175H (22, 23) was constructed by A. Bren (Weizmann Institute, Rehovot, Israel) and G. Blandino (Regina Elena Cancer Institute, Rome, Italy), respectively. Plasmid pM5neo-ecotropic-R, encoding the mouse ecotropic receptor, was provided by S. Benchimol (The Ontario Cancer Institute, Toronto, Canada). pBabe-puro-175H was constructed by removing the p53 open reading frame from pCMV-neo-Bam-p53R175H and inserting it into pBabe-puro.

The EGR1 expression plasmid was constructed by cloning the EGR1 open reading frame, obtained by reverse transcription-PCR with specific primers (sense, CCC GGA TGG CCG CGG CCA AGG; and antisense, GGC CAT CTC CTC CTC CTG TCC) into pcDNA3.1/V5-His-TOPO using the pcDNA3.1/V5-His-TOPO TA expression kit (Invitrogen, Carlsbad, CA). EGR1-luc was constructed by cloning the EGR1 promoter, obtained by

Received 3/31/04; revised 8/12/04; accepted 9/8/04.

Grant support: The Israel-USA Binational Science Foundation, the Israeli Science Foundation, European Commission grants QLG1-1999-00273 and QLK6-2000-00159, the German-Israel Project Cooperation, the Kadoorie Charitable Foundations, and Yad Abraham Center for Cancer Diagnosis and Therapy.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: V. Rotter is the incumbent of the Norman and Helen Asher Professorial Chair Cancer Research at the Weizmann Institute.

Requests for reprints: Varda Rotter, Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel 76100. Phone: 972-8-9344501; Fax: 972-08-9465265; E-mail: varda.rotter@weizmann.ac.il.

genomic PCR with specific primers (sense, GCC ACA CCC GGA AAG ACA C; and antisense, CTG GAC GAG CAG GCT GGA GAG), into the pGL3-Basic luciferase plasmid (Promega, Madison, WI).

Infections, Transfections, and Establishment of Mutant p53-Expressing Cells. Infections were carried out using a standard protocol. In brief, PC3 cells were stably transfected with pM5neo-ecotropic-R, encoding the mouse ecotropic receptor, and selected for 2 weeks in 0.4 mg/mL G418. Concurrently, 293T cells were transfected with the retroviral construct pBabe-puro-175H, using Fugene (Roche, Switzerland) according to the manufacturer's instructions. Virus-containing culture supernatants were harvested 24 to 48 hours post transfection at 6-hour intervals and pooled together. The stably transfected PC3 cells were then infected with the filtered supernatants and selected for 48 hours in 2 μg/mL puromycin, resulting in cell populations stably expressing mutant p53R175H. Transfections were carried out using Fugene (Roche) at a ratio of 2 μg of DNA:4 μL of Fugene, according to the manufacturer's instructions. H1299 cells were stably transfected with p53R175H followed by G418 selection (0.4 mg/mL) for 3 weeks to achieve single-cell clones.

Apoptosis Assays and Fluorescence-Activated Cell Sorter Analysis. PC3 cells, either noninfected or infected with mutant p53R175H, were plated on 6-cm dishes. Cells were then transfected with various plasmid combinations, with the aid of Fugene 6 (Roche), using a standard protocol. A plasmid encoding H2B-GFP was included in all transfections. Twenty-four hours after transfection, cells were treated with either 10 μmol/L etoposide or 1 μg/mL cisplatinum for either 48 or 72 hours. Cells were subsequently trypsinized and fixed in 80% methanol/20% PBS for at least 25 minutes. Cells were then rehydrated for at least 30 minutes in PBS, washed, resuspended in PBS containing 25 μg/mL propidium iodide (Sigma) and 10 μg/mL RNase A, and subjected to fluorescence-activated cell sorter-based cell cycle analysis. Only green fluorescent protein (GFP)-positive cells, expected to represent the successfully transfected subpopulation of the culture, were included in the analysis.

Nuclear Extracts. Nuclear extracts were prepared as described previously (24). Cells (10^6~10^7) were washed twice with cold PBS and harvested. Cell pellets were resuspended by gentle pipetting in 400 μL of buffer A [10 mmol/L HEPES-KOH (pH 7.9), 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, 0.2 mmol/L phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor mixture (Roche)]. After 15 minutes of incubation on ice, 25 μL of 10% NP40 were added, and vortexing was performed vigorously for 10 minutes. After centrifugation, cell pellets were resuspended in approximately 150 to 300 μL of buffer C [20 mmol/L HEPES-KOH (pH 7.9), 25% glycerol, 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 0.2 mmol/L PMSF, and protease inhibitors mixture (Roche)]. Tubes were transferred to a rotating platform for 15 minutes at 4°C and then centrifuged, and the protein content of the supernatant was determined by the Bradford procedure. Aliquots were stored at −70°C.

Western Blot Analysis. Cells were lysed in passive lysis buffer (Promega). Lysate aliquots were resolved by SDS-PAGE on a 7.5% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed sequentially with EGR1 antibody (sc-189; Santa Cruz Biotechnology, Santa Cruz, CA), a mixture of the p53-specific monoclonal antibodies PAb1801 and DO-1, and finally either antivinculin or antitubulin antibody (Sigma). Membranes were then reacted with secondary goat-antimouse and goat-antirabbit horseradish peroxidase–conjugated antibodies (1:10,000; Jackson) and developed using the ECL kit (Amersham Biosciences, Uppsala, Sweden).

Southwestern Analysis. Increasing amounts of each DNA sample were mounted onto a nitrocellulose membrane. The membrane was washed with PBS, dehydrated at 60°C overnight, and then washed twice in PBS and blocked with 5% skim milk in PBS for 3 hours. Nuclear extracts were prepared as described and diluted to a protein concentration of 0.5 mg/mL in gel-shift buffer [12.5 mmol/L Tris-HCl (pH 7.9), 3.1 mmol/L $MgCl_2$, 25 mmol/L KCl, 0.5 mmol/L dithiothreitol, 10% glycerol, 0.25 mmol/L EDTA, 0.2 mmol/L PMSF, protease inhibitors mixture (Roche), and 1 mg/mL pGL3-Basic plasmid DNA for blocking]. Nuclear extracts were incubated overnight at 4°C with the membrane, followed by one 5-minute wash in PBS-Tween. The membrane was next incubated for 1 hour with a p53-specific polyclonal serum under standard Western blot analysis conditions, followed by three consecutive 5-minute washes in PBS-Tween and incubation for 30 minutes with horseradish peroxidase–conjugated goat-antirabbit secondary antibody. Membranes were developed with an ECL kit (Amersham Biosciences).

Chromatin Immunoprecipitation Analysis. Formaldehyde (Merck, Darmstadt, Germany) was added directly into cell culture medium to a final concentration of 1%. Fixation proceeded at room temperature for 10 minutes and was stopped by addition of glycine to a final concentration of 0.125 mol/L. Dishes were rinsed with cold PBS and incubated with 5 mL of 20% trypsin-EDTA (Gibco) in PBS. Cells were removed by scraping, collected by centrifugation, and washed in cold PBS. Cells were incubated on ice for 20 minutes in 5 mmol/L Pipes (pH 8.0), 85 mmol/L KCl, and protease inhibitors mixture; nuclei were dounced and collected by microcentrifugation at 4,000 rpm, resuspended in nuclei lysis buffer [1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl (pH 8.1), and protease inhibitors mixture], and incubated on ice for 10 minutes. Samples were sonicated on ice to an average length of 600 to 1,000 bp and then microfuged at 14,000 rpm. The chromatin solution was precleared by incubation with protein A beads for 2 hours at 4°C. Before use, protein A beads were blocked with 1 μg/mL sheared herring sperm DNA and 4% bovine serum albumin for at least 4 hours at 4°C. Precleared chromatin from 2.5×10^7 cells was diluted 1:10 in dilution buffer (0.01%SDS, 1% Triton X-100, 1.2 mmol/L EDTA,167 mmol/L NaCl, and protease inhibitors mixture) and divided into 600-μL aliquots. Each aliquot was mixed with 30 μL of protein A beads cross-linked by dimethyl pimelimidate to anti-p53 polyclonal antibody and rotated at 4°C for 12 hours. Beads subjected to a similar process in the absence of antibody were used as negative control. Immunoprecipitates were washed twice with dilution buffer, twice with washing buffer [100 mmol/L Tris-Cl (pH 9.0), 500 mmol/L LiCl, 1% NP40, 1% deoxycholic acid, and PMSF], and once with Tris EDTA buffer. Elution of immune complexes was carried out by addition of 50 μL of elution buffer (50 mmol/L $NaHCO_3$ and 1% SDS). The samples were diluted again to 0.5 mL, and a second round of immunoprecipitation, washing, and elution was performed under the same conditions. Proteins and RNA were removed by addition of 10 μg of RNase A per sample for 30 minutes, followed by addition of 30 μg of proteinase K for 2 hours at 42°C. Cross-links were reversed by incubation at 65°C overnight. DNA was extracted once with phenol:chloroform and once with chloroform and precipitated with 0.1 volume of 3 mol/L NaAc and 2.5 volume of ethanol. Pellets were collected by microcentrifugation, resuspended in 30 μL of water, and analyzed by PCR. Total input samples were resuspended in 100 μL of water and then diluted 1:10. PCR reactions contained 2 μL of immunoprecipitated or diluted total input, 50 ng of each primer (EGR1 forward, 5′-GCG GTA CCG GGC AGC ACC TTA TTT GGA G; EGR1 reverse, 5′-GCG GTA CCC ACT CCC GGT TCG CTC TCA; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5′-GTA TTC CCC CAG GTT TAC AT; and GAPDH reverse, 5′-TTC TGT CTT CCA CTC ACT CCT), 10% dimethyl sulfoxide, and Ready mix PCR master mix (Promega) in a total volume of 20 μL. After 35 cycles of amplification, PCR products were run on a 1.5% agarose gel and analyzed by ethidium bromide staining.

Luciferase Assays. Cells were seeded in 24-well culture dishes. Each well was transfected with a reporter plasmid expressing the firefly *luciferase* gene under the transcriptional control of the different gene promoters, together with increasing amounts of various expression plasmids and β-galactosidase (β-gal) plasmid. Luciferase activity was assayed 48 hours post transfection. Each plasmid combination was transfected into three identical wells. Luciferase assays were performed using (D)-luciferin (Roche). Luminescence was determined with the aid of a Rosys-Anthos Lucy 3 luminometer. The luciferase values were normalized to β-gal activity.

Real-Time Reverse Transcription-Polymerase Chain Reaction Analysis. Total RNA was extracted using the Trireagent kit (Macherey Nagel, Germany). Two μg of each RNA sample were reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) and random hexamer primers. Real-time PCR was performed on an ABI 7000 machine (Applied Biosystems) using Sybr Green PCR mastermix (Applied Biosystems), EGR1-specific primers (sense, TTT GCC AGG AGC GAT GAA C; antisense, CCG AAG AGG CCA CAA CAC TT) and p53-specific primers (sense, CCC AAG CAA TGG ATG ATT TGA; antisense, GGC ATT CTG GGA GCT TCA TCT). cDNA levels were normalized to GAPDH amplified with appropriate primers (sense, ACC ACA GTC GCC ATC AC; antisense, TCC ACC ACC CTG TTG CTG TA).

RESULTS

Establishment of Mutant p53 Producer Cell Lines. Isogenic mutant p53 producer cell lines were established from well-characterized p53-null cell lines. To that end, we used H1299 lung adenocarcinoma cells and PC3, a prostate cancer–derived cell line. Mutant p53-overexpressing cell lines were generated by either transfection or infection of the p53-null parental cells with a vector encoding mutant p53 protein. We chose to focus on p53R175H, a common p53 mutant previously shown to possess a marked anti-apoptotic gain of function in H1299 cells (11). Single cell–cloned H1299 derivatives and PC3 polyclonal pools were selected in the presence of the corresponding drugs, and levels of p53 protein were determined by Western blot analysis (Fig. 1*A* and *B*). Ten individual H1299-derived single-cell clones expressing p53R175H were selected for additional analysis. PC3-derived cell populations were analyzed as polyclonal pools without additional subcloning.

Identification of Mutant p53-Regulated Genes by Expression Microarray Analysis. To search for specific mutant p53-regulated genes, gene expression patterns of p53-null and derivative mutant p53-producing H1299 cells were analyzed. Differential expression patterns were determined by the use of Affymetrix DNA microarrays. To avoid clonal variability and improve the statistical strength of the study, equal amounts of RNA obtained from individual clones were pooled together before cDNA synthesis. cDNA was subsequently prepared and subjected to hybridization with DNA microarrays. Data obtained were analyzed by the Affymetrix analysis criteria. Genes that are either up-regulated or down-regulated after expression of mutant p53 were identified (Table 1) and postulated to represent genes directly affected by mutant p53 as well as genes whose expression is modulated by secondary events occurring after mutant p53 expression. One of the genes whose expression was consistently and prominently induced by mutant p53 was *EGR1*, and this gene was therefore chosen for additional study. Quantitative reverse transcription-PCR analysis confirmed that *EGR1* mRNA levels were indeed elevated to a similar extent in both H1299 and PC3 cells as a consequence of mutant p53 overexpression (Fig. 1*C*). It should be noted, however, that this increase in basal levels of *EGR1* mRNA was not always easy to detect: Although in some experiments it was quite prominent, in others, it was only minor or even undetectable (data not shown). As discussed later, a more unequivocal effect of mutant p53 on *EGR1* mRNA levels became apparent under conditions that lead to stronger stimulation of *EGR1* gene expression.

Table 1 *Representative example of genes differentially expressed in H1299 cells overexpressing the p53R175H mutant*

Name	Accession no.	Fold change
Down-regulated genes		
Human succinyl CoA:3-oxoacid CoA transferase	U62961	13
Human annexin III (ANX3)	L20591	3.5
Fibronectin receptor α subunit	X06256	4.5
Human interleukin-13 receptor	U70981	14
plasminogen activator inhibitor type 1	X04729	7.5
Homo sapiens galactocerebrosidase (GALC)	L23116	8.4
H. sapiens glycogen synthase kinase 3	L40027	2.5
A-myb = DNA-binding transactivator	S75881	2.5
MHC-encoded proteasome subunit gene LAMP7-E1	Z14982	2.6
H. sapiens hbrm	X72889	3.6
Human transforming growth factor-β–induced gene product (BIGH3)	M77349	2.6
Urokinase-type plasminogen activator receptor gene	U09937	3.4
Human MEST	D78611	3.7
Human insulin-like growth factor binding protein 6 (IGFBP6)	M62402	9
Up-regulated genes		
Human down syndrome critical region 1 (DSCR1)	U85267	7.5
Human aldehyde dehydrogenase 6	U07919	2.7
Zyxin 2	X95735	2.5
Adrenomedullin	D14874	2.7
Asparagine synthetase mRNA	M27396	2.7
Human NF-IL6-β protein	M83667	3
Human heparin-binding EGF-like growth factor	M60278	3
Aldose reductase	X15414	3
Human skeletal muscle LIM-protein SLIM1	U60115	3.7
H. sapiens NF-H gene	X15306	4.5
Fibronectin	X02761	6.5
Elongation factor 1 α-2	X70940	3
Calretinin	X56667	4.7
Homeotic Protein Gbx2	HG3123	5
Human heparin-binding EGF-like growth factor	M60278	4.4
Early growth response protein 1 (hEGR1)	X52541	15
Oncogene c-*fos*	V01512	23

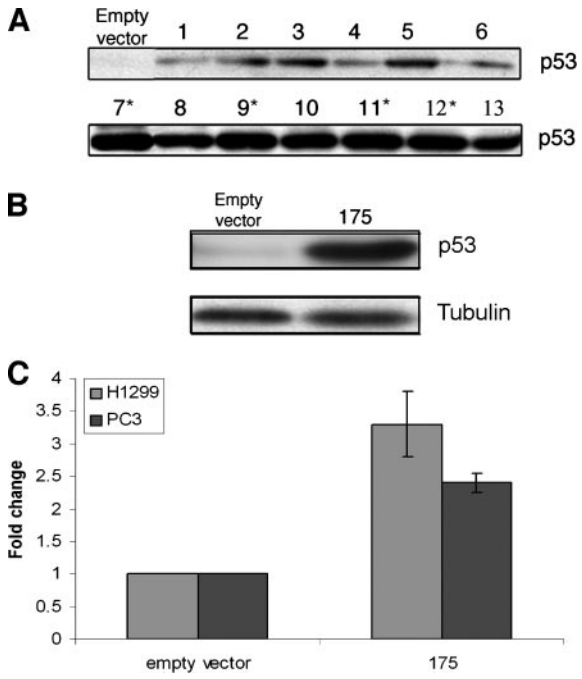

Fig. 1. *EGR1* mRNA levels are up-regulated in cells overexpressing mutant p53. *A*. H1299 cells were transfected with p53R175H expression plasmid or empty vector plasmid as a control. After 2 weeks of drug selection, single-cell clones were isolated. Mutant p53 expression levels in individual clones were determined by Western blot analysis. The clones shown are a representative sample from a larger group of positive clones. Clones marked by ∗ were chosen for additional analysis, along with additional clones not shown in the figure. *B*. PC3 cells were infected with either p53R175H recombinant retroviruses or a control retrovirus. Polyclonal cell pools were analyzed by Western blotting for mutant p53 expression. *C*. H1299 cells were transfected with the p53R175H expression plasmid or empty vector plasmid as a control. PC3 cells were infected with recombinant retroviruses encoding either p53R175H or control retrovirus (*empty vector*). Two days post transfection, total RNA was isolated, and RNA levels of *EGR1* were determined by quantitative real-time reverse transcription-PCR. All EGR1 values were normalized for GAPDH in the same RNA samples. Fold increase in EGR1 mRNA was calculated relative to the level of expression of EGR1 mRNA in control virus-infected H1299 and PC3 cells, respectively. The values are the average of several independent experiments for each cell line; the *SEM* is indicated.

Mutant p53 Binds the *EGR1* Promoter and Up-Regulates Its Expression. To examine the possibility that up-regulation of *EGR1* expression is mediated by transactivation of the *EGR1* promoter by mutant p53, we cloned the *EGR1* promoter sequence upstream to a luciferase reporter gene and transfected it into cells together with mutant p53 expression plasmids. H1299 cells were transfected with the EGR1-luciferase plasmid and increasing concentrations of R175H and several other tumor-associated p53 hot spot mutants. As seen in Fig. 2*A* (*left panel*), mutants R175H, R248W, R273H, and R281G elicited a dose-dependent increase in luciferase activity, whereas no increase was noticed with H179E. For most mutants, comparable protein levels were produced in the transfected cells (Fig. 2*B*). These results suggest that p53R175H, as well as most of the common

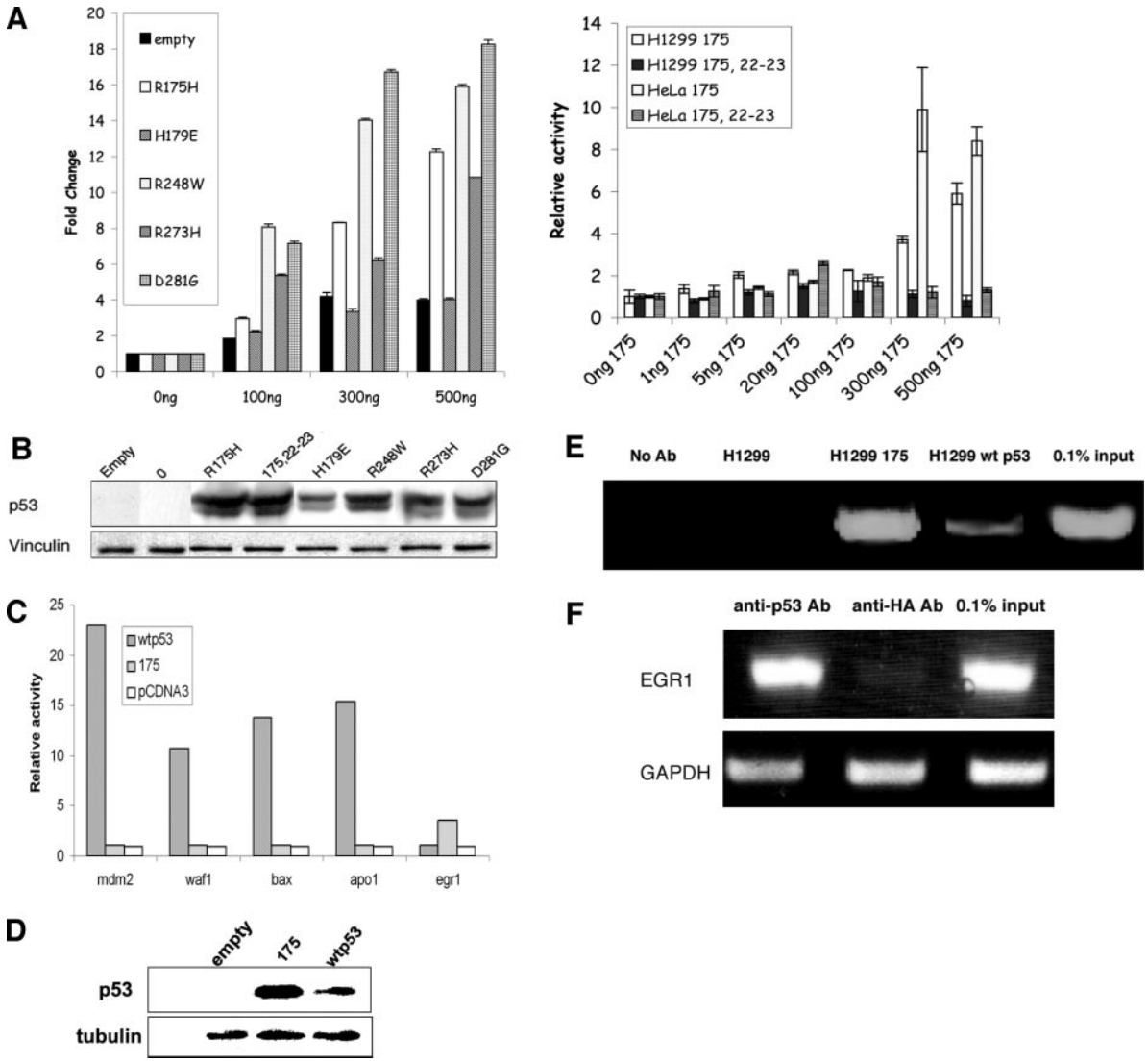

Fig. 2. The p53R175H mutant binds and up-regulates the EGR1 promoter. *A*. *Left panel*. H1299 cells were transiently transfected with EGR1-luciferase (200 ng) along with increasing amounts (indicated at the bottom of the figure) of expression plasmids encoding the indicated p53 mutants. A β-gal expression plasmid was also included in all transfections, and luciferase activity was normalized to β-gal activity. *Right panel*. H1299 and HeLa cells were transiently transfected with EGR1-luciferase (200 ng) along with the indicated increasing amounts of expression plasmids encoding either p53R175H or p53R175H,22-23. A β-gal expression plasmid was also included in all transfections, and luciferase activity was normalized to β-gal activity. *B*. Western blot analysis of p53 expression in H1299 cells transfected by the different p53 mutants used in *A*. Extracts were prepared from cells transfected with 300 ng of expression plasmid DNA. The nitrocellulose membrane was probed with p53-specific antibodies. *C*. H1299 cells were transiently transfected with 200 ng of the indicated promoters upstream to a luciferase reporter gene along with p53R175H (*175*), wtp53, or empty vector control (*pCDNA3*). A β-gal expression plasmid was also included in all transfections. Luciferase activity was determined 48 hours after transfection, and readings were normalized to β-gal. Luciferase activities were calculated for each promoter, relative to the activity obtained with pCDNA3, which was assigned a value of 1.0. *D*. Western blot analysis of p53 expression in the wtp53 or p53R175H-transfected H1299 cells used in *C*. *E*. H1299 cells were transiently transfected with wtp53 expression plasmid. Two days post transfection, a chromatin immunoprecipitation experiment was performed for these cells along with H1299 and H1299 stably expressing the p53R175H mutant: After cross-linking of proteins to DNA, DNA was fragmented, and the p53 protein was immunoprecipitated with specific antibody or with no antibody as a control (*No Ab*). PCR analysis was performed on the immunoprecipitated DNA samples using *EGR1*-specific primers. *F*. SKBR3 cells were subjected to chromatin immunoprecipitation analysis as in *E*, except that p53-bound chromatin was immunoprecipitated with either p53-specific antibody or anti-hemagglutin antibody as a control. PCR analysis was performed on the immunoprecipitated DNA samples using *EGR1*-specific primers or *GAPDH*-specific primers as a control.

cancer-associated p53 mutants, can up-regulate *EGR1* expression via transactivation of the *EGR1* promoter.

We reported previously (13) that core mutant p53 protein with additional mutations at residues 22 and 23 in the transactivational domain (p53R175H,22-23) was unable to block drug-induced apoptosis, suggesting that such p53 mutants require an intact transactivation domain for their anti-apoptotic "gain of function" activity. It was therefore of interest to examine whether such transcription-deficient p53 mutants have also lost their capacity to transactivate the *EGR1* promoter sequence. As seen in Fig. 2*A* (*right panel*), overexpression of p53R175H,22-23 in two different cell lines did not elicit a significant increase in luciferase activity, even though it was expressed at levels comparable with those of p53R175H (Fig. 2*B*). This implies that the activation of the *EGR1* promoter by mutant p53 requires the integrity of the transactivation domain. Of note, the *EGR1* promoter behaved very differently from classical p53-responsive promoters, which are transactivated preferentially by wtp53 but not by mutant p53 (Fig. 2*C*). A Western blot representing the relative p53 protein levels in this experiment is shown in Fig. 2*D*.

The increase in *EGR1* mRNA levels and EGR1-luciferase activity suggests that mutant p53 specifically transactivates the *EGR1* gene. We therefore wished to determine whether mutant p53 could interact physically with *EGR1* promoter sequences. To that end, chromatin immunoprecipitation analysis was performed on H1299 cells transfected with either p53R175H or wtp53; nontransfected cells served as a control. As seen in Fig. 2*E*, *EGR1* promoter sequences were selec-

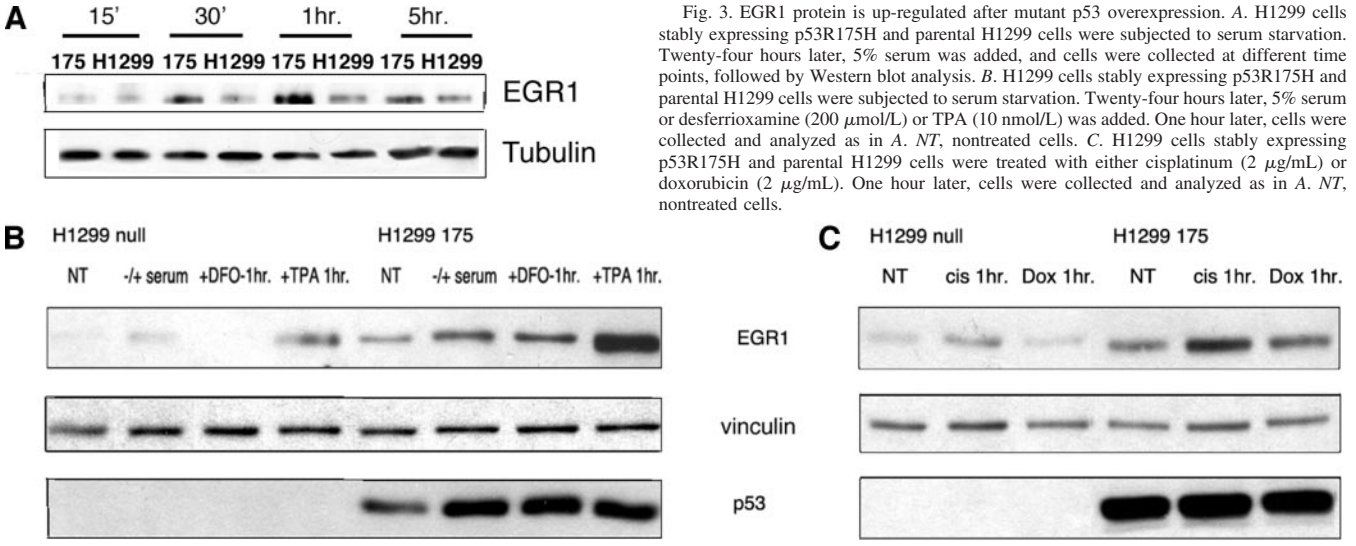

Fig. 3. EGR1 protein is up-regulated after mutant p53 overexpression. *A*. H1299 cells stably expressing p53R175H and parental H1299 cells were subjected to serum starvation. Twenty-four hours later, 5% serum was added, and cells were collected at different time points, followed by Western blot analysis. *B*. H1299 cells stably expressing p53R175H and parental H1299 cells were subjected to serum starvation. Twenty-four hours later, 5% serum or desferrioxamine (200 μmol/L) or TPA (10 nmol/L) was added. One hour later, cells were collected and analyzed as in *A*. *NT*, nontreated cells. *C*. H1299 cells stably expressing p53R175H and parental H1299 cells were treated with either cisplatinum (2 μg/mL) or doxorubicin (2 μg/mL). One hour later, cells were collected and analyzed as in *A*. *NT*, nontreated cells.

tively immunoprecipitated with mutant p53. Thus, mutant p53 is selectively associated *in vivo* with the *EGR1* promoter. A markedly fainter signal was obtained with wtp53; whether this reflects a real, albeit weak interaction between the *EGR1* promoter and wtp53 is presently under investigation. To further confirm the results in cells expressing endogenous mutant p53, we performed chromatin immunoprecipitation analysis on SKBR3 cells naturally harboring the R175Hp53 mutant. As seen in Fig. 2*F*, *EGR1* promoter sequences were immunoprecipitated specifically by the anti-p53 antibodies but not by the irrelevant control anti-hemagglutin antibodies.

Mutant p53 Up-Regulates EGR1 Protein Levels. Western blot analysis was performed to confirm that the increase in *EGR1* mRNA is also accompanied by a corresponding increase in EGR1 protein. It should be noted however, that detection of EGR1 protein in nonstressed cells is difficult (25, 26). Therefore, to facilitate additional analysis, we subjected p53R175H-producing cells and their empty vector controls to serum starvation for 24 hours, followed by serum re-addition. As seen in Fig. 3*A*, EGR1 protein levels increased gradually within the 1st hour after serum addition. This increase was significantly more pronounced in cells expressing p53R175H compared with control H1299 cells (Fig. 3*A*). Thus, mutant p53 increases not only *EGR1* mRNA but also EGR1 protein levels.

To confirm that the effect of mutant p53 on EGR1 levels can be seen also under other stress conditions that lead to EGR1 elevation, we treated cells with desferrioxamine, which is a hypoxia-mimetic drug; phorbol ester 12-myristate 13-acetate (phorbal myristate acetate or 12-O-tetradecanoylphorbol-13-acetate), which is a PKC activator; and several DNA damaging agents (cisplatin, etoposide, and doxorubicin) and monitored the relative levels of EGR1 protein. As seen in Fig. 3*B* and *C*, under all of those conditions, cells expressing mutant p53 showed significantly elevated EGR1 levels compared with cells that do not express any p53.

Knockdown of Endogenous EGR1 Expression Compromises Mutant p53 Gain of Function Effects. Our findings indicate that EGR1 is a specific mutant p53 target gene. To find out whether EGR1 induction is a downstream event that contributes to mutant p53 gain of function activity, we asked whether knockdown of EGR1 expression by RNA interference (RNAi) would compromise such gain of function activity. To that end, an appropriate EGR1-specific sequence was inserted into the pSUPER plasmid, which directs the synthesis of

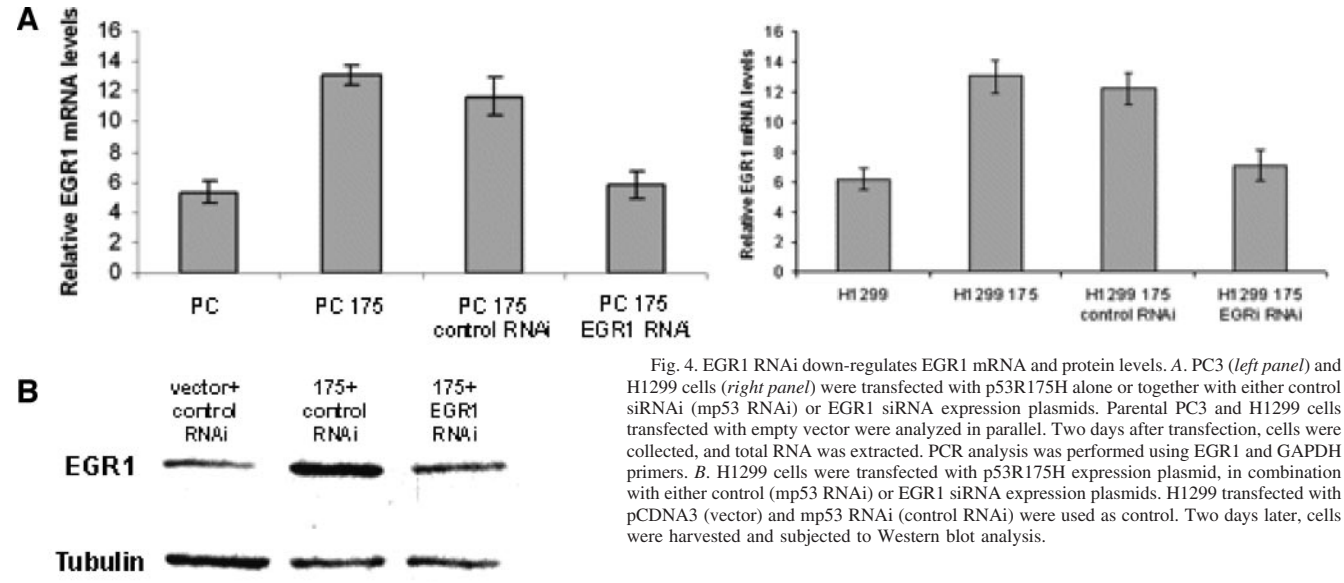

Fig. 4. EGR1 RNAi down-regulates EGR1 mRNA and protein levels. *A*. PC3 (*left panel*) and H1299 cells (*right panel*) were transfected with p53R175H alone or together with either control siRNAi (mp53 RNAi) or EGR1 siRNA expression plasmids. Parental PC3 and H1299 cells transfected with empty vector were analyzed in parallel. Two days after transfection, cells were collected, and total RNA was extracted. PCR analysis was performed using EGR1 and GAPDH primers. *B*. H1299 cells were transfected with p53R175H expression plasmid, in combination with either control (mp53 RNAi) or EGR1 siRNA expression plasmids. H1299 transfected with pCDNA3 (vector) and mp53 RNAi (control RNAi) were used as control. Two days later, cells were harvested and subjected to Western blot analysis.

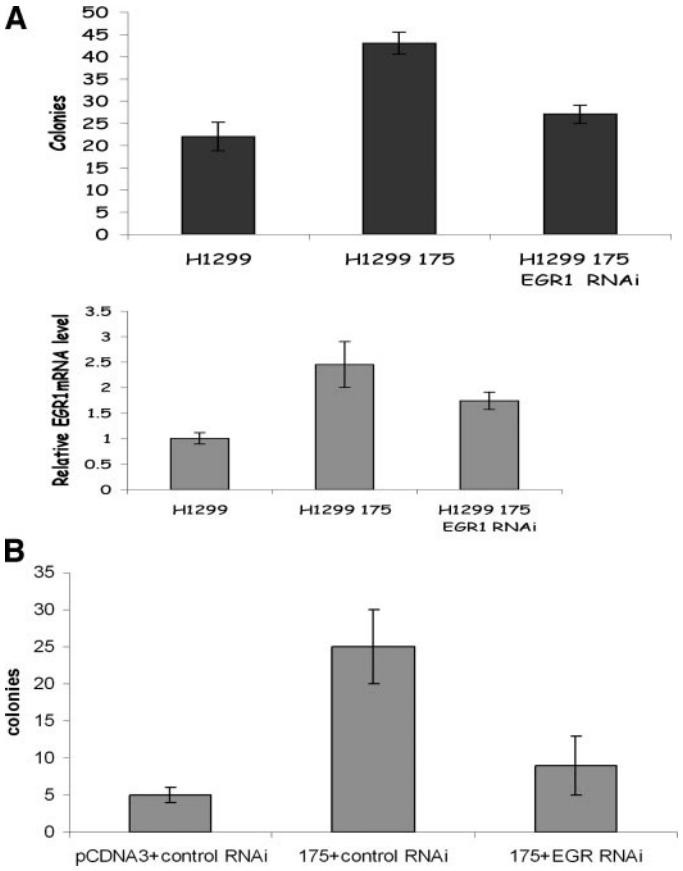

Fig. 5. EGR1 down-regulation reduces the cloning efficiency of human tumor cells overexpressing mutant p53. *A*. *Top panel*. H1299 cells were transiently transfected with p53R175H along with pSUPER vector control (*middle column*) or EGR1 RNAi (*right column*). Two days post transfection, cells were seeded in soft agar. One month later, colonies were counted. H1299, parental H1299 cells, seeded in parallel. Values represent the average of four independent experiments ± *SEM*. *Bottom panel*. H1299 cells were transiently transfected with p53R175H along with pSUPER vector control (*middle column*) or EGR1 RNAi (*right column*). Two days post transfection, mRNA was extracted from cells, and relative *EGR1* mRNA was measured by quantitative real-time PCR. Values are shown in arbitrary units, after correction for GAPDH in the same RNA samples. *B*. H1299 cells were cotransfected with p53R175H and either EGR1 RNAi plasmid or mp53 RNAi plasmid as a specificity control. H1299 cells transfected with pCDNA3 and mp53 RNAi plasmid (*control RNAi*) were used as control. Two days later, cells were seeded at low density and selected with puromycin. Two weeks later, colonies were fixed and stained with crystal violet.

small interfering RNA (siRNA) in mammalian cells. Similar plasmids directing the synthesis of either p53 siRNA carrying three mismatches (mp53 RNAi) or mouse p63 siRNA served as specificity controls. Transfection of the EGR1 siRNA plasmid (EGR RNAi) caused a specific, albeit partial, down-regulation of endogenous EGR1 mRNA (Fig. 4*A*) and protein (Fig. 4*B*) in cells overexpressing mutant p53; no such down-regulation was seen when either pSUPER empty vector (data not shown) or mp53 RNAi (Fig. 4*A* and *B*) was used as a control.

Next, we asked whether knockdown of EGR1 expression has an effect on mutant p53 biological activities associated with growth control and apoptosis. In the first set of experiments, we compared the efficiency of soft agar colony formation by H1299 cells subjected to various genetic manipulations. Cells were transfected either with an empty vector or with p53R175H, with or without EGR1 RNAi. Two days post transfection, cells were seeded in soft agar, and colonies were counted 1 month later. As seen in Fig. 5*A* (*top panel*), expression of mutant p53 in H1299 cells elevated significantly the number of colonies, whereas transfection of EGR1 RNAi compromised this elevation. To confirm that the RNAi expression plasmid was functional in this system, we performed real-time PCR analysis of EGR1 mRNA 2 days after transfection. As shown in Fig. 5*A* (*bottom panel*), levels of EGR1 mRNA were significantly reduced in H1299 175H cells transfected with the EGR1 RNAi plasmid.

To further assess the role of EGR1 in mutant p53 gain of function, the effect of EGR1 knockdown on the plating efficiency of tumor cells overexpressing mutant p53 was measured. As seen in Fig. 5*B*, overexpression of mutant p53 in p53-null H1299 cells resulted in a marked increase in plating efficiency. Importantly, EGR1 RNAi reversed to a great extent the enhancement of colony formation by mutant p53. This further supports the conjecture that elevation of EGR1 expression by mutant p53 plays a role in the gain of function activities of mutant p53, as reflected in the ability of cells to grow under stress.

Anti-apoptotic Effects of EGR1. One of the well-documented facets of mutant p53 gain of function is an increased resistance of tumor cells to stress-induced apoptosis (11–13). In view of the proposal that EGR1 is a downstream target of mutant p53, we investigated the effect of EGR1 expression levels on cell survival under different kinds of stress, particularly those that result in elevation of EGR1 (see Fig. 3).

Parental PC3 cells or PC3 cells overexpressing p53R175H were transfected with a plasmid expressing either EGR1 siRNA or a siRNA control plasmid (mouse p63 siRNA). A plasmid encoding H2B-GFP was included in all transfections to enable identification of successfully transfected cells. After DNA damage (1 μg/mL cisplatinum for 48 hours), GFP-positive cells were analyzed by fluorescence-activated cell sorter for cell cycle distribution. As seen in Fig. 6*A*, cells expressing mutant p53 underwent apoptosis at consistently lower rates than parental p53-null cells. On the other hand, both parental and mutant p53-expressing cells exhibited an increase in apoptosis after transfection with an EGR1 RNAi plasmid. Of note, EGR1 knockdown in mutant p53 overexpressors restored apoptosis to a rate comparable with that seen in parental p53-null cells expressing basal levels of EGR1.

To determine whether EGR1 can contribute to cell survival also in tumor cells harboring endogenous mutant p53, we used SKBR3 human breast cancer-derived cells carrying a mutation at position 175 of their endogenous p53. Knockdown of either p53 or EGR1 led to a significant increase in cell death when the cultures were treated with increasing concentrations of desferrioxamine (Fig. 6*B*).

Real-time reverse transcription-PCR analysis confirmed that EGR1 mRNA in transfected cells was down-regulated by both EGR1 RNAi and p53 RNAi, but not by RNAi directed specifically against mouse p63, serving as an irrelevant control (Fig. 6*C*). In contrast, p53 mRNA levels were reduced only when a p53 RNAi plasmid was transfected (Fig. 6*D*). It is noteworthy that the apparent extent of reduction is most certainly an underestimate of the true knockdown efficacy, because the transfection efficiency was well below 100%, and thus the RNA analyzed is derived from a mixture of transfected and nontransfected cells.

Moreover, as seen in Fig. 7 (*top panel*), down-regulation of either p53 or EGR1 in SKBR3 cells resulted in a significant increase in cell death after exposure to DNA-damaging anticancer drugs; the observed increase is probably an underestimate, because transfection efficiency was no more than 50%, as assessed with the aid of a cotransfected GFP expression plasmid (data not shown). Real-time reverse transcription-PCR analysis confirmed that the relative EGR1 mRNA levels were reduced when cells were transfected with EGR1 RNAi or p53 RNAi plasmids compared with cells transfected with control RNAi plasmid (Fig. 7, *bottom panel*). Here, too, the actual extent of knockdown is likely to be markedly higher than apparent from the data, owing to the limited transfection efficiency. These data confirm the notion that the endogenous mutant p53 in such tumor cells contributes, at least to some extent, to increased resistance of the cells

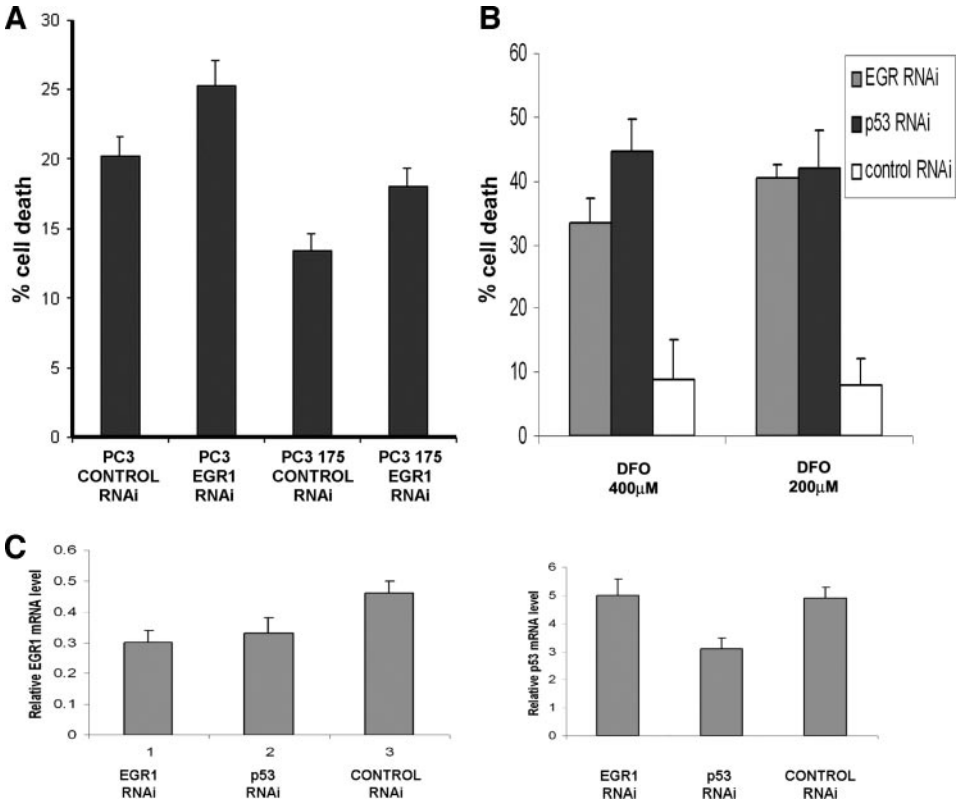

Fig. 6. Knockdown of EGR1 results in increased apoptosis after genotoxic stress. *A*. Parental PC3 cells or p53R175H-overexpressing cells (*PC3 175*) were cotransfected with 250 ng of H2B-GFP expression plasmid and 1.25 μg of either control RNAi plasmid (mouse p63 RNAi) or EGR1 RNAi plasmid. Twenty-four hours post transfection, cells were treated with 1 μg/mL cisplatinum for 48 hours. Cells were then fixed in methanol, rehydrated, and analyzed for DNA content after propidium iodide staining, as described in Materials and Methods. *B*. SKBR3 cells were transfected with EGR1 RNAi or p53 RNAi plasmids or a control (mouse p63 RNAi) plasmid. Cells were subjected 2 days later to treatment with increasing amounts of desferrioxamine. After 2 days, cells were counted, and relative cell death was calculated; values were normalized for cell death of nontreated cells. *C*. SKBR3 cells were transfected with RNAi plasmids specific for EGR1, p53, or mouse p63, the latter serving as a control. Cells were subjected 2 days later to treatment with 200 μmol/L desferrioxamine. After 8 hours, total cellular RNA was extracted, and relative levels of p53 and EGR1 mRNA were determined by quantitative real-time reverse transcription-PCR. Values are shown in arbitrary units, after correction for GAPDH in the same RNA samples. *SEM* values are shown.

to chemotherapeutic agents. Taken together, our observations strongly suggest that EGR1 knockdown effectively decreases the anti-apoptotic gain of function effect of mutant p53. Hence, the induction of EGR1 expression by mutant p53 is proposed to play a significant role in this gain of function.

Vascular Endothelial Growth Factor Is Up-Regulated in Mutant p53-Expressing Cells. VEGF, a major regulator of the response to hypoxia, was previously reported to be up-regulated by mutant p53 (27, 28). At least according to some studies, VEGF expression is also positively regulated by EGR1, albeit probably not in a direct manner (26). We therefore compared the induction of VEGF mRNA by desferrioxamine in H1299 cells and in their mutant p53-overexpressing derivatives. Although basal VEGF mRNA levels were not appreciably higher in cells overexpressing mutant p53, the extent of VEGF induction by desferrioxamine was remarkably higher in these cells compared with the parental, p53-null cells (Fig. 8*A*). The up-regulation of VEGF expression by mutant p53 is in agreement with earlier findings (27, 28). Furthermore, in SKBR3 cells expressing endogenous p53R175H, knockdown of either the endogenous mutant p53 or EGR1 resulted in a mild but reproducible drop in the concentration of VEGF protein secreted into the cell culture medium (Fig. 8*B*). The effect of mutant p53 knockdown was somewhat more pronounced than that of EGR1 knockdown; this might imply that EGR1 up-regulation may be only one of several mediators of the effect of mutant p53 on VEGF expression. These data are consistent with the notion that induction of EGR1 gene expression by mutant p53 mediates, at least in part, the ability of mutant p53 to up-regulate VEGF promoter activity. This is further supported by DNA binding analysis: As shown in Fig. 8*C*, the p53R175H protein did not bind to a plasmid carrying the VEGF promoter while exhibiting good interaction with the CD95/Fas promoter, which was recently found to be a target for binding and transcriptional repression by mutant p53 (29). Hence, the VEGF promoter is not targeted directly by mutant p53. Our finding that RNAi-mediated down-regulation of EGR1 expression leads to a

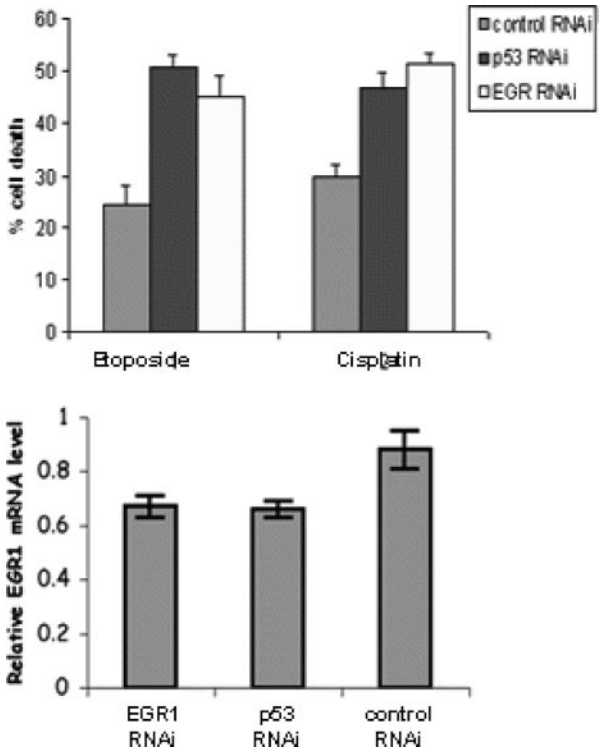

Fig. 7. Knockdown of EGR1 or endogenous mutant p53 predisposes to DNA damage-induced cell death. *Top panel*. SKBR3 cells were transfected with EGR1 RNAi plasmid or p53 RNAi plasmid or a control plasmid (mp53 RNAi). The entire culture was subjected 2 days later to treatment with either cisplatin (1 μg/mL) or etoposide (10 μmol/L). After 2 days, cells were counted, and the relative survival rates were calculated; values were normalized for survival of nontreated cells. *Bottom panel*. SKBR3 cells were transfected with EGR1 RNAi plasmid or p53 RNAi plasmid or a control plasmid (mp53 RNAi). Cultures were subjected 2 days later to treatment with 1 μg/mL cisplatin. Eight hours later, total cellular RNA was extracted, and relative EGR1 mRNA levels were determined by quantitative real-time reverse transcription-PCR. Values are shown in arbitrary units, after correction for GAPDH in the same RNA samples.

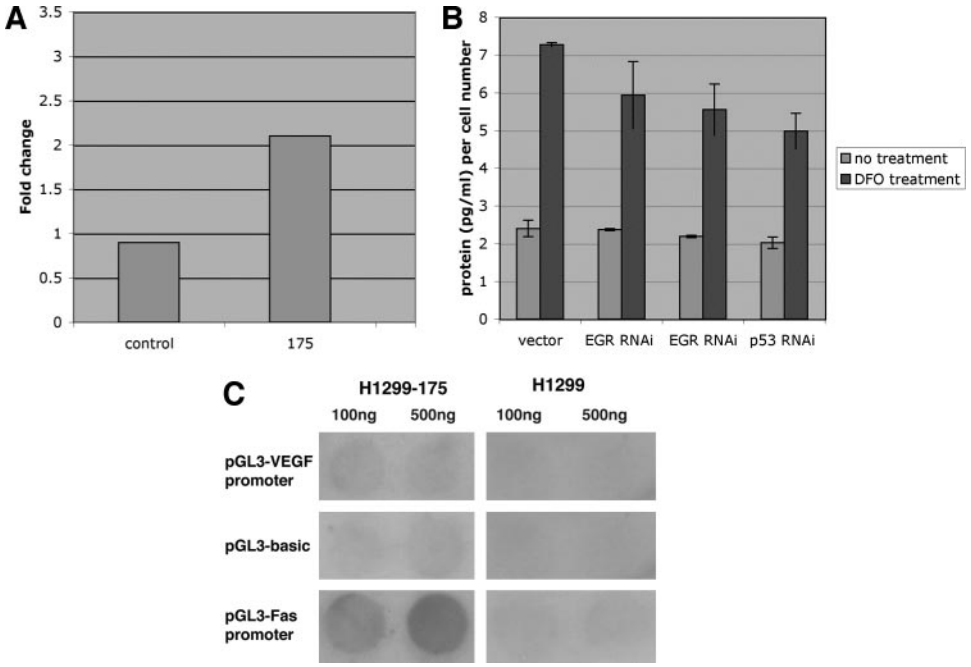

Fig. 8. EGR1-dependent induction of VEGF by mutant p53. *A*. H1299 cells stably expressing p53R175H (*175*) and parental H1299 cells (*control*) were treated with 200 μmol/L desferrioxamine overnight. Total RNA was isolated, and *VEGF* mRNA levels were determined by quantitative reverse transcription-PCR. The values shown represent the fold induction of *VEGF* mRNA by desferrioxamine in each cell line, relative to the basal level in the same line (nontreated). *B*. SKBR cells were transiently transfected with EGR1 RNAi (0.5 and 1 μg in the second and third pair, respectively), p53 RNAi, or pSUPER (*vector*) as a control. One day post transfection, cells were either exposed to desferrioxamine (200 μmol/L) for an additional 12 hours or left untreated. VEGF protein levels in the culture medium were determined with the aid of a human VEGF enzyme-linked immunosorbent assay kit (Oncogene Research Products, San Diego, CA). *C*. Different amounts (nanograms) of VEGF-luciferase plasmid (*top row*), control empty plasmid (*middle row*), and CD95/Fas-luciferase plasmid (*bottom row*) were mounted on a nitrocellulose membrane. The membrane was dehydrated at 60°C, followed by hybridization with either nuclear extract of H1299 cells or H1299 cells overexpressing the p53R175H mutant. The membrane was subjected to Western blot analysis using anti-p53 antibodies.

reduction in the expression of VEGF is in agreement with a previous report (26) that down-regulation of EGR1 in mice leads to a significant reduction in VEGF after hypoxic stress. It should be noted, however, that other studies do not support the conclusion that EGR1 is a significant regulator of VEGF expression (30, 31). This discrepancy might imply that EGR1 can regulate VEGF expression only in certain cell types or under specific experimental conditions.

All together, the data discussed above strongly suggest that mutant p53 employs EGR1 as a mediator of some of its downstream gain of function tumor-promoting effects.

DISCUSSION

In the present study, we examined the involvement of EGR1 in mutant p53 gain of function. Using expression microarray analysis, EGR1 was identified as one of the prominent genes up-regulated in tumor cells overexpressing the p53R175H mutant. This up-regulation, seen with a number of different cancer-associated p53 hotspot mutants, was also confirmed at the EGR1 protein level. Moreover, promoter analysis and chromatin immunoprecipitation assays indicated that the *EGR1* gene is a direct transcriptional target of p53R175H. The EGR1 protein induced by p53R175H mutant p53 is biochemically active, as manifested by its ability to mediate the up-regulation of VEGF expression (Fig. 8), as well as of two other EGR1 target genes, *fibronectin 1* and *TGF-β1* (data not shown). It is of particular note that the two p53 mutants most active in up-regulation of the *EGR1* promoter, R248W and D281G (Fig. 2*A*), were also found to be the most potent in biological *in vitro* and *in vivo* assays for mutant p53 gain of function (7). Although only correlative, this might imply that the oncogenic gain of function of mutant p53 is closely coupled with its ability to up-regulate *EGR1* gene expression. For the sake of simplicity, we chose to focus on data from experiments employing p53R175H. However, a parallel albeit somewhat more limited analysis was also performed with another common p53 mutant, p53R273H; overall, p53R273H was found to behave qualitatively in a similar manner to p53R175H with regard to EGR1 activation and its functional consequences (data not shown). It is of note that p53R175H and p53R273H represent different classes of p53 mutants: p53R175H is a gross conformational mutant, whereas p53R273H is a DNA contact site mutant. Our conclusions may thus be relevant to a broad range of p53 mutants implicated in human cancer.

EGR1 is a protein encoded by an immediate early growth response gene, which is rapidly and transiently induced by various factors including growth factors, DNA damaging agents, and stress mediators (32). Up-regulation of EGR1 expression may result in apparently contradictory activities including mitogenesis (33, 34), differentiation (35, 36), tumor suppression (37), apoptosis, and protection from apoptosis (38, 39). It was suggested that EGR1 exerts its activity by regulating the expression of different genes involved in various pathways. EGR1 regulates the insulin-like growth factor-II, platelet-derived growth factor-A and platelet-derived growth factor-B genes, which are known to be involved in cell proliferation (40–42); BCL-2, fibronectin, and nuclear factor-κB, which are associated with survival and cell differentiation (43–45); as well as p53, PTEN, and tumor necrosis factor-α, which are involved in apoptosis (39, 46, 47). Additional relevant targets are VEGF and tissue factor, which are associated with tumor progression (32, 48), and p57/KIP2 and TGFβ1, which induce growth inhibition in a cell type–dependent manner (49, 50). The fact that EGR1 itself is up-regulated by several growth factors and oncogenes supports the notion that it functions as a growth-promoting protein. Although its induction is generally transient and greatly dependent on the nature of the various inducers, it appears to be sustained in a high proportion of prostate cancer cell lines and tumors, suggesting that EGR1 stimulates tumor cell growth in certain types of cancer (51). Indeed, EGR1 overexpression promotes growth in several systems, including kidney and endothelial cells (52). In contrast, in breast, lung, and brain tumors, EGR1 is down-regulated and when re-expressed results in growth suppression and apoptosis (53–55). EGR1 was also observed to be required for apoptosis in normal and tumor cells and to stimulate differentiation (56). It seems that EGR1 lies at a convergence point, and its effects depend on the signals transduced and the cell context.

Because EGR1 is strongly and frequently up-regulated in prostate tumors, several studies have addressed the importance of this protein in the development of prostate cancer. It was shown that in the

prostate tumor mouse model (TRAMP mice), tumor progression depends on EGR1 expression (57). Studies of human prostate tumors show strong correlation between the level of EGR1 expression and tumor grade (51). Furthermore, after hypoxia in tumor cell masses that become depleted of oxygen, EGR1 is induced and transactivates growth factors, proteases, and matrix molecules that are responsible for tumor cell proliferation, survival, and development of new blood vessels that determine tumor progression (30, 58, 59). Altogether, although the contribution of EGR1 to cancer is complex and seemingly even contradictory, it is evident that in some types of cancer, it has a substantial positive role in tumor promotion.

As suggested by our findings, induction of EGR1 contributes in a number of ways to the gain of function effects of mutant p53, and down-regulation of EGR1 attenuates the oncogenic and anti-apoptotic effects of overexpressed mutant p53. Thus, in some tumors naturally overexpressing mutant p53, the predicted induction of EGR1 is likely to contribute to tumor progression. This may pertain particularly to types of cancer in which elevated EGR1 expression is known to be associated with tumor progression, such as prostate cancer (22). In this regard, it is of note that many of the experiments described here used PC3 prostate carcinoma cells. Our data raise the possibility that a similar functional relationship between mutant p53 gain of function and EGR1 activation may also exist in some cases of additional tumor types, as represented by the H1299 and SKBR3 cell lines.

The picture appears to be practically opposite in tumor cells retaining wtp53 expression. In fact, numerous studies indicate that EGR1 can enhance the growth inhibitory and anti-apoptotic effects of wtp53 (46, 60). EGR1 can directly activate the p53 gene promoter and induce an increase in p53 mRNA (46). In cells retaining wtp53 expression, this might restrict the growth-promoting and anti-apoptotic activities of EGR1 and serve as an effective functional negative feedback loop. In this regard, it is noteworthy that EGR1 appears to exert pro-oncogenic effects in lung cancer cells overexpressing mutant p53 (this study), whereas elevated expression of EGR1 was found to correlate positively with apoptosis in H460 human lung carcinoma cells (61), which express wtp53.

Our findings suggest that the contribution of EGR1 is reversed when tumor cells undergo p53 gene mutations. In such cells, mutant p53 will actually drive a positive feedback loop. The activation of the EGR1 promoter by mutant p53 will lead to elevated EGR1 protein, which in turn will activate the p53 promoter and contribute to the maintenance of constitutive high levels of mutant p53.

It is noteworthy that EGR1 can stimulate not only p53 expression but also p73 expression, and the latter may contribute to the pro-apoptotic effects of EGR1 (62). Furthermore, joint activation of wtp53 and p73 by EGR1 may have a synergistic apoptotic outcome, because these two p53 family members cooperate in the transactivation of pro-apoptotic target genes (63). Conversely, in tumor cells naturally overexpressing mutant p53, the possible induction of p73 by EGR1 is expected to be functionally neutralized through the physical binding and subsequent inactivation of p73 by the mutant p53 protein (10, 64, 65). Hence, one important aspect of p53 mutations in cancer may be the switching of EGR1 from a transformation-restrictive, tumor-suppressing molecule into an overt positive contributor to oncogenesis.

In conclusion, our data reveal a novel connection between p53 and EGR1, and imply that p53 status in a given tumor may serve as a critical determinant in directing EGR1 either toward antiproliferative effects in cells expressing wtp53 or toward tumor-promoting effects in cells harboring mutant p53.

REFERENCES

1. Cadwell C, Zambetti GP. The effects of wild-type p53 tumor suppressor activity and mutant p53 gain-of-function on cell growth. Gene 2001;277:15–30.
2. Janus F, Albrechtsen N, Dornreiter I, Wiesmuller L, Grosse F, Deppert W. The dual role model for p53 in maintaining genomic integrity. Cell Mol Life Sci 1999;55: 12–27.
3. Hussain SP, Harris CC. Molecular epidemiology of human cancer. Recent Results Cancer Res 1998;154:22–36.
4. Deppert W, Gohler T, Koga H, Kim E. Mutant p53: "gain of function" through perturbation of nuclear structure and function? J Cell Biochem Suppl 2000;(35): 115–22.
5. Roemer K. Mutant p53: Gain of function oncoproteins and wild-type p53 inactivators. Biol Chem 1999;380:879–87.
6. Halevy O, Michalovitz D, Oren M. Different tumor-derived p53 mutants exhibit distinct biological activities. Science 1990;250:113–6.
7. Dittmer D, Pati S, Zambetti G, et al. Gain of function mutations in p53. Nat Genet 1993;4:42–6.
8. Hsiao M, Low J, Dorn E, et al. Gain-of-function mutations of the p53 gene induce lymphhematopoietic metastatic potential and tissue invasiveness. Am J Pathol 1994; 145:702–14.
9. Iwamoto KS, Mizuno T, Ito T, Tsuyama N, Kyoizumi S, Seyama T. Gain-of-function p53 mutations enhance alteration of the T-cell receptor following X-irradiation, independently of the cell cycle and cell survival. Cancer Res 1996;56:3862–5.
10. Bergamaschi D, Gasco M, Hiller L, et al. p53 polymorphism influences response in cancer chemotherapy via modulation of p73-dependent apoptosis. Cancer Cell 2003; 3:387–402.
11. Blandino G, Levine AJ, Oren M. Mutant p53 gain of function: differential effects of different p53 mutants on resistance of cultured cells to chemotherapy. Oncogene 1999;18:477–85.
12. Li R, Sutphin PD, Schwartz D, et al. Mutant p53 protein expression interferes with p53-independent apoptotic pathways. Oncogene 1998;16:3269–77.
13. Matas D, Sigal A, Stambolsky P, et al. Integrity of the N-terminal transcription domain of p53 is required for mutant p53 interference with drug-induced apoptosis. EMBO J 2001;20:4163–72.
14. Lin J, Chen J, Elenbaas B, Levine AJ. Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2 and the adenovirus 5 E1B 55-kD protein. Genes Dev 1994;8:1235–46.
15. Lin J, Teresky AK, Levine AJ. Two critical hydrophobic amino acids in the N-terminal domain of the p53 protein are required for the gain of function phenotypes of human p53 mutants. Oncogene 1995;10:2387–90.
16. Frazier MW, He X, Wang J, Gu Z, Cleveland JL, Zambetti GP. Activation of C-*myc* gene expression by tumor-derived p53 mutants requires a discrete C-terminal domain. Mol Cell Biol 1998;18:3735–43.
17. Margulies L, Sehgal PB. Modulation of the human interleukin-6 promoter (IL-6) and transcription factor C/EBPb (NF-IL6) activity by p53 species. J Biol Chem 1993; 268:15096–100.
18. Tsutsumi-Ishii Y, Todakoro K, Hanaoka F, Tsuchida N. Response of heat shock element within the human HSP70 promoter to mutated p53 genes. Cell Growth Differ 1995;6:1–8.
19. Ludes-Meyers JH, Subler MA, Shivakumar V, et al. Transcriptional activation of the human epidermal growth factor receptor promoter by human p53. Mol Cell Biol 1996;16:6009–19.
20. Subler MA, Martin DW, Deb S. Activation of the human immunodeficiency virus type 1 long terminal repeat by transforming mutants of human p53. J Virol 1994;68: 103–10.
21. Deppert W. Binding of MAR-DNA elements by mutant p53: possible implications for its oncogenic functions. J Cell Biochem 1996;62:172–80.
22. Adamson ED, Mercola D. Egr1 transcription factor: multiple roles in prostate tumor cell growth and survival. Tumour Biol 2002;23:93–102.
23. Thiel G, Cibelli G. Regulation of life and death by the zinc finger transcription factor Egr-1. J Cell Physiol 2002;193:287–92.
24. Offer H, Wolkowicz R, Matas D, Blumenstein S, Livneh Z, Rotter V. Direct involvement of p53 in the base excision repair pathway of the DNA repair machinery. FEBS Lett 1999;450:197–204.
25. Quinones A, Dobberstein KU, Rainov NG. The egr-1 gene is induced by DNA-damaging agents and non-genotoxic drugs in both normal and neoplastic human cells. Life Sci 2003;72:2975–92.
26. Yan SF, Fujita T, Lu J, et al. Egr-1, a master switch coordinating upregulation of divergent gene families underlying ischemic stress. Nat Med 2000;6:1355–61.
27. Kieser A, Weich HA, Brandner G, Marme D, Kolch W. Mutant p53 potentiates protein kinase C induction of vascular endothelial growth factor expression. Oncogene 1994;9:963–9.
28. Narendran A, Ganjavi H, Morson N, et al. Mutant p53 in bone marrow stromal cells increases VEGF expression and supports leukemia cell growth. Exp Hematol 2003; 31:693–701.
29. Zalcenstein A, Stambolsky P, Weisz L, et al. Mutant p53 gain of function: repression of CD95(Fas/APO-1) gene expression by tumor-associated p53 mutants. Oncogene 2003;22:5667–76.
30. Fahmy RG, Dass CR, Sun LQ, Chesterman CN, Khachigian LM. Transcription factor Egr-1 supports FGF-dependent angiogenesis during neovascularization and tumor growth. Nat Med 2003;9:1026–32.
31. Gille J, Swerlick RA, Caughman SW. Transforming growth factor-α-induced transcriptional activation of the vascular permeability factor (VPF/VEGF) gene requires AP-2-dependent DNA binding and transactivation. EMBO J 1997;16:750–9.
32. Yan SF, Pinsky DJ, Mackman N, Stern DM. Egr-1: is it always immediate and early? J Clin Investig 2000;105:553–4.
33. Kaufmann K, Thiel G. Epidermal growth factor and platelet-derived growth factor induce expression of Egr-1, a zinc finger transcription factor, in human malignant glioma cells. J Neurol Sci 2001;189:83–91.

34. Kaufmann K, Thiel G. Epidermal growth factor and thrombin induced proliferation of immortalized human keratinocytes is coupled to the synthesis of Egr-1, a zinc finger transcriptional regulator. J Cell Biochem 2002;85:381–91.
35. Baron V, De Gregorio G, Krones-Herzig A, et al. Inhibition of Egr-1 expression reverses transformation of prostate cancer cells in vitro and in vivo. Oncogene 2003;22:4194–204.
36. Nguyen HQ, Selvakumaran M, Liebermann DA, Hoffman B. Blocking c-Myc and Max expression inhibits proliferation and induces differentiation of normal and leukemic myeloid cells. Oncogene 1995;11:2439–44.
37. Huang RP, Liu C, Fan Y, Mercola D, Adamson ED. Egr-1 negatively regulates human tumor cell growth via the DNA-binding domain. Cancer Res 1995;55: 5054–62.
38. Virolle T, Adamson ED, Baron V, et al. The Egr-1 transcription factor directly activates PTEN during irradiation-induced signalling. Nat Cell Biol 2001;3:1124–8.
39. Virolle T, Krones-Herzig A, Baron V, Gregorio GG, Adamson ED, Mercola D. Egr1 promotes growth and survival of prostate cancer cells: identification of novel Egr1 target genes. J Biol Chem 2003;278:11802–10.
40. Bae SK, Bae MH, Ahn MY, et al. Egr-1 mediates transcriptional activation of IGF-II gene in response to hypoxia. Cancer Res 1999;59:5989–94.
41. Khachigian LM, Lindner V, Williams AJ, Collins T. Egr-1-induced endothelial gene expression: a common theme in vascular injury. Science 1996;271:1427–31.
42. Wang ZY, Madden SL, Deuel TF, Rauscher FJ III. The Wilms' tumor gene product, WT1, represses transcription of the platelet-derived growth factor A-chain gene. J Biol Chem 1992;267:21999–2002.
43. Cogswell PC, Mayo MW, Baldwin AS Jr. Involvement of Egr-1/RelA synergy in distinguishing T cell activation from tumor necrosis factor-α-induced NF-κ B1 transcription. J Exp Med 1997;185:491–7.
44. Huang RP, Fan Y, Peng A, et al. Suppression of human fibrosarcoma cell growth by transcription factor, Egr-1, involves down-regulation of Bcl-2. Int J Cancer 1998;77: 880–6.
45. Liu C, Yao J, Mercola D, Adamson E. The transcription factor EGR-1 directly transactivates the fibronectin gene and enhances attachment of human glioblastoma cell line U251. J Biol Chem 2000;275:20315–23.
46. Nair P, Muthukkumar S, Sells SF, Han SS, Sukhatme VP, Rangnekar VM. Early growth response-1-dependent apoptosis is mediated by p53. J Biol Chem 1997;272: 20131–8.
47. Yao J, Mackman N, Edgington TS, Fan ST. Lipopolysaccharide induction of the tumor necrosis factor-α promoter in human monocytic cells: regulation by Egr-1, c-Jun, and NF-κB transcription factors. J Biol Chem 1997;272:17795–801.
48. Mechtcheriakova D, Wlachos A, Holzmuller H, Binder BR, Hofer E. Vascular endothelial cell growth factor-induced tissue factor expression in endothelial cells is mediated by EGR-1. Blood 1999;93:3811–23.
49. Liu C, Adamson E, Mercola D. Transcription factor EGR-1 suppresses the growth and transformation of human HT-1080 fibrosarcoma cells by induction of transforming growth factor β 1. Proc Natl Acad Sci USA 1996;93:11831–6.
50. Svaren J, Ehrig T, Abdulkadir SA, Ehrengruber MU, Watson MA, Milbrandt J. EGR1 target genes in prostate carcinoma cells identified by microarray analysis. J Biol Chem 2000;275:38524–31.
51. Eid MA, Kumar MV, Iczkowski KA, Bostwick DG, Tindall DJ. Expression of early growth response genes in human prostate cancer. Cancer Res 1998;58:2461–8.
52. Scharnhorst V, Menke AL, Attema J, et al. EGR-1 enhances tumor growth and modulates the effect of the Wilms' tumor 1 gene products on tumorigenicity. Oncogene 2000;19:791–800.
53. Calogero A, Arcella A, De Gregorio G, et al. The early growth response gene EGR-1 behaves as a suppressor gene that is down-regulated independent of ARF/Mdm2 but not p53 alterations in fresh human gliomas. Clin Cancer Res 2001;7:2788–96.
54. Huang RP, Fan Y, de Belle I, et al. Decreased Egr-1 expression in human, mouse and rat mammary cells and tissues correlates with tumor formation. Int J Cancer 1997; 72:102–9.
55. Levin WJ, Press MF, Gaynor RB, et al. Expression patterns of immediate early transcription factors in human non-small cell lung cancer: The Lung Cancer Study Group. Oncogene 1995;11:1261–9.
56. Liu C, Rangnekar VM, Adamson E, Mercola D. Suppression of growth and transformation and induction of apoptosis by EGR-1. Cancer Gene Ther 1998;5:3–28.
57. Abdulkadir SA, Qu Z, Garabedian E, et al. Impaired prostate tumorigenesis in Egr1-deficient mice. Nat Med 2001;7:101–7.
58. Gashler A, Sukhatme VP. Early growth response protein 1 (Egr-1): prototype of a zinc-finger family of transcription factors. Prog Nucleic Acid Res Mol Biol 1995; 50:191–224.
59. Khachigian LM, Collins T. Inducible expression of Egr-1-dependent genes: a paradigm of transcriptional activation in vascular endothelium. Circ Res 1997;81:457–61.
60. Krones-Herzig A, Adamson E, Mercola D. Early growth response 1 protein, an upstream gatekeeper of the p53 tumor suppressor, controls replicative senescence. Proc Natl Acad Sci USA 2003;100:3233–8.
61. Sakaue M, Adachi H, Dawson M, Jetten AM. Induction of Egr-1 expression by the retinoid AHPN in human lung carcinoma cells is dependent on activated ERK1/2. Cell Death Differ 2001;8:411–24.
62. Pignatelli M, Luna-Medina R, Perez-Rendon A, Santos A, Perez-Castillo A. The transcription factor early growth response factor-1 (EGR-1) promotes apoptosis of neuroblastoma cells. Biochem J 2003;373:739–46.
63. Flores ER, Tsai KY, Crowley D, et al. p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. Nature 2002;416:560–4.
64. Di Como CJ, Gaiddon C, Prives C. p73 function is inhibited by tumor-derived p53 mutants in mammalian cells. Mol Cell Biol 1999;19:1438–49.
65. Irwin MS, Kondo K, Marin MC, Cheng LS, Hahn WC, Kaelin WG. Chemosensitivity linked to p73 function. Cancer Cell 2003;3:403–10.

Transactivation of the *EGR1* Gene Contributes to Mutant p53 Gain of Function

Lilach Weisz, Amir Zalcenstein, Perry Stambolsky, et al.

Cancer Res 2004;64:8318-8327.