N-Myc downstream-regulated gene 2 is involved in p53-mediated apoptosis

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ABSTRACT

The tumor suppressor, p53, is a transcription factor which can modulate the transcription of a number of target genes that are involved in cell-cycle arrest and apoptosis. However, the apoptotic pathway mediated by p53 is not fully understood. Here, we showed that N-Myc downstream-regulated gene 2 (NDRG2) is a new target gene that is regulated by p53. NDRG2 mRNA and protein levels can be upregulated in a p53-dependent manner. The first intron of the NDRG2 gene contains a site that binds p53 directly and mediates wild-type p53-dependent transactivation. In addition, silencing of NDRG2 attenuates p53-mediated apoptosis, whereas overexpression of NDRG2 suppresses tumor cell growth, regardless of the presence or absence of p53. Our results indicate that NDRG2 is a novel p53-inducible target that is involved in the p53-mediated apoptosis pathway.

INTRODUCTION

The p53 tumor suppressor has long been regarded as the most important factor for the maintenance of genome integrity (1). In response to cellular stresses, p53 can induce diverse biological responses, including cell-cycle arrest, DNA repair and apoptosis (2). Though it is widely accepted that apoptosis induction is essential to the tumor-suppressive activity of p53 (3–5), the complicated mechanism is not fully understood. Growing evidence has shown that p53 can induce apoptosis through its actions as a transcription factor by transactivating numerous target genes in the apoptotic pathway (1,2,4). Recently, reports have demonstrated that p53 exerts its pro-apoptotic effects through transcription-repressive functions (6–9) as well as transcription-independent activities (10), suggesting that p53 may utilize multiple pathways to initiate apoptotic signals.

Under normal circumstances, p53 is maintained at low levels mainly via the action of murine double minute 2 (MDM2) E3 ubiquitin ligase, which directs p53 ubiquitination and degradation. Upon various stresses, such as hypoxia, heat shock, ionizing and UV light irradiation, treatment with chemotherapeutic agents and viral infection, p53 protein levels rise dramatically and are activated by posttranslational modifications to the protein itself and/or MDM2 (2,11–13). Activated p53 functions as a sequence-specific DNA-binding transcription factor and induces expression of its downstream target genes by recognizing a 20-bp response element, which is generally located in the promoter or intronic regions. The consensus sequence is 5’-PuPuPuC(A/T-A/T)GPyPyPy-N(0–13)-PuPuPuC(A/TA/T)GPyPyPy-3’, and the fourth C and seventh G are highly conserved in the 10-bp half-sites (14). To date, more than a hundred p53 target genes have been identified (10), and some of these proteins are able to mediate p53-induced apoptosis. These proteins include molecules with a pro-apoptotic potential, such as BAX, NOXA, PUMA, BID, FAS/APO1, Pidd and KILLER/DR5 (15–21), as well as some newly identified proteins, which are not part of the canonical intrinsic and extrinsic apoptotic pathways, such as Scotin, voltage-gated sodium channel, type III, beta (SCN3B) and N-Myc downstream-regulated gene 1 (NDRG1) (22–24). Nevertheless, none of these downstream targets fully contribute to the p53-mediated apoptotic response, indicating that p53 may activate a subset of apoptosis-related genes in a cell context- or stress type-dependent fashion (10,25).

Human NDRG2 (also named SYLD/KIAA1248) belongs to the NDRG family and was first cloned in our laboratory from a normal human brain cDNA library by...
subtractive hybridization (GenBank, Accession No. AF 159092) (26). This family consists of four members, \textit{NDRG1}, \textit{NDRG2}, \textit{NDRG3} and \textit{NDRG4}, which share roughly 60% residue identities (27–29). The members have different tissue expression patterns, indicating that they may play distinct roles (27,28), but some exhibit similarities in either expression regulation or biological functions. For example, both \textit{NDRG1} and \textit{NDRG2} can be transcriptionally repressed by Myc (30,31) and upregulated by hypoxia and nickel reagent (32–34). Previous studies demonstrated that both proteins were involved in cellular differentiation (35–38) and in certain human neoplasms. For example, both \textit{NDRG1} and \textit{NDRG2} can be transcriptionally regulated by p53 (39,40). Importantly, \textit{NDRG1} is well-documented to be associated with a tumor-suppression function (37,41,42), and \textit{NDRG2} stands as a necessary contributor during p53-induced caspase activation and apoptosis. This led us to question whether there is a relationship between \textit{NDRG2} and p53, which would help identify the biological function of Ndrg2 and better understand the p53-regulated cellular responses.

In this study, we report that the \textit{NDRG2} gene is transcriptionally regulated by p53. \textit{NDRG2} mRNA and protein synthesis were induced by overexpressing p53 via either an adenovirus infection or stabilization of p53 after DNA damage. Analysis of the \textit{NDRG2} genomic loci revealed one potential p53-binding element in intron 1, which was verified as a functional p53 response element that specifically binds to endogenous or exogenous p53. In addition, our experiments using RNA interference suggest that \textit{NDRG2} is involved in the p53-mediated apoptosis pathway.

MATERIALS AND METHODS

Cell lines and cell culture

The human tumor cell lines H1299 (p53 null), Saos2 (p53 null), MDA-MB-231 (mutant p53), SK-BR-3 (mutant p53), A549 [wild-type (wt) p53], U87 (wt p53) and the human embryonic kidney cell line (HEK293) were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS) (Sijiqing Biological Engineering Materials Co., Hangzhou, China). Cells were incubated at 37°C in the presence of 5% CO2-balanced air.

Real-time PCR

Total RNA was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer’s protocol. Total RNA (2 μg) was reverse transcribed with reverse transcriptase (Promega, WI, USA). The first strand cDNA was used as the template for real-time quantitative PCR analysis. \textit{β-Actin} cDNA was used as an internal control to normalize variances. The following primers were designed using the ABI Primer Express software for \textit{NDRG2}: 5′-GAGATATGCTCTTTAACC ACCGG-3′ (forward) and 5′-GCTGCCCATACTCC AA-3′ (reverse), for \textit{p21}: 5′-GCACCTGAATGCAAG GGAA-3′ (forward) and 5′-GCAATAACGGGAAGG CA-3′ (reverse), and for \textit{β-actin}: 5′-ATCATGTTTTGA GACCTTCAACA-3′ (forward) and 5′-CATCTCTGTGC GAAAGTCCA-3′ (reverse). \textit{NDRG2}, \textit{p21} and \textit{β-actin} mRNAs were detected with SYBR Green PCR Master Mix and an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, UK) using the comparative threshold cycle (CT) method for relative quantification. The PCR reaction consisted of 12.5 μl of SYBR Green PCR Master Mix, 300 nM of the forward and reverse primers and 1.5 μl template cDNA in a total volume of 25 μl. The thermal cycling conditions were as following: 95°C for 5 min, followed by 45 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 45 s. To verify that the primer pair produced a single product, a dissociation protocol was used following thermocycling to determine the dissociation of the PCR products from 65°C to 95°C.

Western blot analysis

Cells were harvested from 60-mm culture dishes and were lysed in 200 μl of RIPA buffer [0.05 M Tris–HCl pH 7.4, 0.15 M NaCl, 0.25% deoxycholic acid, 1% Nonidet P-40 (NP-40), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin and 1 μg/ml leupeptin]. Protein concentrations were measured using the BCA protein assay (Pierce, Rockford, IL, USA). Proteins were separated by SDS-PAGE and transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). Western blot analyses were carried out using the following primary antibodies: anti-p53, anti-Ndrg2, anti-p21 and anti-β-actin antibodies (Santa Cruz Biotechnology, CA, USA), followed by incubation in species-matched horseradish peroxidase (HRP)-conjugated secondary antibodies. The blots were developed with chemiluminescence substrate solution (Pierce) and exposed to X-ray film.

Construction of reporter plasmids

A 24-bp fragment (5′-CGCGTTGCAAGGTCCCGGG GCCGTTGCAAA-3′) that contains the potential p53-binding sites (p53BSs) from \textit{NDRG2} intron 1 was synthesized and cloned into the \textit{MuI} I and \textit{Bgl} II sites of the pGL3-promoter luciferase vector (pGL3-P-luc; Promega). The pGL3-P-luc contains an SV-40 promoter upstream of the luciferase reporter gene. The resulting construct was named pGL3-p-NDRG2. A relative fragment containing four nucleotides point mutations, shown in lowercase (5′-CGCGTTGCAAGGTCCCGGG GCCGTTGCAAA-3′), which are predicated to be critical for p53 binding was also generated and cloned into the reporter vector and named pGL3-p-NDRG2-m.

The 1379 bp (−56/+1323 bp) and the 688 bp (−56/+632 bp) DNA fragments, which either contain or delete the intronic p53BSs of the \textit{NDRG2} intron 1, respectively, were amplified from the A549 genomic DNA
Cells were seeded in a 24-well plate at a density of 

Transfection and reporter gene assay

NDRG2

p53BSs and pGL3-B-

p53BSs-del, 5

m

0

p53BSs-del, 5

m

0

p53BSs: 5

corresponding constructs were named pGL3-B-

NDRG2 basic luciferase vector (pGL3-B-luc; Promega). The

CC-3

by PCR. The following primers were used for NDRG2-

p53BSs: 5'-GCACGGCTGTGCGCCATCTCTCCCA CC-3' (forward) and 5'-GCAGATCTAGGGGAAGTTAA ACCGGGGAGAA-TA-3' (reverse), and for NDRG2-

p53BSs-del, 5'-GCACGGCTGTGCGCCATCTCTCTC CCCACC-3' (forward) and 5'-GCAGATCTCCCGCAG ACCGGGGAGCCAGACC-3' (reverse). These PCR products were cloned into the Mlu I and BgI II sites of the pGL3-

basic luciferase vector (pGL3-B-luc; Promega). The corresponding constructs were named pGL3-B-NDRG2-

p53BSs and pGL3-B-NDRG2-p53BSs-del, respectively.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed according to the EMSA kit procedure (Pierce). Nuclear extracts were prepared from A549 cells treated with or without Adr or from Saos2 cells infected with Ad-p53 or Ad-LacZ. Then endogenous or exogenous p53 protein was prepared using the NE-PER nuclear and cytoplasmic extraction reagents. Briefly, cells (1 \times 10^5) were resuspended in 200 \mu l cytoplasmic extraction reagent (CER) containing protease inhibitors, incubated on ice for 10 min and then 11 \mu l of CER II was added and incubated for 1 min on ice. After centrifugation at 12 000 r.p.m. for 10 min, the cell pellets were resuspended in 50 \mu l nuclear extraction reagent (NER) containing protease inhibitors and rotated at 4°C for 1 h. Supernatants with nuclear proteins were recovered by centrifugation at 12 000 r.p.m. for 15 min. The candidate p53BSs in the NDRG2 intron 1 and the corresponding complementary sequence (5'-TGTGTGCAAGGTTCCCGGGGCTTGGGACCGC-3') were prepared as probes, named NDRG2-wt-p53BSs. A mutant version (5'-TGTGTGCAAGGTTCCCGGGCAGGATCGACCGC-3') was also prepared and named NDRG2-mt-p53BSs. A 5'-biotin modification was included in all probes except for the competitor oligonucleotides, which did not contain any modifications. Nuclear proteins (3 \mu g) were incubated with 10 fmol of biotin-labeled NDRG2-wt-p53BSs or NDRG2-mt-p53BSs, and the DNA-protein complexes were separated on a 6% nondenaturing polyacrylamide gel in 0.5 x TBE with 2.5% glycerol and transferred to positively charged nylon membranes. The membranes were blocked with blocking buffer and incubated with HRP-conjugated streptavidin. The blots were developed with chemiluminescence substrate solution (Pierce) and exposed to X-ray film. For competition assays, 100- or 200-fold excess of unlabeled probes were added to the reaction mixture for 1 h at room temperature before the addition of the labeled probe.

Adenovirus infection

Adenoviruses carrying wt-p53 (Ad-p53) or the negative control Lac Z (Ad-lac Z) were purchased from Benyuan Zhengyang Company (Beijing, China). The multiplicity of infection (MOI) was determined for the Saos2 and H1299 cell lines.

Gene transfection

All transfections were performed with lipofectamine 2000 (Invitrogen Life Technologies), according to the manufacturer's instructions. For stable transfection, single colonies were obtained after selection with G418

Transfection and reporter gene assay

Cells were seeded in a 24-well plate at a density of 1 \times 10^5 cells/well and transfected the next day using Lipofectamine 2000 (Invitrogen Life Technologies), according to the manufacturer's protocol. Briefly, for HEK 293 cells, 0.1 \mu g of the reporter vector was cotransfected with 1 \mu g of the pcDNA3.1(+) control vector or a vector that expresses wt or mutant p53. The Renilla luciferase vector (0.01 \mu g, pRL-CMV, Promega) was also cotransfected as an internal control. Luciferase activity was measured 36 h after transfection using the dual-luciferase reporter assay system. For A549, MDA-MB-231 and Saos2 cells, 1 \mu g of the reporter genes were cotransfected with 0.01 \mu g of pRL-CMV. Then 12 h after transfection, the cells were treated with 0.4 \mu g Adr for 24 h and the luciferase activity was measured.

Treatment of cells with siRNA for p53 and NDRG2

The siRNA pSilencer™3.1-H1 neo vector system was utilized and the siRNA motifs were selected from the human p53 or NDRG2 cDNA sequence according to the AA-N19 rule (www.ambion.com/techlib/resources/RNAi). The target sequence for p53 (NM_000546) is localized at positions 734-bp downstream from the start codon. The target sequence for NDRG2 (AF159092) is localized at positions 604-bp downstream from the start codon.

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChIP) analysis was performed using the ChIP Assay kit (Upstate Biotechnology, Charlottesville, VA, USA). After infection with Ad-p53 or Ad-LacZ for 48 h, 2 \times 10^6 Saos2 cells were cross-linked with 1% formaldehyde for 10 min at 37°C. After treatment with 0.4 \mu g Adr or DMSO for 24 h, 6 \times 10^6 A549 cells were cross-linked with 1% formaldehyde for 10 min at 37°C. The cells were then washed, lyzed and sonicated to generate 200–500 bp DNA fragments. The samples were precleared with 60 \mu l of salmon sperm DNA–protein A-agarose and subsequently incubated at 4°C overnight with 5 \mu g anti-p53 antibody (Santa Cruz Biotechnology, CA, USA), with mIgG and no antibody serving as control. Immunocomplexes were recovered, washed thoroughly and eluted with the ChIP elution buffer. Following the reversal of cross-links at 65°C for 4 h, samples were extracted with phenol/chloroform, precipitated with ethanol and then used as templates for PCR amplification, using primers flanking the p53BSs in the NDRG2 intron 1 or the control region. For NDRG2

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(Saos2, 500 μg/ml; A549, 600 μg/ml) for 4 weeks. All clones were analyzed using western blot analysis.

### MTT assay

The cells were seeded into 96-well plates at a starting density of 1 x 10^3 cells/well in triplicate. At each time point, the cells were washed and incubated with tetrazolium salt (MTT, 100 μg/ml, Sigma) at 37°C for 4 h. The supernatant was removed, and 150 μl DMSO was added to each well. The absorbance (OD) of the reaction solution at 570 nm was recorded. Each experiment was performed in triplicate, and the values were reported as the mean ± SD.

### Colony formation assay

Saos2 cells, which were stably transfected with either the empty vector [pcDNA3.1(+)] or the vector carrying Flag-tagged NDRG2 [pcDNA3.1(+)-F-2 and pCDNA 3.1(+)-F-4], were seeded into 6-cm dishes at a density of 200 cells/dish. The cells were grown for 4 weeks in selective culturing medium (G418, 500 μg/ml). Then, the colonies were fixed and stained with Coomassie blue.

### Detection of apoptosis

Apoptosis was detected using flow cytometry analysis and the terminal uridine deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. For flow cytometry, cells were harvested following mild trypsinization and were washed with phosphate-buffered saline (PBS). Cell death was measured using two-color analysis of fluorescence isothiocyanate-labeled annexin V (Roche Applied Science) binding and propidium iodide (PI) uptake using a Becton Dickinson fluorescence-activated cell sorter (FACS) apparatus. For the TUNEL assay, the Apoptag Direct in situ Apoptosis Detection Kit-Fluorescence (Roche Applied Science, Basel, Switzerland) were used to detect DNA fragmentation in apoptotic cells by direct end-labeling of cellular genomic DNA with a fluorescein-conjugated nucleotide using the terminal deoxynucleotidyltransferase enzyme. Cells were washed, fixed in 4% formaldehyde in PBS for 30 min at room temperature and stored dry on aminoaalkylsilane-coated slides at −20°C before processing. 4′,6-Diamidino-2-phenylindole (DAPI) was used for counterstaining. The cells fixed on slides were observed using a fluorescence microscope.

### Statistical analysis

Data are expressed as the mean ± SD. Statistical analyses were performed with the SPSS software (version 10.0; SPSS, Chicago, IL, USA) by using the t-test for independent groups. Statistical significance was based on a value of P < 0.05.

## RESULTS

### Induction of NDRG2 by DNA-damaging agents or stress is p53 dependent

Adr, as a topoisomerase II inhibitor, is able to induce double-strand DNA breaks, and consequently stabilize and activate p53, which results in the induction of p53 target genes (13,49). To explore the relationship between NDRG2 and p53, we first examined the changes of NDRG2 mRNA and protein levels in response to p53 activation by Adr. Adr treatment resulted in increased expression of NDRG2 and p21 in both A549 and U87 cells as measured by real-time PCR (Figure 1A). The increase in NDRG2 mRNA began after 3 h and was significant 6 h following Adr treatment. Additionally, Ndrg2 protein expression increased in A549 cells as detected by western blot (Figure 1B). Two other classical p53 inducible treatment (MMS and UV) can also upregulate the expression of NDRG2 in mRNA and protein level (Figure 1C and D). In contrast, the effect of Adr on NDRG2 expression was not observed in either the mutant p53-bearing cell lines, including MDA-MB-231 and SK-BR-3, or the H1299 p53 null cell line (Figure 1E and F). To further confirm that upregulation of Ndrg2 is p53-dependent, we transfected A549 cells with pSilencer3.1-p53 to knockdown endogenous p53, treated them with Adr and then observed the change of Ndrg2 protein level. As shown in Figure 1G, reduction of endogenous p53 markedly attenuated Adr-induced increase of Ndrg2 (Figure 1G). These data strongly suggest that the elevated expression of NDRG2 by DNA-damaging agents or stress is p53 dependent.

### Ectopic expression of p53 results in upregulation of NDRG2

To directly investigate whether p53 can upregulate NDRG2 expression, we examined the effect of exogenously introduced p53 via either adenoviral infection or gene transfection on the expression of NDRG2 in p53 null H1299 cells. We found that the Ndrg2 protein level was upregulated due to the increase in exogenous p53 following adenovirus infection, similar to p21, which is a known p53 target gene (Figure 2A and B). We also detected that the NDRG2 mRNA level increased with p53 overexpression (Figure 2C), suggesting that the effect occurred at the transcriptional level. Meanwhile, transfection of only the wt p53, but not the mutant forms (175H and 248W), upregulated the expression of NDRG2 (Figure 2D), indicating that NDRG2 may be a p53 target gene.

### The putative p53-binding site in NDRG2 intron 1 is responsive to p53

It is well known that the upstream regulatory regions and intronic regions of p53-regulated genes may contain p53BSs (50). By analyzing the genomic sequence of NDRG2, we identified a putative sequence for p53BSs in intron 1 (5′- GTCGCAAGGTCCTGGGGCcGCTTGCA-3′) (Figure 3A). The sequence contains mismatches (shown in lower case) in the nonconserved positions within the consensus p53BS.

To determine whether the putative binding site in NDRG2 was responsive to p53, a fragment containing this element was synthesized and cloned into a pGL3-promoter luciferase vector (pGL3-p-NDRG2). A mutant reporter vector, pGL3-p-NDRG2-m, was also generated by replacing the four nucleotides in the p53BSs (see Materials and methods section). The empty reporter vector, pGL3-p, served as the control (Figure 3A). Each reporter vector
was cotransfected into HEK293 cells with either a pcDNA3.1(+) control vector or a vector expressing wt p53 or mutant p53 (R175H). We found that the wt p53 was capable of increasing the luciferase activity of pGL3-p-NDRG2 markedly, while the mutant p53 and empty vector did not. The luciferase activity was nearly 4-fold higher after transfection with pGL3-p-NDRG2 than after transfection with the control vector.

Figure 1. Induction of NDRG2 by DNA-damaging reagents or stress depends on the presence of wt p53. (A) The levels of the NDRG2 and p21 transcripts were assessed by real-time PCR assays in A549 cells and U87 cells that were treated with Adr (0.2 μg/ml) for 0, 3, 6, 12 and 24 h. (B) The levels of the p53, Ndr2 and p21 proteins were assessed by western blot in A549 cells that were treated with 0.2 μg/ml Adr for 0, 3, 6, 12 and 24 h. (C) The levels of the NDRG2 and p21 transcripts were assessed by real-time PCR assays in A549 cells that were treated with methyl methanesulfonate (MMS, 120 μg/ml) or irradiated with UV (7.5 J/m²) for 0, 3, 6, 12 and 24 h. (D) The levels of the Ndr2 and p21 proteins were assessed by western blot in A549 cells that were treated with MMS (120 μg/ml) or irradiated with UV (7.5 J/m²) for 0, 3, 6, 12 and 24 h. (E) The levels of the p53, Ndr2 and p21 proteins were assessed by western blot in MDA-MB-231 and SKBR-3 cells that were treated with or without 0.2 μg/ml Adr for 24 h. (F) The levels of the NDRG2 transcripts were measured by real-time PCR assays in H1299 cells that were treated with 0.2 μg/ml Adr for 0, 3, 6, 12 and 24 h. (G) A549 cells were transiently transfected with pSilencer3.1-p53 or pSilencer3.1-X and 24 h later, cells were treated with or without 0.2 μg/ml Adr for 24 h. The levels of the p53 and Ndr2 proteins were assessed by western blot. In (B), (D), (E) and (G), actin was used as a loading control. The experimental details are described in the Materials and methods section.
p53BSs were mutated (pGL3-p-NDRG2-m), p53-mediated transactivation was significantly diminished (Figure 3B), implying that the p53BSs in intron 1 of NDRG2 are required for responsiveness to exogenous p53.

We then examined the effect of activation of endogenous p53 via Adr treatment on the transactivation of the reporter vectors. We found that the luciferase activity of pGL3-p-NDRG2, rather than pGL3-p-NDRG2-m or pGL3-p, was markedly increased in wt p53-bearing A549 cells after treatment with 0.2 μg or 0.4 μg Adr (Figure 3C). In contrast, Adr treatment failed to increase the transcriptional activity of pGL3-p-NDRG2 in either mutant p53-bearing MDA-MB-231 cells or p53 null Saos2 cells (Figure 3D). Together, these results indicated that the p53BSs in the NDRG2 intron 1 is responsible for mediating p53-induced gene transactivation.

Next, we examined whether the intronic p53BSs in NDRG2 are required for driving p53-induced transactivation of its own promoter. We amplified the DNA fragments consisting of the NDRG2 promoter, exon 1 and intron 1 containing p53BSs and its corresponding mutant counterpart that lacked p53BSs, cloned them into pGL3-B-luc, and named the vectors pGL3-B-NDRG2-p53BS and pGL3-B-NDRG2-p53BS-del, respectively (Figure 3A). The pGL3-B vector served as the control. When each of these reporter vectors was cotransfected into HEK293 cells with either the pcDNA3.1(+) control vector or vectors expressing the wt p53 or mutant p53 (R175H), we observed that the luciferase activity of pGL3-B-NDRG2-p53BSs could be induced only by wt p53, and not by the mutant p53 or empty vector. Moreover, neither the wt nor the mutant p53 was capable of increasing the transcriptional activity of pGL3-B-NDRG2-p53BSs-del (Figure 3E), which strongly suggests that the intronic p53BSs are indispensable for p53-mediated transactivation of NDRG2.

p53 binds directly to the p53-binding sites in NDRG2 intron 1

To further verify that NDRG2 is a p53 target gene, ChIP and EMSA were performed to examine whether p53 directly binds to the p53BSs in intron 1 of NDRG2. First, we demonstrated that both exogenous and endogenous p53 can bind the p53BSs in intron 1 of NDRG2 using the in vivo ChIP assay. As shown in Figure 4A, in Saos2 cells upon Ad-p53 infection and in A549 cells upon Adr treatment, the fragment (+889/+1082 bp) containing the p53BSs in NDRG2 intron 1 was amplified by PCR from DNA–protein complexes that were coimmunoprecipitated.
using the anti-p53 antibody. In contrast, a DNA sequence (-235/-62 bp) far from the p53BSs failed to be coimmunoprecipitated and amplified.

Next, we used EMSA to confirm this interaction in vitro. As shown in Figure 4B and C, when the labeled wt probe for NDRG2-p53BSs was incubated with extracts from Ad-p53-infected Saos2 cells or Adr-treated A549 cells, we detected a clear band shift that did not occur with Ad-LacZ infection (Figure 4B, lane 4 versus 9) or vehicle treatment (Figure 4C, lane 4 versus 9).

However, when using the mutant NDRG2-p53BSs probe, we did not observe band shifts with extracts from either Ad-p53-infected Saos2 cells (Figure 4B, lane 2) or Adr-treated A549 cells (Figure 4C, lane 2). The unlabeled wt NDRG2-p53BSs probe, rather than the mutant version, is able to reduce the protein–DNA complex formation in a dose-dependent manner when included in the reaction as a competitor (Figure 4B and C, lane 4 versus lanes 5 and 6; and lane 7 versus 8). Taken together, these results indicate that NDRG2 is a direct target gene of p53.

Figure 2. Exogenous, overexpressed p53 induces upregulation of NDRG2. (A) The levels of the p53, Ndr2 and p21 proteins were assessed by western blot in H1299 cells that were infected with Ad-LacZ or different MOI Ad-p53 for 48 h. (B) The levels of the p53, Ndr2 and p21 proteins were assessed by western blot in H1299 cells that were infected with 40 MOI Ad-p53 for 0, 24, 48 and 72 h. (C) The levels of the NDRG2 transcripts were measured by real-time PCR assays in H1299 cells that were infected with Ad-LacZ for 24 h or with 40 MOI Ad-p53 for 0, 3, 6, 12, 24 and 48 h. (D) H1299 cells were transfected with pcDNA3.1(+), pcDNA3.1(+)-wt-p53, pcDNA3.1(+)-175H or pcDNA3.1(+)-248W, respectively. Twenty-four hours after transfection, the protein levels of p53, Ndr2 and p21 were determined by western blot. In (A), (B) and (D), actin was used as a loading control.
**Figure 3.** The putative p53-binding site in intron 1 of NDRG2 is responsive to p53 and is required for p53-dependent transactivation of NDRG2 promoter. (A) Shown are the wt putative p53-binding site located within NDRG2 intron 1, its corresponding mutant version and a schematic diagram of the various reporter vectors used in the luciferase assays. (B) Activation of the p53-binding site in NDRG2 intron 1 by exogenous wt p53. HEK293 cells were individually cotransfected with an equal amount (0.1 µg) of reporter vectors (pGL3-p, pGL3-p-NDRG2 or pGL3-p-NDRG2-m) and either empty vector pcDNA3.1(+) or a vector expressing wt p53 [pcDNA3.1(+)-wt-p53] or mutant p53 [pcDNA3.1(+)-175H]. Luciferase activity was determined 36 h following transfection. (C) Activation of the p53-binding site in NDRG2 intron 1 by endogenous p53 in response to DNA damage in A549 cells. A549 cells were transfected with pGL3-p, pGL3-p-NDRG2 or pGL3-p-NDRG2-m, and 12 h later, the cells were treated with the indicated amount of Adr for 24 h, and luciferase activity was determined. (D) Responsiveness of the p53-binding site in NDRG2 intron 1 to endogenous p53 depends on the presence of wt p53. A549, MDA-MB-231 or Saos2 cells were individually transfected with pGL3-p-NDRG2, 12 h later, cells were treated with the indicated amount of Adr for 24 h, and luciferase activity was determined. (E) The reporter vectors (pGL3-B, pGL3-B-NDRG2-P53BSs or pGL3-B-NDRG2-P53BSs-del) were cotransfected into HEK293 cells with either pcDNA3.1(+) or a vector expressing wt p53 [pcDNA3.1(+)wt-p53] or mutant p53 [pcDNA3.1(+)175H], and 36 h later, luciferase activity was determined. In (B–D), Renilla luciferase vector pRL-CMV was also cotransfected as an internal control vector. Luciferase and Renilla activity was assayed using the Dual-Luciferase Reporter Assay System (Promega), and luciferase fold activation values were measured relative to the levels of Renilla activity. The histograms represent the average of three independent experiments with error bars showing the SD. *P < 0.05 among each compared group.
Figure 4. p53 binds to the putative p53-binding site of NDRG2 intron 1 in vivo and in vitro. (A) Binding of exogenous or endogenous p53 to the p53-binding site in NDRG2 intron 1 in vivo by the ChIP assay. Cell lysates from Saos2 cells that were infected with Ad-p53 or Ad-LacZ for 48 h (left, upper and lower panel) or from A549 cells that were pretreated with 0.4 μg/ml Adr or DMSO for 24 h (right, upper and lower panel) were prepared, cross-linked and immunoprecipitated in the presence or absence of p53-specific antibody. The immunoprecipitated samples were analyzed by PCR amplification using the primers flanking the p53BSs in NDRG2 intron 1 or the control region, as indicated in the Materials and methods section. Genomic DNA and input chromatin (input), which represent portions of sonicated chromatin before immunoprecipitation, were both used as positive controls. (B and C) Binding of exogenous or endogenous p53 to the p53-binding site in NDRG2 intron 1 in vitro was measured by EMSA. Nuclear extracts of Saos2 cells that were infected with Ad-p53 or Ad-LacZ for 48 h (B) or of A549 cells that were pretreated with or without Adr for 24 h (C) were prepared and incubated with biotin-labeled wt or mutant probes for p53BSs in NDRG2 intron 1 [NDRG2-wt-p53BSs (lanes 3–9); NDRG2-mt-p53BSs (lanes 1 and 2)], or together with competitive unlabeled probes [NDRG2-wt-p53BSs (lanes 5 and 6); NDRG2-mt-p53BSs (lane 8)]. DNA–protein complexes were separated on a 6% TBE gel, transferred to nylon membranes and shifted probes were detected with HRP-conjugated streptavidin.
Silencing of NDRG2 inhibits p53-mediated apoptosis

To investigate the functional role of NDRG2 in the p53-regulated pathway, Saos2 (p53 null) cells and A549 (wt-p53) were transfected with siRNA vectors that target NDRG2 (pSilencer-NDRG2) or control vectors (pSilencer-X) and stable cell clones were obtained. As shown in Figure 5A (left panel), the expression of NDRG2 was amplified after p53 introduction (Saos2) or Adr treatment (A549) in the control cells, whereas such an effect was obviously compromised in the NDRG2-siRNA transfecants.

To determine whether NDRG2 contributed to p53-mediated apoptosis, we first examined the pro-apoptotic...
effect of p53 introduction in Saos2 cells, where \textit{NDRG2} expression was either knocked down or not. As shown in upper panel of Figure 5B, control cells transfected with pSilencer-X underwent extensive p53-triggered apoptosis, as indicated by the proportion of annexin V positive cells (74.8%). In contrast, the p53-induced apoptotic effects were greatly compromised by \textit{NDRG2} knockdown in pSilencer-\textit{NDRG2} transfected cells (44.2%). Next, we observed whether Ndrg2 knockdown also had an effect on the process of endogenous p53-mediated apoptosis in A549 cells that were treated with Adr. As shown in lower panel of Figure 5B, Adr treatment caused more apoptosis in the cells transfected with pSilencer-X than pSilencer-\textit{NDRG2} (72.1 + 5.3% versus 65.1 + 2.1%, \(P < 0.01\)). Finally, we confirmed these results by the TUNEL assay. As shown in Figure 5C, there are significantly more apoptotic cells in the pSilencer-X transfectants than in the pSilencer-\textit{NDRG2} transfectants following Ad-p53 infection in Saos2 cells/or Adr treatment in A549 cells. These data indicate that Ndrg2 is required for p53-mediated apoptosis.

**Overexpression of \textit{NDRG2} suppresses tumor cell growth**

To further evaluate the biological role of Ndrg2 in tumor cells, we generated two Saos2 cell lines that were stably transfected with pcDNA3.1(+)−Flag-\textit{NDRG2} [pcDNA3.1(+)−F-2 and pcDNA3.1(+)−F-4], and stable empty vector-transfected clones [pcDNA3.1(+)−] were used as the control. Ndrg2 overexpression was confirmed by western blot analysis (Figure 6A).

To determine whether Ndrg2 has any effect on cell growth, we performed the MTT assay (Figure 6B). The results demonstrated that enhanced expression of Ndrq2 inhibited cell growth in comparison to the control, implying that Ndrq2 may play an inhibitory role in cell proliferation. We then performed colony formation assays to evaluate the proliferating ability of these cells. As shown in Figure 6C, overexpressed Ndrq2 significantly inhibited the colony-forming ability of Saos2 cells, consistent with the results of the MTT assay.

Considering that stable clones generated by selective pressure may differ from their parental cells, we also transiently transfected pcDNA3.1(+)−Flag-\textit{NDRG2} into Saos2 cells and observe its effect on cell growth and apoptosis. The results showed that enhanced expression of Ndrq2 inhibits cell growth in comparison to the control (Figure S1B). However, Ndrq2 overexpression does not promote cell apoptosis (Figure S1C), implying that Ndrq2 suppresses cell growth through inhibiting proliferation rather than inducing apoptosis.
To further examine whether Ndrg2 has an antiproliferation effect on wt p53-bearing tumor cells, we infected A549 cells with Ad-NDRG2 or Ad-LacZ and found that Ndrg2 overexpression suppressed the growth of A549 cells using the MTT assay (data not shown). Collectively, these data suggest that Ndrg2 is involved in suppression of cell proliferation, regardless of the presence or absence of p53.

**DISCUSSION**

As one of the most commonly altered genes in human cancer, research on p53 has been intense for many years. It is well established that most of the biological effects of p53 are due to its function as a transcription factor. Although a variety of p53 downstream target genes have been identified, which are involved in apoptosis (e.g. BAX, DR5, BID, APAF-1, Caspase-6, PIGs, Perp, p53AIP, SCN3B and Scotin), knockout of any one individual gene has failed to protect cells completely from p53-induced death. These findings suggest that the p53 response requires a network of collaborating genes (10,13,17,19,21–23,25,50–54). In addition, several studies have shown that some genes with antiapoptotic activity, such as HB-EGF, DcR1 and DcR2, can be transcriptionally activated by p53 (55–57), while others, such as survivin and PTG/G1, are repressed by p53 (6,7). Thus, the p53 pathway is complicated, and identification of additional p53 targets is critical for understanding the pathway. Here, our results reveal that NDRG2 is a p53-inducible target gene that is transactivated by p53 and is involved in p53-mediated apoptosis.

Increased NDRG2 mRNA and protein levels occurred not only after treatment with the DNA-damaging agents or stress, which activates endogenous p53, but also following adenovirus-mediated ectopic expression of p53 in p53 null cells. Neither DNA damage in p53 mutant and p53 null cell lines nor introduction of mutant p53 could enhance NDRG2 expression; moreover, knockdown of endogenous p53 markedly attenuated Adr-induced increase of Ndrg2, indicating that the effect requires intact p53. Considering the previous finding that NDRG1, a member of NDRG family, is a direct transcriptional target gene of p53, we hypothesized that NDRG2 could also be a p53 target gene.

While NDRG1 has a p53 responsive element in its promoter, we identified one candidate sequence for p53BSs within intron 1 of NDRG2. The NDRG2 element contained all the conserved residues, of the consensus p53BS. It is well documented that p53 target genes harbor p53BSs in either their promoters or intronic regions, and sequences with different mismatched bases may vary considerably in their transcriptional activation function (52,58). Interestingly, some response elements whose structures are quite different from the canonical p53 consensus site can also be recognized by p53 (52).

Our experiments demonstrate that the putative p53BS within NDRG2 intron is a functional one in response to exogenous or endogenous wt p53, as shown in reporter assays. Reporter constructs carrying the endogenous NDRG2 promoter region, exon 1 and intron 1 containing p53BSs exhibited increased transcriptional activity upon p53 activation. Meanwhile, the corresponding counterpart...
lacking p53BSs showed no response to p53, indicating that the intronic p53BSs are essential for p53-mediated trans-activation of NDRG2. Using ChiP and EMSA, we demonstrated that the p53BSs form protein–DNA complexes in Ad-p53-infected p53 null cells or Adr-treated wt p53-bearing cells, suggesting that the intronic NDRG2 p53BSs can bind to p53 after its activation and DNA damage. Collectively, we provide compelling evidence that p53 directly transactivates NDRG2 via intronic p53BSs.

As a member of the NDRG family, the NDRG2 gene has been mapped to chromosome 14q11.2, and encodes a 41 kDa protein, which is expressed in various tissues, particularly in the brain, heart and skeletal muscle (28). Although the precise function of Ndr2 is still unclear, it has been shown to be intimately involved in numerous biological processes, such as cell differentiation (30,35,38), neurodegeneration (39), stress responses (34), as well as carcinogenesis and cancer progression (44,46–48). Mounting evidence has indicated that NDRG2 mRNA levels are downregulated or undetectable in a number of human cancers and cancer cell lines, including glioblastoma, squamous cell carcinoma, pancreatic cancer, gastric cancer and colorectal carcinoma, compared to their cognate noncancerous counterparts (44,46–48). Moreover, NDRG2 expression has been inversely correlated with tumor grade and aggressive tumor behavior (43,45). In two recent reports, NDRG2 was even identified as a gene whose expression in high-grade primary astrocytomas or gastric cancers was positively correlated with survival (47,48). These findings strongly implicate Ndr2 is a tumor suppressor. However, to date, only a few studies have explored the role of Ndr2 in tumor suppression. Ndr2 overexpression in a human glioblastoma cell line was shown to markedly inhibit cell proliferation (43). Another study from our laboratory demonstrated that Ndr2 translocates from the cytosol into the nucleus upon NiCl₂ treatment, where it interacts with the MSP58 oncoprotein and inhibits MSP58-induced proliferation of HeLa cells (59). Most recently, Choi et al. (47) demonstrated that NDRG2 silencing can decrease Fas-mediated cell death via a slight downregulation of Fas expression in SNU-620 gastric cancer cell lines.

Here, we showed that Ndr2 is required for the full p53-mediated apoptotic response. Additionally, we showed that NDRG2 knockdown using siRNA markedly attenuated the p53-mediated apoptotic effect. It is possible that the attenuation was incomplete because the silencing efficiency in our current experiment was not high enough to entirely block the NDRG2 expression. Alternatively, it is likely that other apoptosis-related proteins that are downstream of p53 play a compensatory role in the case of NDRG2 knockdown by contributing to the apoptotic effect. In this regard, the function of Ndr2 may be similar to many known p53 targets, such as BAX, BID, CD95, APAF-1 and DR5, since when each of these genes is silenced or removed from a particular model system, a partial resistance to p53-induced apoptosis is observed (17–19,21,25,54). Therefore, our results together with two recent reports (23,34) support the idea that p53-dependent apoptosis is a complex process, reflecting the combined effect of a number of p53-inducible targets. As for the role of Ndr2 in the p53-initiated apoptosis pathway, it is unlikely that Ndr2 functions as a pro-apoptotic factor because we did not observed Ndr2-promoted apoptosis following its overexpression in p53-null Saos2 cells and p53-intact A549 cells (34). Another possibility is that Ndr2 may play a regulatory role either by facilitating apoptosis choice over cell-cycle arrest for p53 in response to stresses, or by regulating p53 downstream targets that are implicated directly in apoptosis, such as Bax, PUMA and NOXA. Further studies are needed to prove or disprove these hypotheses. Interestingly, our previous data showed that NDRG2 also participates in hypoxia-evoked apoptotic response in A549 cells at least partially by acting as a hypoxia-inducible HIF-1 target gene (34), provided that hypoxia-induced p53 accumulation (60) and subsequent induction of NDRG2 may play a role during this process. Collectively, it appears that NDRG2 can act as a stress-responsive gene to facilitate cell death in order to eliminate hazardous cells.

We have also shown that NDRG2 overexpression led to inhibition of cell proliferation, regardless of the absence or presence of p53, which is consistent with previous reports (43,59). These results suggest that Ndr2 has an inhibitory role on proliferation that might be p53 independent. Nevertheless, in case of p53-intact cells, whether the proliferation-inhibitory role of Ndr2 influences its positive role in apoptosis and vice versa still remains to be determined.

In summary, this study demonstrated for the first time that NDRG2, a novel p53-inducible gene, is implicated in the p53-mediated apoptosis pathway in response to DNA damage and also plays a proliferation-inhibitory role that is independent of p53 status. These findings contribute insights for the function of Ndr2, particularly its stress-responsive activity and tumor-repressive effects.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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