p73 or p53 Directly Regulates Human *p53* Transcription to Maintain Cell Cycle Checkpoints

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Abstract

Whereas the p53 tumor suppressor protein plays a central role in cellular checkpoints that respond to damage or stress to prevent tumorigenesis, the transcriptional control of the *p53* gene has remained unclear. We show that chemotherapeutic agents induce *p53* transcription and that p73 or p53 transactivates endogenous *p53* expression through direct binding to the *p53* promoter. Silencing of p53 or p73 by RNA interference significantly suppresses *p53* transcription under physiologic conditions or in response to cellular stress. Mutational analysis of the human *p53* promoter localized a p53 DNA-binding site, which confers p53- or p73-dependent *p53* promoter activation. Importantly, impaired p53-mediated autoregulation of *p53* transcription by inducible-interfering RNA results in aberrant cell cycle regulation and suppression of p53-mediated apoptosis. Thus, a positive feedback loop regulates human *p53* expression and involves p73 and p53. Disruption of *p53* transcription contributes to defective checkpoint control. (Cancer Res 2006; 66(14): 6982-9)

Introduction

The tumor suppressor p53, frequently mutated in a wide variety of tumors, plays an important role in maintaining genomic integrity (1–5). Exposure of a normal cell to genotoxic stress leads to an increase in p53 protein levels. The increase in p53 protein results in an increase in p53-dependent transcription of p53 target genes, which subsequently leads to cell cycle arrest or apoptosis (6–9). The practical implication of these facts is that when a cell undergoes alterations that predispose it to become cancerous, p53 is activated to trigger checkpoints that either take care of the damage through its DNA repair function or eliminate the affected cells through induction of apoptosis, thereby preventing the development of tumors (4, 10).

Regulation of p53 activity is therefore critical to allow both normal cell growth and tumor suppression. The current dogma is that p53 regulation in DNA damage–activated cell cycle checkpoints occurs at the level of protein degradation and protein stability. This includes regulation of p53 protein stability, posttranslational modifications, protein-protein interactions, and subcellular localization. These mechanisms keep a strong check on p53 in normal circumstances but allow rapid activation in response to cellular stress that might be caused by or contribute to oncogenic progression (3, 4). However, little is known about the transcriptional regulation of the *p53* gene and the contribution of this transcriptional control of p53 itself to DNA damage–induced cell cycle checkpoints.

p53 is known to be transcriptionally up-regulated by the homeobox protein HOXA5 (11). Several reports have raised the possibility that the p53 response to genotoxic stress may also be regulated at the transcriptional level (12, 13). However, the underlying mechanism or functional consequences have remained unclear. Recently, Bcl6 oncoprotein was found to suppress *p53* expression through binding two specific sites within the *p53* promoter region (14). In the current study, we found that p53 mRNA can be induced by multiple chemotherapeutic DNA-damaging agents and that this induction seems to occur at the transcriptional level. Deletional analysis of the *p53* promoter localized a region that was shown to be required for the transactivation of *p53* in response to DNA-damaging agents and either p53 or p73 overexpression. Ectopic expression of p53 or p73 augmented *p53* promoter reporter activity as well as endogenous p53 mRNA levels. Knockdown of endogenous p53 or p73 by short hairpin RNA dramatically prevent the activation of *p53* transcription under physiologic conditions or in response to cellular stress. We showed the direct interaction between p53 or p73 and the genomic *p53* locus by yeast one-hybrid assays and chromatin immunoprecipitation assays and precisely defined the elements in the human *p53* genomic locus for this transcriptional regulation. Our results identify a previously unknown positive feedback loop regulating human *p53* expression. Interestingly, we provide evidence that disruption of p53-mediated autoregulation of *p53* transcription by inducible gene silencing can lead to defects in cell cycle regulation and suppression of p53-mediated apoptosis.

Materials and Methods

Cell lines and cell culture. The human cell lines H460 and Calu 6 (human non–small-cell lung cancer), HCT116 and SW480 (human colon cancer), and U2OS and Saos2 (osteosarcoma) were from American Type Culture Collection (Manassas, VA) and cultured under the recommended conditions. Stable H460/E6 cells in which wild-type p53 is degraded by Ad-E6 were maintained as previously described (15).

Generation of doxycycline-inducible cell lines for p53 silencing. To generate pSuperior-puro-p53 for inducible expression of small interfering RNA targeting p53, the annealed oligonucleotides (GATCCCC*GACTCCAGTGGTAATCTAC*TTCAAGAGA*GTAGATTACCACTGGAGTC*TTTTTGGAAA and AGCTTTTCCAAAAA*GACTCCAGTGGTAATCTAC*TCTCTTGAA*GTAGATTACCACTGGAGTC*GG G) were ligated into the pSuperior-puro vector (OligoEngine, Seattle, WA). HCT116 cells, which stably express PG13, a p53 reporter plasmid, and renilla luciferase reporter plasmid were transfected with a Tet repressor–expressing vector pcDNA6/TR and pSuperior-puro-p53 or control pSuperior-puro vector and selected with 0.5 μg/mL puromycin (Sigma, St. Louis, MO) and 20 μg/mL blasticidin hydrochloride

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

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(Sigma). The annealed oligonucleotides for silencing p73 were cloned into the pSuperior-puro vector and the sequences are available on request.

Plasmid construction. The human *p53* promoter-pGL2 basic luciferase plasmids containing the 2.4-kb *Xba*I fragment and the 356-bp *Xba*I-*Bam*HI fragment of the *p53* promoter were obtained from Saraswati Sukumar (Johns Hopkins Oncology Center, Baltimore, MD). *p53* promoter fragments −344 to +12, −188 to +12, −88 to +12, and −38 to +12 (relative to the first transcription initiation site) were amplified by PCR and cloned into the pGL2 basic reporter vector, and the sequence of each plasmid was verified.

PCR-directed mutagenesis. The primers for PCR-directed mutagenesis in the potential half-binding sites for p53, located at −170 to −85 upstream of the *p53* transcription start site, were M1F, 5′-CCTCCGGCAGGCGGATTAATTTCCCTTACTTGTC-3′; M1R, 5′-CGCCATGACAAGTAAGGGAAATTAATCCGCCTGCC-3′; M2F, 5′-TTACTTGCCCTTACTTGTAATTGCGACTGTCCAG-3′; M2R, 5′-ACAAAGCTGGACAGTCGCAATTACAAGTAAGGGC-3′; M3F, 5′-TTACTTGTCATGGCGACTGTACATCTTTGTGCCAG-3′; and M3R, 5′-CGAGGCTCCTGGCACAAAGATGTACAGTCGCCATG-3. *Xba*I-*Bam*HI fragment (356 bp) of the *p53* promoter plasmid was used a template.

Adenovirus infections. The human Ad-p73α,β were obtained from Takashi Tokino (Sapporo Medical University School of Medicine, Sapporo, Japan). The recombinant adenoviruses containing green fluorescent protein (GFP), p53, and Myc-GFP were generated with the Ad-Easy System.

Flow cytometric analysis. Cells were harvested after the indicated treatments and time periods, stained with propidium iodide, and analyzed by flow cytometry as previously described (16, 17).

Luciferase assays. Cells were cotransfected with the *p53* promoter-reporter (firefly luciferase) plasmid and renilla luciferase control reporter plasmid. At 24 hours after transfection, the cells were harvested and luciferase activity was measured with the dual-luciferase reporter assay system (Promega, Madison, WI). Light units were normalized to renilla luciferase activity.

***In vivo* bioluminescence imaging.** Cells were cotransfected with the indicated *p53* promoter-reporter plasmids and renilla luciferase reporter plasmid. At 24 hours after transfection, 150 μg/mL D-luciferin or 10 μg/mL coelenterazine (Biotium, Hayward, CA) was added to each well. Bioluminesence imaging was done with the cooled CCD camera in the *In Vivo* Imaging System (Xenogen, Alameda, CA).

Chromatin immunoprecipitation assays. The chromatin immunoprecipitation assays were done with the chromatin immunoprecipitation assay kit (Upstate Biotechnology, Inc., Lake Placid, NY). H460 cells (1×10^7) were treated with 30 μg/mL CPT-11 for 16 hours, transfected with pFlag-p73, or infected with Ad-GFP, Ad-p53, Ad-Myc-GFP, or Ad-p73α for 24 hours. U2OS cells (1×10^7) were treated with 7.5 μg/mL *cis*-diammine-dichloroplatinum (Sigma), 0.2 μg/mL Adriamycin, or 30 μg/mL CPT-11 for 20 hours. The DNA fragments containing the *p53* promoter region were amplified in the samples immunoprecipitated with antibodies against p53 (monoclonal antibody DO-1, Santa Cruz Biotechnology, Santa Cruz, CA; Ab-1, Oncogene, San Diego, CA) or p73 (monoclonal antibody Ab-3, Oncogene; polyclonal antibody sc-7237, Santa Cruz Biotechnology). Immunoglobulin G (IgG) antibody was used as a negative control. PCR products were subjected to Southern blotting with a 356-bp fragment of the *p53* promoter as a probe to verify the authenticity of the amplified DNA. Oligonucleotide sequences for PCR primers were 5′-CAGAGTGATAAGGGTTGTGAAGGAG-3′ and 5′-AAAACCCCAATCCCATCAACC-3′ in the *p53* promoter region upstream of exon 1.

Reverse yeast-one hybrid assays. Yeast one-hybrid assays were carried out with the BD Matchmaker Library Construction and Screening kits (Clontech, Palo Alto, CA). The 356-bp *Xba*I-*Bam*HI fragment of the human *p53* promoter was cloned into the pHIS2 one-hybrid reporter vector and used as target DNA. Yeast strain Y187 cells were cotransformed with pGAD-Rec2-p53 and pHIS2-356-bp *Xba*I-*Bam*HI fragment of *p53* promoter. p53HIS2, which carries three tandem copies of p53 consensus binding sites, and pHIS2 were used as positive and negative controls. The transformed Y187 cells were spread on SD/−His/−Leu/−Trp plates containing 20 mmol/L 3-aminotriazole and incubated at 30°C for 3 to 6 days to select for one-hybrid interactions.

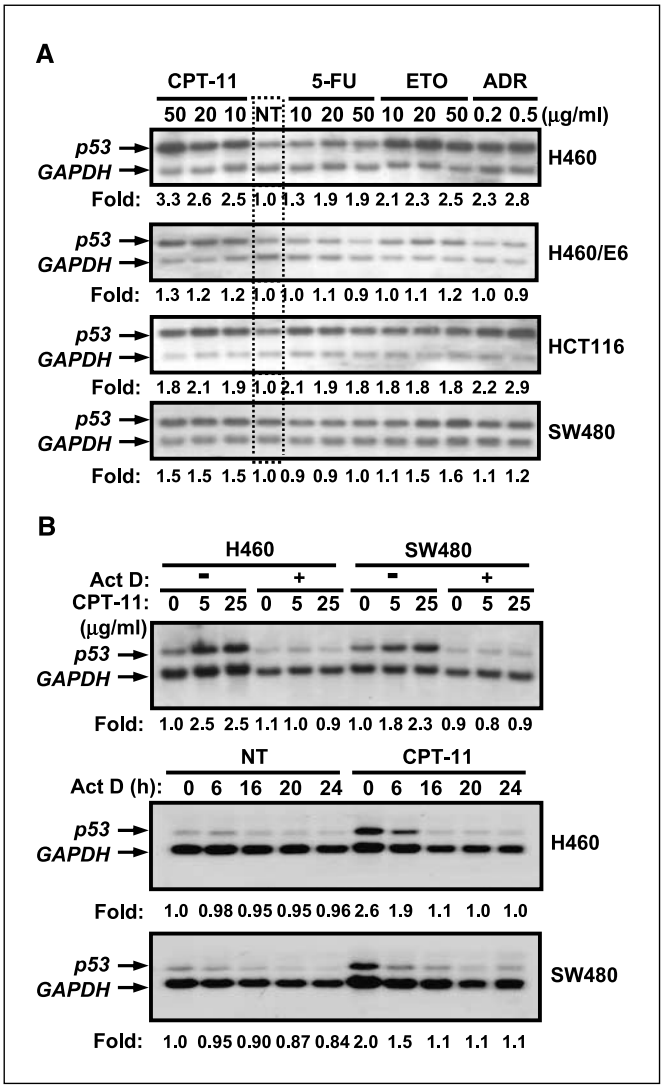

Figure 1. Chemotherapeutic agents induce transactivation of *p53* expression. *A,* cells were treated with multiple DNA-damaging chemotherapeutic agents at the indicated concentrations for 16 hours. *NT,* no treatment control. RNAs isolated from each point were examined by Northern blotting. *B,* cells were pretreated with or without 0.2 μg/mL actinomycin D for 2 hours, followed by CPT-11 at the indicated concentrations for 14 hours (*top*). Cells were treated with 30 μg/mL CPT-11 for 8 hours, followed by 0.2 μg/mL actinomycin D treatment at the indicated time points (*bottom*). *Middle* and *bottom,* time 0 refers to when the CPT-11 treatment ended. RNAs from each point were analyzed by Northern blots. The density of each band was quantified with NIH image 1.63 software. Fold values were calculated relative to GAPDH controls.

Western blotting. Western blotting was carried out essentially as previously described (16, 17) with mouse anti-human p53 monoclonal antibody (DO-1, Santa Cruz Biotechnology), mouse anti-human p73 monoclonal antibody (Ab-3, Oncogene), and mouse anti-human Ran antibody (BD Transduction Laboratories, San Diego, CA).

Northern blotting. Total RNA was isolated with the RNeasy total miniprep kit (Qiagen, Valencia, CA) following the instruction of the manufacturer. Northern blotting was carried out as previously described (16, 17). Full-length *p53* cDNA was used as a probe. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control.

Quantitative reverse transcription-PCR. Quantitative reverse transcription-PCR (RT-PCR) was carried out with the TaqMan reverse transcription reagents and TaqMan PCR core reagent kit (Applied

Biosystems, Foster City, CA). The primers were human p53-F, 5′-TGTCCCTTCCCAGAAAACCTACC-3′; human p53-R, 5′-CCACTCGGATAAGATGCTGAGGAG-3′; mouse p53-F, 5′-CCTCTGAGCCAGGAGACATTTTC-3′; mouse p53-R, 5′-AAGCCCAGGTGGAAGCCATAGTTG-3′; human GAPDH-F, 5′-AACGGATTTGGTCGTATTGGG-3′; human GAPDH-R, 5′-TGGAAGATGGTGATGGGATTTC-3′; mouse GAPDH-F, 5′-TTGCCATCAACGACCCCTTC-3′; and mouse GAPDH-R, 5′-AGACTCCACGACATACTCAGCACC-3′.

Results and Discussion

DNA-damaging chemotherapeutic agents induce transcriptional activation of the *p53* gene. p53 has been observed to be induced after DNA damage but the pathway that regulates its transcription has remained unclear (12, 18–20). We show here that p53 mRNA is significantly induced by multiple DNA-damaging chemotherapeutic agents (CPT-11, 5-fluorouracil, etoposide, or Adriamycin) in p53 wild-type human tumor cell lines (H460 non–small-cell lung cancer or HCT116 colon carcinoma; Fig. 1*A*). In p53-deficient (H460-E6 where wild-type p53 is degraded or SW480 colon carcinoma cells where both p53 alleles are mutated) tumor cell lines, the induction of p53 mRNA by these chemotherapeutic agents was less effective as compared with the cells carrying wild-type p53 (Fig. 1*A*). We also observed the induction of p53 mRNA on Adriamycin treatment in a time- and concentration-dependent manner in wild-type p53 mouse embryonic fibroblasts (Supplementray Fig. S1). These results suggest that p53 itself might be involved in the induction of p53 mRNA by chemotherapeutic agents. While further investigating the mechanisms of this regulation, with actinomycin D to block transcription, we found that the induction of p53 mRNA by the chemotherapeutic agent CPT-11 mainly occurs at the transcriptional level (Fig. 1*B*).

DNA-damaging agents induce *p53* promoter activation and p53 or p73 is involved in the regulation of *p53* transcription. To further analyze control of *p53* transcription, we investigated the sequences within the *p53* promoter that may regulate *p53* after genotoxic stress or oncogene activation. Deletional analysis of the human *p53* promoter showed that the 100 bp located at −88 to +12 (relative to the first transcription initiation site) promoter region contains the minimal promoter elements responsible for basal promoter activation. The 200-bp (−188 to

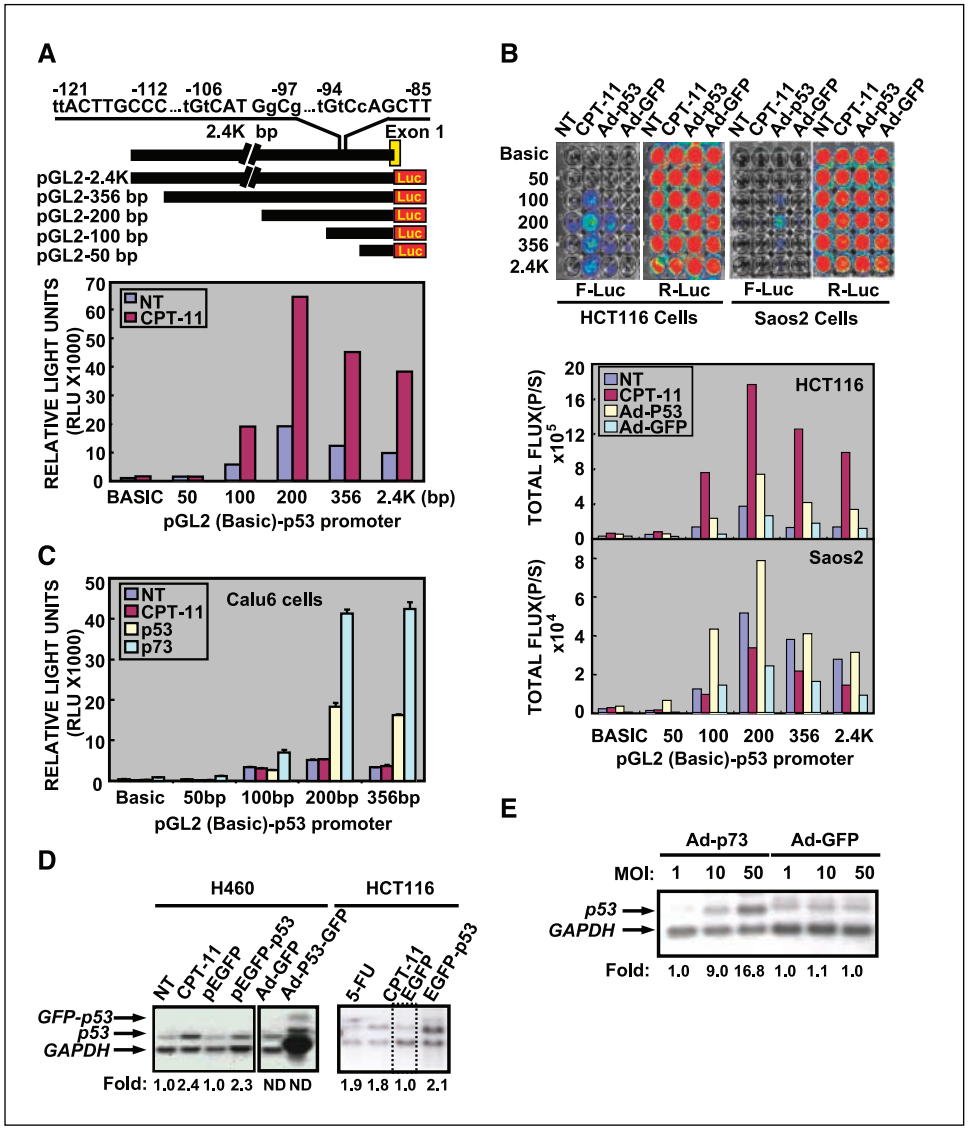

Figure 2. Chemotherapeutic agents induce *p53* promoter activation and p73 or p53 transactivates *p53* expression. *A,* responsiveness to genotoxic stress of the 5′deletion mutants of the *p53* promoter. H460 cells were cotransfected with deletion mutants of the *p53* promoter and a control renilla luciferase reporter plasmid for 24 hours and treated with or without 30 μg/mL CPT-11 for 16 hours. The *p53* promoter-reporter firefly luciferase activity is indicated relative to the activity of renilla luciferase control. Candidate p53 DNA-binding sites are shown on top. *B,* ectopic expression of p73 or p53 transactivates endogenous *p53* expression. HCT116 (wild-type p53) or Saos2 ($p53^{-/-}$) cells were cotransfected with deletion mutants of the *p53* promoter firefly luciferase reporter and a renilla luciferase control plasmid for 24 hours and treated with 30 μg/mL CPT-11 for 16 hours or infected with Ad-GFP or Ad-p53 for 16 hours. D-Luciferin (150 μg/mL) or coelenterazine (10 μg/mL) was added to the cells. The *p53* promoter luciferase activity was determined by the bioluminescence imaging system. F-Luc indicates firefly luciferase activity and R-Luc represents renilla luciferase control. The intensities of bioluminescence signals were measured (histograms). *C,* Calu 6 ($p53^{-/-}$) cells were cotransfected with deletion mutants and renilla luciferase reporter plasmid for 24 hours and treated with 30 μg/mL CPT-11 for 16 hours or infected with Ad-p53 or Ad-p73 for 16 hours. The *p53* promoter reporter luciferase activity is indicated relative to the activity of renilla luciferase control. *D,* HCT116 cells were treated with 30 μg/mL CPT-11 or 25 μg/mL 5-fluorouracil for 16 hours or transfected with p-EGFP-p53 plasmid for 24 hours. H460 cells were treated with 30 μg/mL CPT-11, transfected with p-EGFP-p53 plasmid, or infected with Ad-EGFP-p53 for 24 hours. *E,* U2OS cells were infected with Ad-GFP or Ad-p73α for 24 hours at the indicated multiplicities of infection (*MOI*).

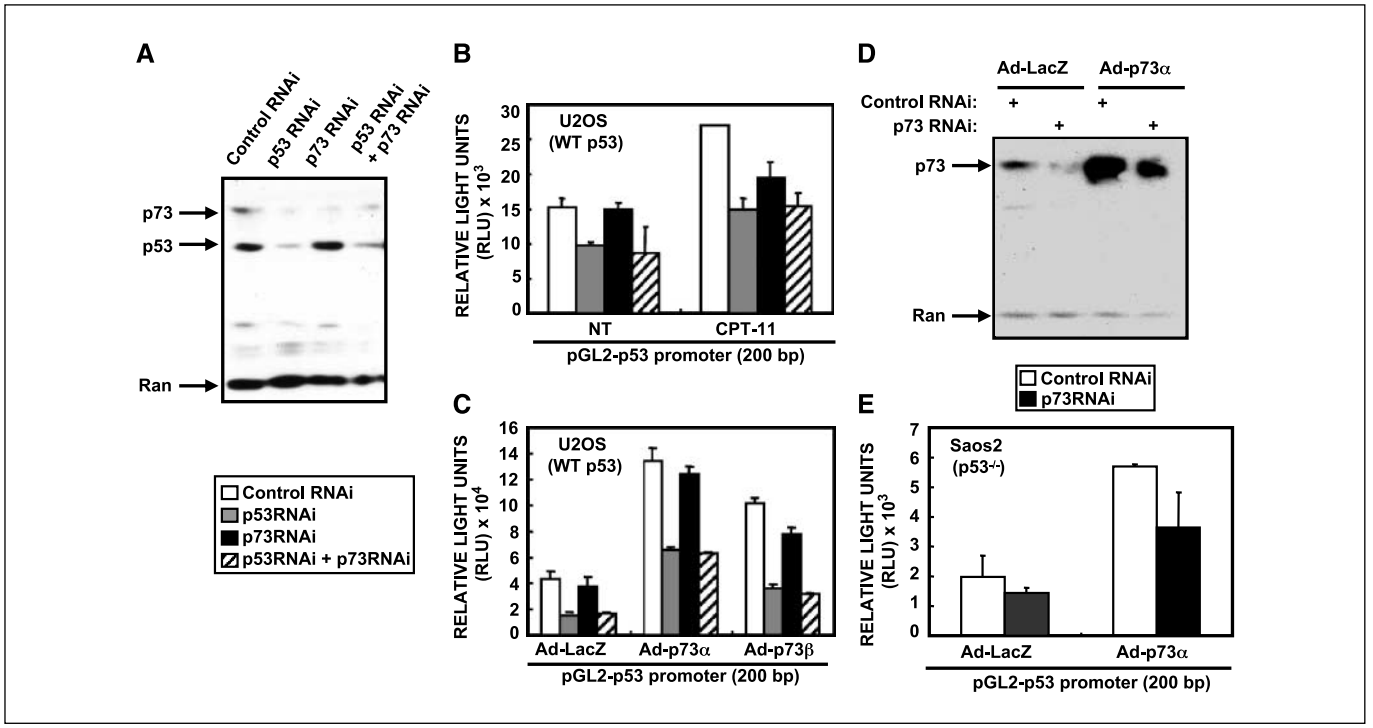

Figure 3. Silencing of endogenous *p53* or p73 suppresses *p53* promoter activation. *A,* U2OS cells were transfected with pSuperior-control, pSuperior-p53, pSuperior-p73, or pSuperior-p53 and pSuperior-p73 for 24 hours. Cell lysates were subjected to Western blot analysis. *B* and *C,* U2OS cells were cotransfected with pSuperior-control, pSuperior-p53, pSuperior-p73, or pSuperior-p53 and pSuperior-p73 with the *p53* promoter firefly luciferase reporter (200 bp) plasmid and a renilla luciferase control plasmid for 24 hours, and then treated with or without 30 μg/mL CPT-11 for 16 hours or infected with Ad-LacZ or Ad-p73α,β for 16 hours. *D,* Saos2 cells were transfected with pSuperior-control or pSuperior-p73 plasmid for 24 hours and infected with Ad-LacZ or Ad-p73α for 16 hours. Cell lysates were analyzed by Western blot. *E,* Saos2 cells were cotransfected with pSuperior-control or pSuperior-p73 with the *p53* promoter firefly luciferase reporter (200-bp fragment of *p53* promoter) plasmid and a renilla luciferase control plasmid for 24 hours and infected with Ad-LacZ or Ad-p73α for 16 hours.

+12) proximal promoter retains full basal activity and shows an enhanced response to the DNA-damaging agent CPT-11 (Fig. 2*A*). However, further deletion to 50 bp (−38 to +12) results in a 4- to 5-fold reduction in the basal promoter activity and complete unresponsiveness to cellular stress (Fig. 2*A*). These results also indicate that the *p53* promoter region from −346 to −188 may contain *cis*-element(s) that may repress the promoter.

Most chemotherapeutic agents stabilize and activate p53 as well as its family member p73. We therefore asked whether chemotherapy-induced *p53* promoter activation is mediated by p53 or p73 using HCT116 (wild-type p53), Saos2 ($p53^{-/-}$), or Calu 6 ($p53^{-/-}$) cells for the analysis. Overexpression of p73 or p53 in these cells led to a significant increase in p53 promoter-reporter luciferase activity. The 200-bp promoter region–driven reporter showed the highest induction, implying that this region contains the necessary element(s) that are responsible for p73- or p53-dependent regulation (Fig. 2*B* and *C*). Interestingly, CPT-11 induced *p53* promoter activation in HCT116 cells (wild-type p53) but less effectively in $p53^{-/-}$ Saos2 or Calu 6 cells, suggesting that p53 or p73 might be critical for CPT-11-mediated *p53* promoter activation (Fig. 2*B* and *C*).

We investigated the effects of p73 or p53 on p53 mRNA levels. Ectopic expression of p53 in H460 or HCT116 cells led to a significant increase in endogenous p53 mRNA (Fig. 2*D*). The size of the exogenous p53-GFP mRNA was distinguishable from that of endogenous p53 mRNA. Infection of U2OS cells with Ad-p73α also dramatically induced p53 mRNA as compared with Ad-GFP infection (Fig. 2*E*). These results suggest that expression of p73 or p53 activates endogenous p53 mRNA expression.

To further study the role of p53 or p73 in the regulation of *p53* transcription, we determined the effect of silencing endogenous p53 or p73 by short hairpin RNA on *p53* promoter activation. p53 or p73 short hairpin RNA effectively knocked down endogenous p53 or p73 protein in wild-type p53–expressing U2OS and $p53^{-/-}$ Saos2 cells (Fig. 3*A* and *D*). Loss of p53 or p73 significantly prevented the activation of the *p53* promoter either in the absence or in response to cellular stresses in U2OS cells (Fig. 3*A-C*). *p53* promoter activation by Ad-p73 is more significantly blocked by p53 RNAi than by p73 RNAi (Fig. 3*B* and *C*). To directly analyze the contribution of p73 to the control of *p53* transcription, we silenced p73 expression in the p53-null Saos2 cells and found that silencing of p73 suppressed *p53* promoter activation (Fig. 3*D* and *E*).

Direct interaction of p73 or p53 protein with the p53 promoter. p53 autoregulation of its transcription has been studied by several different groups, who reported conflicting results. Ginsberg et al. (21) showed that p53 protein down-regulated its transcription. Hudson et al. (22) suggested that p53 autoregulates its transcription indirectly and in a cell type–specific manner. Deffie et al. (23) showed that p53 transactivates its own promoter and identified in the murine *p53* promoter a domain responsive to wild-type, but not mutant, p53 although they did not show the binding of the p53 protein to the promoter *in vivo*. To determine whether p53 is a direct target of transcriptional activation by p73 or p53, we searched for potential p53 responsive elements by aligning the *p53* promoter with the consensus p53 DNA-binding site (24). We found three adjacent potential half-binding site decamers for p53 located

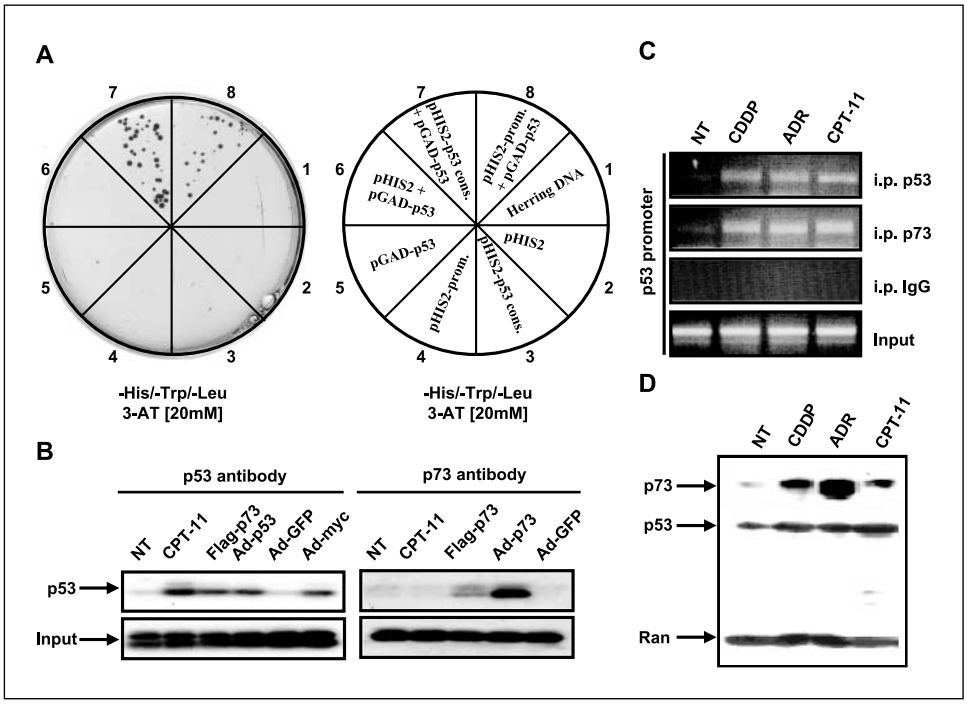

Figure 4. p53 or p73 protein directly binds to the *p53* promoter. *A,* yeast one-hybrid analysis reveals direct binding of p53 to its own promoter. Yeast strain Y187 cells were cotransformed with pGAD-Rec2-p53 and pHIS2-p53-356 bp promoter yeast one-hybrid reporter plasmid (pHIS2-prom.). pHIS2 or p53HIS2 consensus binding sites (*pHIS2-cons.*) reporter plasmids were used as negative or positive controls. The yeast transformants were spread on selective plates containing 20 mmol/L 3-aminotriazole (*3-AT*). *B,* chromatin immunoprecipitation assays reveal direct binding of p53 or p73 protein to the *p53* promoter under cellular stress. The DNA fragments containing the *p53* promoter region were amplified in the samples immunoprecipitated with antibodies against p53 or p73. Immunoprecipitates in the absence of p53 or p73 antibodies were used as a negative control (data not shown). PCR products were subjected to Southern blot analysis with a 356-bp fragment of the *p53* promoter as a probe. *C* and *D,* U2OS cells were treated with 7.5 μg/mL *cis*-diammine-dichloroplatinum (*CDDP*), 0.2 μg/mL Adriamycin, or 30 μg/mL CPT-11 for 20 hours. The DNA fragments containing the *p53* promoter region were amplified in the samples immunoprecipitated with antibodies against p53, p73, or IgG. Cell lysates were analyzed by Western blot.

within positions −121 to −85 upstream of exon 1 in the human *p53* genomic locus. To study whether p53 protein selectively binds to any of these candidate sequences *in vivo*, we first did direct yeast one-hybrid assays. We generated yeast one-hybrid reporter plasmid by cloning the 356-bp *p53* promoter fragment into the pHIS2 reporter plasmid (pHIS2-p53 prom.) and then cotransformed the yeast strain Y187 cells with pHIS2-p53-prom. reporter plasmid and pGAD-Rec2-p53 encoding p53-GAL4 AD fusion. We used pHIS2-p53 cons. reporter plasmid, which carries three tandem copies of a consensus DNA-binding site for p53 as a positive control. The results revealed that the double transformants (pHIS2-p53 prom. and pGAD-Rec2-p53) formed colonies on the selective plates although the size and number of the colonies were relatively smaller and less than those of the positive control transformants (pHIS2-p53 cons. and pGAD-Rec2-p53). However, transformants with pHIS2-p53 cons., pHIS2-p53 prom., or pGAD-Rec2-p53 alone did not form any colonies (Fig. 4*A*). These results provide evidence that p53 protein specifically binds to the *p53* promoter. To determine the physiologic relevance of this finding, we did chromatin immunoprecipitation assays with H460 cells infected with Ad-p53, Ad-c-Myc, or Ad-GFP, or transfected with pFlag-p73, or treated with CPT-11. p53 protein was dramatically stabilized by CPT-11 treatment, c-Myc overexpression, or p73 overexpression (Supplementray Fig. S2). Immunoprecipitation of DNA-protein complexes with antibody against p53 was done on extracts from the treated cells. We measured the abundance of candidate sequences (−40 to −469) within the immunoprecipitated complexes by PCR amplification. The chromatin immunoprecipitation assays indicated that p53 protein reproducibly resides at a DNA fragment containing the candidate p53 DNA-binding sites in H460 cells (Fig. 4*B*) and that the binding of p53 protein to the *p53* promoter is significantly enhanced following cellular stress, which is likely due to the stabilization of p53 protein under these conditions (Fig. 4*B*). Because p73 protein can potentially bind to p53 DNA-binding sequences (25, 26), we then determined whether p73 can also directly interact with the *p53* promoter region. Interestingly, we found that p73 protein selectively associated with the candidate p53 DNA-binding site(s) in the *p53* promoter as revealed by chromatin immunoprecipitation assays in H460 and U2OS cells and that the binding of p73 protein to the *p53* promoter was also enhanced in response to cellular stress (Fig. 4*B-D*). In addition to the direct binding of p73 protein to the p53 promoter, p73 also activates *p53* transcription through stabilization of p53 protein (Supplementray Fig. S2), which in turn activates its own transcription through this positive feedback loop. Taken together, these results suggest that p73 or p53 protein directly binds to the *p53* promoter and therefore can transactivate endogenous p53 expression under either physiologic conditions or in response to cellular stress.

Mutational analysis of the p53 or p73 responsive elements in the genomic *p53* locus. The sequence of the response elements Benoit at al. (27) identified by electrophoretic mobility shift assay (*in vitro* assays) is partially identical with those we found in the present study, although we extended the site and fully characterized the three potential half-binding sites for p53. Our data suggested that there are three potential half-binding sites for p53 located at −121 to −85 upstream of exon 1 (Fig. 5*A*). To examine whether these potential p53-binding sites are responsive to p53- or p73-mediated p53 promoter-reporter activation, we generated a series of mutants for each of these potential p53 response elements predicted to be critical for the p53-binding within the 200-bp (−188 to +12) promoter region. Mutation of (−121) ttACTTGCCC (−112) into ttAATTTCCC led to complete loss of response to p53 or p73 overexpression; however, mutations in the other two p53 half-binding site decamers did not produce any effect on the responsiveness to p53 or p73α overexpression (Fig. 5*A*). We next directly focused on the p53 binding sites located at −121 to −85 and generated mutants bearing mutations

in the conserved nucleotides that are critical for p53 binding. The wild-type p53 binding site reporter showed significant induction on p53 or p73α overexpression but not c-Myc overexpression, showing the specificity of the *p53* DNA-binding site for p53 or p73α (Fig. 5*B, bottom*). The human *p53* promoter region contains a putative E-box motif, located at nucleotides −33 to −38, which is a recognition site for Myc. We found that Myc overexpression resulted in a dramatic increase in the 200-bp promoter reporter luciferase activity, indicating that Myc transactivates *p53* expression (Fig. 5*B, top*). Importantly, mutation of the dinucleotides C and G into A and T in the first half-binding site for p53 resulted in unresponsiveness to p53 or p73α overexpression (Fig. 5*B*). To analyze the physiologic significance of the second and third half-site to p53 promoter activation, we generated a mutant p53 promoter-reporter plasmid bearing mutations in both the second and third half-sites (with the first half-site intact). The results indicate that mutation of both the second and third half-sites partly inhibits the responsiveness to p53 but not to p73 overexpression (Supplementray Fig. S3). Therefore, our data suggest that p53 or p73 regulation of the p53 promoter occurs minimally through the sequence ttACTTGCCC located at −121 to −112 upstream of the *p53* transcription initiation site and that the dinucleotides C and G in the first half-binding site for p53 are essential for p73- or p53-mediated transcriptional regulation of human *p53*. The second and third half-sites might also play a mutually redundant role for p53 protein to recognize the *p53* promoter.

p53-mediated autoregulation of *p53* transcription contributes to DNA damage–induced cell cycle checkpoints. To determine the biological significance of these findings, we investigated whether p53-mediated regulation of *p53* transcription might influence cell cycle checkpoints in response to DNA damage. To this end, we used stable clones harboring p53-inducible gene silencing in which p53 can be conditionally silenced on addition of doxycycline (Supplementray Fig. S4). We first pretreated the cells with CPT-11 for 4 hours and removed the CPT-11 thereafter so that there was no DNA-damaging signal from time 0 to 44 hours in the presence or absence of doxycycline. As shown in Fig. 6, 4 hours of pretreatment of the cells with CPT-11 resulted in a significant increase in p53 protein level in the presence (2-fold; Fig. 6*A, right, second lane*) or absence (2-fold; Fig. 6*A, left, second lane*) of doxycycline. In the absence of doxycycline (Fig. 6*A* and *B, left*), the stabilized p53 protein (2-fold; Fig. 6*A, second lane*) induced by CPT-11 treatment for 4 hours activated p53 transcription, leading to the synthesis of new p53 mRNA (observed at 8-28 hours; Fig. 6*B, lanes 4-6*) and the newly synthesized mRNA resulted in a sustained increase in p53 protein expression (2.8- to 3.2-fold) at later time points (20-44 hours; Fig. 6*A, lanes 5-8*). In the presence of doxycycline (*right*), doxycycline-induced p53 short hairpin RNA suppressed the induction of p53 transcription by stabilized p53 protein (Fig. 6*A, right, second lane*) following CPT-11 treatment for 4 hours and therefore abolished the increase of p53 protein at later time points (20-44 hours; Fig. 6*A, right, lanes 5-8*). Close examination of the cellular response of the CPT-11-treated cells with or without doxycycline by fluorescence-activated cell sorting (FACS) revealed that doxycycline-treated cells exhibited a significantly higher S-phase population accompanied by a slight decrease in both the G_1 and G_2-M phases (from 28 to 44 hours after CPT-11 treatment) as compared with the cells without doxycycline after induction of genotoxic stress. Less cell death was observed in the doxycycline-treated cells at 44 hours after CPT-11 treatment (Fig. 6*C*). Moreover, the inducible silencing of p53 that disrupted the p53-mediated positive feedback loop affected the cell cycle distribution with accelerated DNA synthesis in the doxycycline-treated cells (from 28 to 44 hours) as compared with the cells without doxycycline (Supplementray Fig. S4). It has been known for some time (28, 29) that p53 mRNA levels are tightly regulated during the cell cycle with its transcription being induced before DNA synthesis with peak production at the G_1-S boundary of the cell cycle. However, the mechanisms underlying this regulation are not well defined. Our data are consistent with this observation that p53 mRNA reaches a maximal level when most cells enter S phase (8 hours after CPT-11 treatment) and further

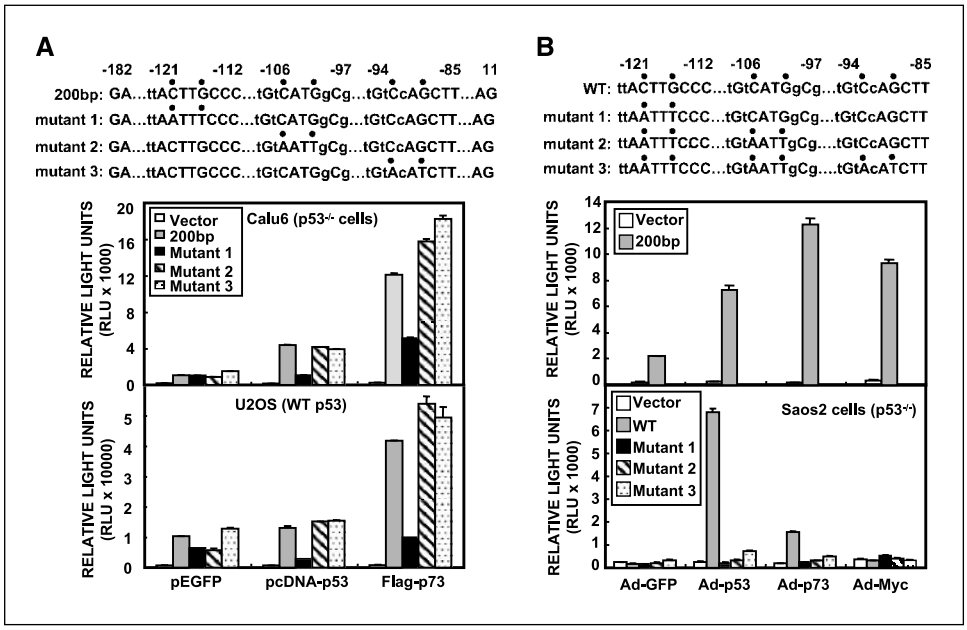

Figure 5. Mutational analysis of the p53 response elements in the *p53* promoter. *A,* U2OS or Calu 6 cells were cotransfected with wild-type (*WT*; 200-bp p53 promoter fragment) or mutant reporter plasmids, with renilla luciferase plasmid and pEGFP, pcDNA-p53, or pFlag-p73 for 24 hours. *B,* Saos2 cells were cotransfected with wild-type (37-bp p53 binding sites) or mutant reporter plasmids with renilla luciferase control plasmid and then infected with Ad-GFP, Ad-p53, Ad-p73α, or Ad-GFP-Myc for 24 hours (*bottom*). Saos2 cells were cotransfected with wild-type (200-bp p53 promoter fragment) reporter plasmid with renilla luciferase control plasmid and then infected with Ad-GFP, Ad-p53, Ad-p73α, or Ad-GFP-Myc for 24 hours (*top*). The promoter reporters and the sequences of the wild-type or mutant p53 promoter reporters are shown on top.

suggest that p53 protein itself is involved in this regulation of *p53* transcription during the cell cycle. Interestingly, our results further reveal that p53 can directly transactivate expression of the *p53* gene itself, which leads to synthesis of new p53 protein, and that the newly synthesized protein can be stabilized on DNA damage or directly activates p53 downstream target genes, thereby sustaining and amplifying stress-induced checkpoint responses. Disruption of p53-mediated autoregulation of *p53* transcription suppresses a p53-mediated apoptotic response to DNA damage and accelerates DNA synthesis within cells (Fig. 6*D*).

Taken together, our current study indicates that DNA-damaging chemotherapeutic agents induce p53 expression that, in part, occurs at the transcriptional level and that p73 or p53 is critical for chemotherapy-induced promoter-reporter activation. p73 or p53 expression transactivates endogenous p53 expression through direct binding of p73 or p53 protein to the *p53* promoter, thereby forming a previously unknown positive feedback loop regulating human p53 expression. Importantly, interference with p53-mediated autoregulation of *p53* transcription results in aberrant cell cycle regulation and abolishes a p53-mediated apoptotic response to DNA damage. These findings have fundamental importance for understanding the regulation and maintenance of DNA damage checkpoint responses. Induction of p53 mRNA can sustain and amplify stress-induced checkpoint responses and may contribute over time to a greater activation of

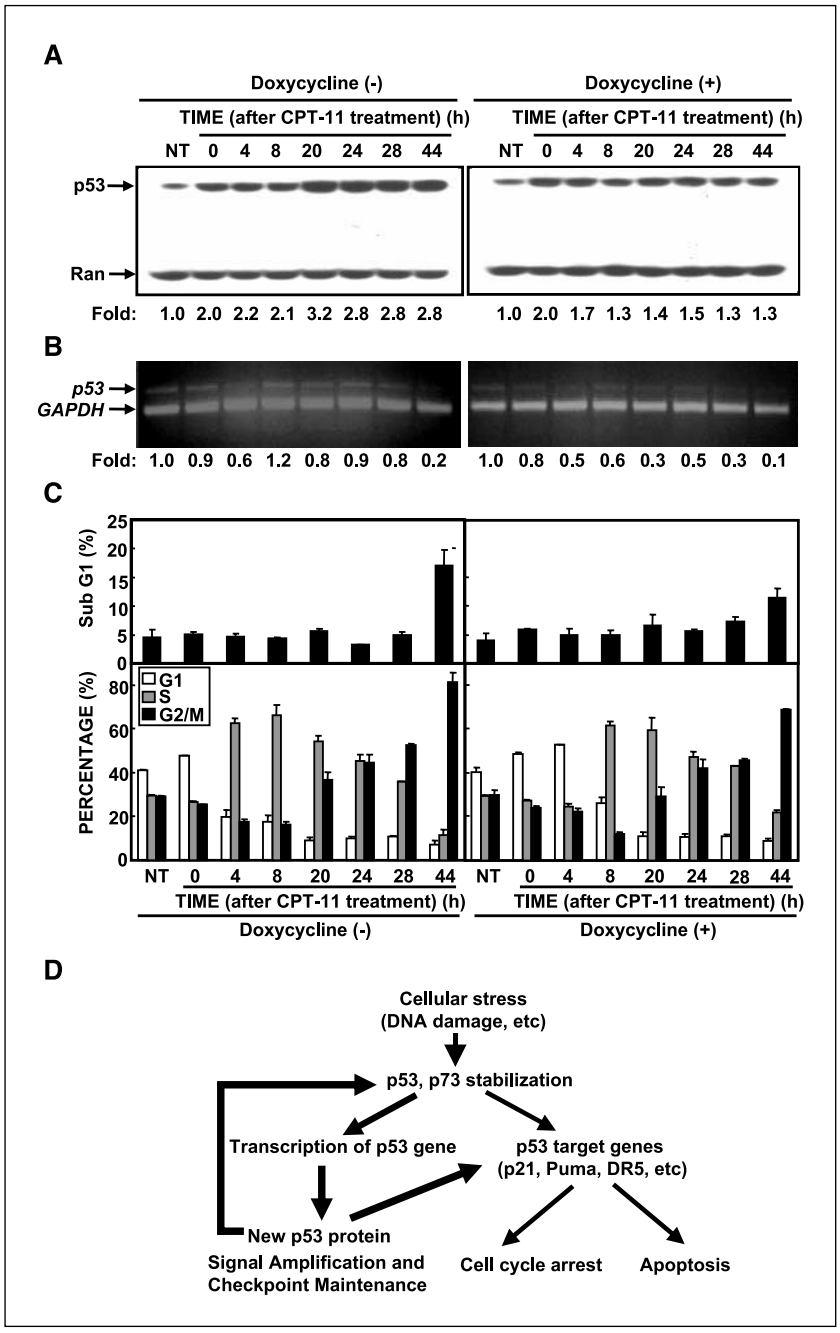

Figure 6. p53-mediated autoregulation of *p53* transcription contributes to cell cycle checkpoints induced by DNA damage. Clones with inducible p53 silencing were treated with 50 μg/mL CPT-11 for 4 hours in the presence or absence of doxycycline (1 μg/mL) and then the medium was replaced with fresh medium with or without doxycycline. The cells were harvested at the indicated time points. *A,* cell lysates at each time point were used for Western blot analysis. *B,* RNAs were subjected to quantitative RT-PCR analysis. *C,* cells were harvested and analyzed by FACS. *D,* schematic model showing the contribution of p53- or p73-mediated regulation of *p53* transcription to the maintenance of cell cycle checkpoints. In response to DNA damage, p53 protein is stabilized and the increase in p53 protein results in an increase of p53-dependent gene transcription, which in turn leads to cell cycle arrest or apoptosis through transactivation of downstream target genes such as *p21, Puma*, and *DR5*, etc. Our results indicate that p53 or p73 protein can also directly transactivate expression of the *p53* gene itself, which leads to synthesis of new p53 protein. The newly synthesized protein can be stabilized on DNA damage or directly activate p53 downstream target genes, thereby sustaining and amplifying stress-induced checkpoint responses. p53- or p73-mediated *p53* transcription may contribute over time to a greater activation of downstream p53 effector genes.

downstream p53 effector genes. We believe that the impact of our study is in modifying the well-established current models that notably lack this level of regulation, which we find is essential in maintaining and ultimately executing checkpoints induced by cellular stress.

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