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p16^{INK4A} enhances the transcriptional and the apoptotic functions of p53 through DNA-dependent interaction

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Abstract

p16^{INK4A} and p53 are two important tumor suppressor proteins that play essential roles during cell proliferation and aging through regulating the expression of several genes. Here, we report that p16^{INK4A} and p53 co-regulate a plethora of transcripts. Furthermore, both proteins colocalize in the nucleus of human primary skin fibroblasts and breast luminal cells, and form a heteromer whose level increases in response to genotoxic stress as well as aging of human fibroblasts and various mouse organs. CDK4 is also present in this heteromeric complex, which is formed only in the presence of DNA both in vitro using pure recombinant proteins and in vivo. We have also shown that p16^{INK4A} enhances the binding efficiency of p53 to its cognate sequence presents in the *CDKN1A* promoter in vitro, and both proteins are present at the promoters of *CDKN1A* and *BAX* in vivo. Importantly, the fourth ankyrin repeat of p16^{INK4A} and the C-terminal domain of p53 were necessary for the physical association between these two proteins. The physiologic importance of this association was revealed by the inability of cancer-associated p16^{INK4A} mutants to interact with p53 and to transactivate the expression of its major targets *CDKN1A* and *BAX* in the p16-defective U2OS cells expressing either wild-type or mutated p16^{INK4A}. Furthermore, the association between p16^{INK4A} and p53 was capital for their nuclear colocalization, the X-ray-dependent induction of p21 and Bax proteins as well as the induction of apoptosis in various types of cells. Together, these results show DNA-dependent physical interaction between p16^{INK4A} and p53.

Keywords

aging; apoptosis; heterocomplex; p16^{INK4A}; p53

1 | INTRODUCTION

p16^{INK4A} (p16) and p53 are two important tumor suppressor proteins that belong to two different, yet overlapping tumor suppressor molecular pathways p16^{INK4A}/pRB/E2F1, and ARF/p53/p21^{WAF1}, which are deregulated in most, if not all, human tumors.¹ p16 and p53 are involved in various physiological processes and are frequently inactivated in many tumor types.^{2,3} Furthermore, p16 and p53 loss cooperate to increase the rate of tumorigenesis and expand the spectrum of tumor types.⁴ Both proteins are major regulators of cell proliferation and are strongly up-regulated during aging, and are both essential for the establishment and maintenance of senescence.^{5,6} In mouse models, deletion of p16 or p53 abrogated senescence and enabled tumor formation.⁷ These findings suggest the presence, at least in some physiological processes, of functional interactions between p16 and p53.

p16 is a cyclin-dependent kinase inhibitor (CDKI) encoded by the *CDKN2A* gene.^{8–10} In response to various stresses p16 causes cell cycle arrest in the G1 phase, by binding to CDK4/6 and preventing their activation by D-type cyclins. As a result, CDK-phosphorylation of the pRB protein and the E2F-mediated transcription of genes required for DNA synthesis are blocked.^{11,12} Additionally, p16 controls the expression of different miRNAs and genes involved in various cellular processes.^{13–15} p16 is also a major player in the cellular response to DNA damage, which leads to cell cycle arrest,¹⁶ senescence,^{17,18} and/or apoptosis.^{19,20}

The transcription factor p53 is a sequence specific DNA binding protein, which promotes the expression of genes involved in cell cycle control, DNA repair, apoptosis, and cellular responses to various stresses.^{21–23} Indeed, p53 plays a major role in the transcriptional response to ionizing radiation (IR) through the up-regulation of a plethora of genes involved in various physiological processes.²⁴ The p53 tetramer binds to its response element and recruits various transcriptional coregulators.²³ p21^{WAF1} (p21), the universal CDKI, is a crucial transcriptional target of p53 and mediator of p53-related cell cycle arrest and senescence.²⁵ In addition to its transactivator function, p53 can also repress several cellular and viral genes.²⁶ p53 activity can be influenced by various binding partners or coactivators to induce differential transactivation of target genes and outcomes.^{23,27} The importance of *TP53* as tumor suppressor is exemplified by the fact that this gene is the most frequently mutated in human cancers.²⁸ Like p16, p53 is also at the crossroad between cancer and aging.²¹

The number of functional similarities between p16 and p53 raised an important question on the possible direct interaction between these two major tumor suppressor proteins. In the present study, we addressed this possibility and have shown that p16 and p53 coregulate the expression of several genes and form a heteromeric complex in a DNA-dependent manner.

2 | MATERIALS AND METHODS

2.1 | Cell lines, cell culture, and chemicals

Mouse embryonic fibroblasts (MEFs) (p16^{+/+} and p16^{-/-}) were a generous gift from Dr. R.A DePinho. Mouse skin fibroblasts (MSFs) (p53^{+/+} and p53^{-/-}),²⁹ the p16 defective

osteosarcoma U2OS cell line and its isogenic derivative EH1 cell line, which express *CDKN2A* under the control of an IPTG inducible promoter, were a generous gift from Dr. G. Peters, and HFSN1 (primary normal human skin fibroblast) were routinely cultured in DMEM-F12 medium supplemented with 10% FCS. MCF10A cells were obtained from ATCC (Manassas, VA) and were cultured following the instructions of the company. Normal human primary luminal cells (NBL-10) were obtained from reduction mammoplasty and were cultured and characterized as previously described.³⁰ All supplements were purchased from Gibco (Grand Island, NY). Cells were maintained at 5% CO₂ and 37°C humidified incubator. Hydrogen Peroxide (H₂O₂) was purchased from Sigma-Aldrich (St Louis, MO).

2.2 | Animals and homogenization of tissues

The breeding, care, and sacrifice of the animals were in accordance with the protocols approved by the Animal Care and Use Committee of the King Faisal Specialist Hospital and Research Centre. Frozen tissues were mechanically homogenized using a mortar and pestle. Three young (1 month) and three old (6 months) female mice (C57BL/6 J) were sacrificed and spleens as well as lungs were extracted, frozen in liquid nitrogen and kept at -80°C. Samples were then solubilized in the lysis buffer containing 9 M urea, 2 M thiourea, 1 mM EDTA, 65 mM DTT, 0.2 mM PMSF, 0.8 mM benzamidine, 25 mM CHAPS, 5% Resolyte (pH 4–8), and 5% Nonidet P40. The mixture was incubated on a shaker at room temperature for 3 h, and then centrifuged at 12 000 rpm for 15 min to remove any insoluble material. Protein concentration was determined using the Bradford method.

2.3 | Transfection

CDKN2A-shRNA expressed in pRNAT-U6/Neo vector (GenScript Corporation) and the corresponding control plasmid were used at 1 µg/mL for transfection using human dermal fibroblast nucleofactor kit (Amaxa Biosystems) following the protocol recommended by the manufacturer.

pIRES-Puro2 encoding FLAG-tagged human p16 wild-type or PLVX-Puro-Flag-tagged mutants,³¹ pC53SN3 encoding wild-type human p53³² or pCMVHup53Cdelta30 encoding mutant p53 (C 30)³³ and their control plasmids were used at 1 µg/mL for transfection using Lipofectamine 2 000 following the manufacturer's instructions (Invitrogen, Carlsbad, CA).

2.4 | Cellular lysate preparation

Cells were washed and scraped in lysis buffer (50 mM Tris.Cl pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% NP40, and protease inhibitor cocktail [Roche]). Lysates were clarified by centrifugation at 14 000 rpm for 30 min. The supernatant was removed, aliquoted and stored at -80°C.

2.5 | Immunoblotting

SDS-PAGE was performed using 12% separating minigels. Equal amounts of protein extract (30 µg) from different samples were placed in boiling water for 5 min in the presence of SDS gel sample buffer (0.5M Tris pH 6.8, 10% glycerol, 10% SDS, 5% 2-mercaptoethanol, 1% bromophenol) and electrophoresed for 2 h at 125 V. After transfer onto polyvinylidene difluoride membrane (PVDF), the membrane was incubated overnight with the appropriate

antibodies. Visualization of the secondary antibody was performed using the enhanced chemiluminescence detection system (Amersham Biosciences, UK). The antibodies directed against β -actin (C-11), GAPDH (FL-335), p53 (DO-1), p53 (R-19), p21 (F5), PCNA (PC10) were purchased from Santa Cruz (Santa Cruz, CA), p16 (BD) was purchased from BD Biosciences (San Jose, CA), Bax, Bcl2 (50E3), and PTEN (138G6) were purchased from Cell Signalling Technology (Danvers, MA).

2.6 | Immunofluorescence microscopy

Cells were fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.2% Triton X-100 for 10 min and quenched in 100 mM glycine for 10 min at room temperature. Cells were then blocked in 10% FCS and incubated with p16 antibody (EPR1473) (Abcam, Cambridge, MA) followed by p53 antibody (1C12) (Cell Signalling Technology). Cells were then washed and incubated with the Alexa Fluor-594 or -488 conjugated antibodies, respectively (Life technologies, Carlsbad, CA). Nuclei were stained with DAPI (Vectror laboratories, Inc) and Images were captured using Fliid Cell Imaging Station (Life technologies).

2.7 | RNA purification, PCR, and quantitative RT-PCR

Total RNA was purified using the RNeasy mini kit (Qiagen, UK) according to the manufacturer's instructions and was treated with RNase-free DNase (Qiagen). 1 μ g RNA was used to synthesize cDNA utilizing Advantage RT for PCR kit (Clontech Laboratories, UK). Quantitative RT-PCR was performed using 4 μ L cDNA mixed with 2 $\times$ FastStart Essential DNA Green qPCR mastermix (Roche, Mannheim, Germany) and 0.3 μ M forward and reverse primers. Amplifications were performed utilizing the LightCycler 96 Real-time PCR detection system (Roche) using the following cycle conditions: 95°C for 10 min (1 cycle); 95°C for 10 s, 59°C for 20 s, 72°C for 30 s (45 cycles). *GAPDH* expression levels were used for normalization, and gene expression differences were calculated using the threshold cycle (Ct). At least three independent experiments were performed and the obtained values were plotted as mean $\pm$ SD. The respective primers were:

CDKN1A: 5'-CAGAGGAGGCGCCAAGACAG-3' (forward) and 5'-CCTGACGGCGGAAACGC-3' (reverse); *CDKN2A*: 5'-CAACGCACC-GAATAGTTACG-3' (forward) and 5'-CAGCTCCTCAGCCAGGTC-3' (reverse); *BAX*: 5'-AGGATGCGTCCACCA AGAAGCT-3' (forward) and 5'-TCCGTGTCCACGTCAGCAATC A-3' (reverse); *GAPDH*: 5'-GAGTCCACTGGCGTCTTC-3' (forward) and 5'-GGGGTGCTAAGCAGTTGGT-3' (reverse); *TP63*: 5'-CAGGAAGACAGAGTGTGCTGGT-3' (forward) and 5'-AATTGGACGGCGGTT CATCCCT-3' (reverse); *TP73*: 5'-CATGGAGACGAGGACACGTACT-3' (forward) and 5'-TGCCGATAGGAGTCCACCAGTG-3' (reverse); *PCNA*: 5'-CAAGTAATGTCGATAAAG AGGAGG-3' (forward) and 5'-GTGTCACCGTTGAAGAGAGTG-3' (reverse); *SLAH1*: 5'-TCTTCCTGGTGTGTTGA CTGG-3' (forward) and 5'-CGATTGCGAAGAAGTCTGGTG (reverse); *HPRT1*: 5'-CATTATGCTGAGGATTTGGAAAGG-3' (forward) and 5'-CTTGAG CACACAGAGGGCTACA-3' (reverse).

2.8 | Immunoprecipitation (IP)

The immunoprecipitation experiments were performed as described previously.³⁴ Cell lysates were prepared from confluent cells, and then centrifuged at 14 000 rpm at 4°C. Subsequently, 300 µg of protein extracts were incubated with 2 µg antibody (mouse IgG1 was used as control) and mixed at 4°C for 4 h. Equal volume of protein A agarose was added per immunoprecipitation and mixed overnight at 4°C. After centrifugation, the pellet was washed five times with lysis buffer and placed in boiling water for 10 min in the presence of SDS gel sample buffer (0.5M Tris pH 6.8, 10% glycerol, 10% SDS, 5% 2-mercaptoethanol, 1% bromophenol), and electrophoresed for 2 h at 125 V.

2.9 | Chromatin immunoprecipitation (ChIP) and Re-ChIP assays

Cells (10⁶) were treated with 2% formaldehyde for 10 min at room temperature to cross-link the transcription factor to DNA. The cross-linking was terminated by addition of glycine (0.125 M). After washing with PBS, cells were collected and re-suspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris, pH 8.1) with protease inhibitors. The sonicated lysate was processed using the ChIP assay kit following the manufacturer instructions (Cell Signaling Technology). ChIP experiments were performed using antibodies against p53 (1C12) (Cell Signaling Technology), p16 (PA1-30670) (Thermo Scientific), IgG, and H3. Input is the supernatant of the negative control (mock), and served as a positive PCR control, which represents the starting genomic DNA that was used for normalization. Immunoprecipitated chromatin was analyzed by PCR and qPCR using *GAPDH* promoter as unlinked locus control. Each experiment was performed three times.

The sequences of the primers used were as follow:

CDKN1A promoter (region -2.2): 5'-AGCAGGCTGTGGCTCT-GATT-3' (forward) and 5'-CAAAATAGCCACCAGCCTCTTCT-3;(reverse); *BAX* promoter 5'-AGGCTGAGACGGGGTTATCT-3' (forward) and 5'-AAAGCTCAGCTCAGAGGCCCAAAAT-3' (reverse); *GAPDH* promoter 5'-TACTAGCGGTTTTACGGGCG-3' (forward) and 5'-TCGAACAGGAGGAGCAGAGAGCGA-3' (reverse). For Re-ChIP experiments, after immunoprecipitation with the first antibody, protein-DNA complexes were eluted from protein G-Agarose beads by incubation for 30 min at 37°C in 50 µL of elution buffer (1× TE, 15% dithiothreitol, and protease inhibitors). The second immunoprecipitation was performed as described above using the ChIP assay kit (Cell Signaling Technology).

2.10 | Electrophoretic mobility shift assay (EMSA)

Complementary oligonucleotides representing the wild-type p53 binding site on the *CDKN1A* promoter (5'-GGCCCCTGGGA-GAGGGTGGGAGGCCTAGAGGAG-3') were designed and purified by HPLC. Nuclear extracts were prepared from approximately 8 × 10⁷ cells. For EMSA, nuclear proteins (3 µg) or human recombinant proteins were pre-incubated for 10 min on ice with 1 µg of poly (dI-dC) in a binding buffer (4% Ficoll, 20 mM HEPES (pH 7.9), 1 mM EDTA, 1 mM DTT, 50 mM KCl). Nuclear proteins were then incubated with biotin-labeled single-stranded oligonucleotide probes (20 fmol) for 20 min at room temperature. Fifty-fold excess unlabeled oligonucleotides were used as cold

competitor to test the specificity of the DNA-protein binding. Reactions were then analyzed on 6% polyacrylamide gels and electroblotted onto a positively charged nylon membrane (Pierce, IL). The membrane was UV-cross-linked and the biotin-end labeled probe was detected with streptavidin-horseradish peroxidase using a luminal enhancer substrate (Lightshift Chemiluminescent EMSA kit; Pierce, IL). Light emission was captured using CCD camera (LAS 4000, GE healthcare Biosciences AB). In super-shift assays the protein was pre-incubated with antibody against p16 (BD), p53 (DO-1), or IgG before adding the probe.

2.11 | Dual-luciferase reporter assay

Cells were plated at 1×10^5 cells/well on six-well plates and transfected with 3 μ g of the Gaussia luciferase/Secreted Alkaline Phosphatase reporter vector containing human *CDKN1A* or *BAX* promoters. A no-promoter containing plasmid was used as a control (GeneCopoeia). Transfection was carried out using Lipofectamin 2 000 as recommended by the manufacturer (Invitrogen). At 24 h post-transfection, cells cultured in fresh media and re-incubated for 72 h. Subsequently, media were collected and used to measure the secreted Gaussia luciferase (GLuc) in 96-well plate in triplicates as recommended by the manufacturer (GeneCopoeia, Rockville, MD). The Gaussia luciferase signal was normalized to the secreted alkaline phosphatase signal for each individual analysis. The mean and SEM were calculated from three wells for the promoter activity and presented as percentage over control.

2.12 | Pure proteins interaction

Recombinant human pure proteins p16 (PO1) and p53 (PO2) from Genecopoeia (UK), CDK4 (PO2) from Abnova (Taiwan), p53-CTD (30 AA) from GenScript (Piscataway, NJ) were incubated, separately or in combination, in the presence or absence of oligonucleotides of the p53 consensus binding site of the *CDKN1A* promoter (20 fmol), in the presence of the binding buffer (0.4 M KCl, 25 mM HEPES, 1 mM EDTA, 1 mM DTT, 1% NP40, 10% Glycerol, 5 mM MgCl₂) at room temperature for 2 h. Proteins were resolved in non-denaturing gel containing 6% polyacrylamide (acrylamide:bisacrylamide, 40:2) and samples were subjected to electrophoresis in 75 mM Tris-glycine buffer. The gel was then fixed in 10% methanol and 7% acetic acid for 30 min at room temperature, and then in 100% SYPRO® Ruby protein gel stain overnight at room temperature with light agitation. The gel was then rinsed in 10% methanol and 7% acetic acid four times (30 min each) at room temperature and scanned using Typhoon scanner.

2.13 | TP53 targets PCR array

Total RNA was purified using the RNeasy mini kit (Qiagen, UK) according to the manufacturer's instructions and was treated with RNase-free DNase (Qiagen). 1 μ g RNA was used to synthesize cDNA utilizing Advantage RT for PCR kit (Clontech Laboratories). Quantitative RT-PCR was performed using human and mouse p53 Signaling pathway RT² profiler PCR array plates (Qiagen, UK). The amplifications were performed using the RT² SYBR Green qPCR mastermix (Qiagen) and the LightCycler 96 Real-time PCR detection system (Roche) using the following cycle conditions: 95°C for 10 min (1 cycle); 95°C for 15 s, 60°C for 60 s (45 cycle). *GAPDH* expression levels were used for normalization, and gene

expression differences were calculated using the threshold cycle (Ct). The obtained values were plotted as mean $\pm$ SD.

2.14 | Sub-cellular fractionation

Nuclear and cytoplasmic extracts were prepared as previously described.¹⁶ Briefly, cytoplasmic fractions were obtained by incubating cells in 200 micro-litter of the hypotonic buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM MgCl₂) supplemented with protease inhibitors cocktail on ice, and lysed by addition of 25 micro-litter of buffer A containing 2.5% Nonidet P-40 plus inhibitors. Nuclei were pelleted (14,000 rpm, 5 min, 4°C), and supernatants (Cytoplasmic extracts) were saved. Nuclear pellets were incubated in the extraction buffer C (20 mM HEPES pH 7.9, 0.45 M NaCl, 5 mM EDTA) plus protease inhibitors for 40 min, and then were centrifuged (10 min, 14 000 rpm, 4°C) and supernatants (nuclear extracts) were saved at -80°C.

2.15 | X-ray treatment and apoptosis analysis by Annexin V/PI flow cytometry

Cells were either mock-treated or challenged with X-rays (50 Gy) using X-rays (4MV) linear accelerator. Detached and adherent cells were then harvested after 72 h, centrifuged and re-suspended in 1 mL phosphate buffered saline (PBS). Cells were then stained by propidium iodide (PI) and Alexa Flour 488 annexin V using Vybrant Apoptosis Assay kit (Molecular Probes, Eugene, OR) following the manufacturer's recommendations. Annexin V-stained cells were analyzed by flow cytometry, measuring the fluorescence emission at 530 and >575 nm. The percentage of cells was determined by the FACScadibur apparatus and the Cell Quest Pro software from Becton Dickinson. For each cell culture three independent experiments were performed using 10⁴ cells in each experiment.

2.16 | Quantification of protein expression level

Protein signal intensity of each band was determined using Image-Quant TL Software (GE Healthcare). Next, dividing the obtained value of each band by the values of the corresponding internal control allowed a correction of the loading differences. The fold of induction/ reduction in the protein levels was determined by dividing the corrected values that corresponded to the treated samples by that of the non-treated one (time 0).

2.17 | Statistical analysis

Statistical analysis was performed using student's *t*-test and *P* values of 0.05 and less were considered as significant.

3 | RESULTS

3.1 | p16 controls the expression of several p53 target genes

We have recently shown that p16 modulates the expression of 2,170 genes, with some are known to be part of the p53 regulon.¹⁴ This prompted us to examine the potential role of p16 in controlling the expression of some p53 target genes. To this end, total RNA was prepared from human skin fibroblast (HFSN1) cells expressing either *CDKN2A*-shRNA (HFSN1.p16sh) or a scrambled sequence (HFSN1. Ctl), and the mRNA levels of various p53

target genes were assessed by qRT-PCR utilizing human p53 Signaling pathway RT² profiler PCR array, which profiles the expression of 84 genes related to p53 mediated signal transduction. Interestingly, the levels of 79 transcripts (94%) were affected by p16 down-regulation. Figure 1A shows 15 of the most affected genes: six were strongly down-regulated, and nine others were up-regulated, while five were not affected in p16-deficient human cells as compared to controls. Similar results were obtained utilizing RNA extracted from p16 knockout (p16^{-/-}) mouse embryonic fibroblasts (MEFs) and their corresponding wild-type (p16^{+/+}) cells (Fig. 1B). While five genes were down-regulated, 10 were up-regulated and five others were not affected in p16^{-/-} as compared to control cells (Fig. 1B). It is noteworthy that 14 genes out of 21 (71.4%) were similarly affected in both human and mouse cells (Fig. 1A,B, bolded genes). However, some p53 target genes, such as *PTEN* and *MYC*^{35,36} were significantly affected by p16 down-regulation in human cells but not in mouse cells, while Hif1- α and Hsp90ab1 are p53 target only in mouse cells (Fig. 1A,B).

To confirm these results, we sought to investigate the effect of p16 on the expression of some of these genes at the protein level. Therefore, cell lysates were prepared from HFSN1.Ctl and HFSN1. p16sh cells as well as mouse p16^{-/-} and p16^{+/+}, and were used for immunoblotting analysis using specific antibodies, while GAPDH was used as internal control. Figure 1C shows that, in both human and mouse cells, p16 deficiency was accompanied by a decrease in the level of Bax, while Bcl-2 was up-regulated and the PCNA level changed only slightly (Fig. 1C). However, while p16 down-regulation decreased the level of PTEN in human cells, the effect was only marginal in p16 knock-out mouse cells (Fig. 1C). These results mirror those obtained at the mRNA level (Fig. 1A).

3.2 | p16 and p53 proteins form a heteromer that includes CDK4

The ability of p16 to modulate the expression of several p53 target genes, prompted us to investigate the possible association between these two proteins. To this end, whole cell extracts were prepared from normal mammary human luminal cells (NBL-10), and anti-p16 and anti-p53 antibodies were used for reciprocal immunoprecipitation (IP) reactions. The co-immunoprecipitated proteins were identified by immunoblotting using specific antibodies. p53 was pulled-down with anti-p16 antibody (Fig. 2A). Likewise, p16 was also pulled-down with anti-p53 antibody, while neither protein was observed in an IP with the anti-IgG antibody. CDK4, a major target and partner of p16, was also pulled-down with anti-p16 and anti-p53 antibodies (Fig. 2A). Since both proteins p16 and p53 are up-regulated during aging, we sought to assess the level of their association in young (PD20) and aged (PD40) normal human primary fibroblast cells (HFSN1). Therefore, reciprocal immunoprecipitation using anti-p16 and anti-p53 antibodies and whole cell extracts prepared from young and old HFSN1 cells was performed. Figure 2B (input) shows strong aging related up-regulation of both p16 and p53. Consequently, the levels of the associated proteins also increased at PD40 as compared to PD20 (Fig. 2B). Thus, not only a p16/p53 heterocomplex is formed in vivo, but also its level increases with cellular aging. A similar age-dependent increased levels of p16 and p53, as well as their complex was also observed in the spleens and lungs of mice (Fig. 2C). Therefore, like the monomers, the level of the p16/p53 heteromer increases with aging in human fibroblasts and various mouse organs.

Next, we investigated the effect of oxidative stress induced by hydrogen peroxidase (H_2O_2) on the levels of p16, p53 and their heterocomplex. To this end, HFSN1 and NBL-10 cells were either sham-treated or challenged with H_2O_2 (200 μ M) for 24 h, and then whole cell lysates were prepared and utilized to assess the levels of p16 and p53 by immunoblotting. Figure 2D shows that the levels of both proteins increased post- H_2O_2 treatment in both fibroblast and luminal cells.

Furthermore, reciprocal immunoprecipitation was performed with anti-p16 and anti-p53 antibodies, and the level of p16 and p53 was assessed by immunoblotting. Figure 2E shows increase in the levels of immunoprecipitated p16 and p53 proteins in cells treated with H_2O_2 as compared to the control ones in both HFSN1 and NBL-10 cells. Thus, like p16 and p53, the level of the p16/p53 heterocomplex increased in response to oxidative damage, and in different human cell types.

The above findings prompted us to examine potential cellular colocalization of p16 and p53 spontaneously and in response to H_2O_2 in both fibroblast and luminal cells. Therefore, HFSN1 and NBL-10 cells were treated as above and specific antibodies were used for immunofluorescence analysis. Figure 2F shows clear colocalization of p16 and p53 in the nucleus of both HFSN1 and NBL-10 cells. Interestingly, upon H_2O_2 treatment the size of the spots corresponding to p16 and p53 decreased but their number increased with significant overlapping between both proteins in HFSN1 and NBL-10 cells. This indicates colocalization of p16 and p53 and H_2O_2 -dependent redistribution of the heterocomplex in the nucleus of both fibroblast and luminal cells.

3.3 | The fourth ankyrin repeat of p16 is required for binding to p53

To determine the p16 domain(s) required for the interaction with p53, we made use of mutants with deletions in each of the four ankyrin repeats and the C-terminal 21 amino acids of the p16 protein,³¹ and investigated their binding to p53 in the p16-defective U2OS cells using immunoprecipitation. Like the wild-type, the mutated protein lacking the C-terminal domain bound to p53 (Fig. 3A). Deletion of the ankyrin repeats 1, 2, and 3 reduced, but allowed, p53 binding (Fig. 3A). However, the loss of the ankyrin repeat four completely abolished p16/p53 interaction (Fig. 3A). The mutated p16 proteins were expressed at levels similar to that of the wild-type protein (Fig. 3A, lower panel), indicating that the sharp decrease in the p53 binding to the mutated p16 protein (p16 AR4) was not due to a decrease in its level.

Several p16 mutations have been reported to be located in the ankyrin repeat four of the protein in different human tumours.³⁷ Therefore, we sought to investigate whether such mutations affect p16 interaction with p53. These mutations were specifically generated in vitro in AR4 of human p16 as previously described³¹ and the plasmids bearing them were introduced into U2OS cells. Cell lysates were immunoprecipitated with anti-p16 antibody and the presence of p53 was assessed by immunoblotting. Similar levels of p16 were pulled-down with the anti-p16 antibody (Fig. 3B). Although, all the AR4 mutants interacted weakly with p53, the S140C, A147G, H142R, and A132P mutants completely lost p53 binding like the AR4 (Fig. 3B). Similar results were obtained when WT as well as mutated p16 were ectopically expressed in the p16-defective mammary epithelial MCF10A cells (Fig. 3C).

Interestingly, while anti-p16 pulled-down CDK4 from cells expressing WT-p16 as well as the mutated forms of the protein, anti-p53 immunoprecipitated CDK4 only in cells wherein p53 interacted with p16 (WT-p16-and p16 AR3-expressing cells) but not in those expressing mutated p16 defective in the association with p53 (p16 AR4 and p16S140C) (Fig. 3C). This indicates that the presence of CDK4 in the heterocomplex is p16-dependent and that the p16 AR3 and AR4 domains are not involved in the interaction between p16 and CDK4.

Next, we examined the effect of the p16 mutations that affect the interaction with p53 on the co-localization of both proteins in MCF10A cells expressing either the control plasmid, or plasmids expressing either WT-p16 or the mutated forms p16 AR4, p16S^{140C} and p16 AR3 by immunofluorescence analysis. Figure 3D shows clear colocalization of p16 and p53 in the nucleus of cells expressing WT-p16, but not in those expressing the mutated forms p16 AR4 and p16S^{140C}. Strikingly, in these cells expressing mutated p16, p16, and p53 were localized in well-defined different spots (Fig. 3D). By contrast, a colocalization was also observed between p16 AR3 and p53, which were shown to interact (Fig. 3D). This indicates that nuclear p16/p53 co-localization is due to their association.

3.4 | The C-terminus of p53 contributes to the formation of the p16/p53 complex

Since, the C-terminal domain of the p53 protein (p53-CTD) facilitates its interaction with other proteins such as MDM2 and BRCA1,^{23,38} we decided to test the role of this domain in the interaction of p53 with p16. To this end, cell lysates were prepared from *p53*^{-/-} MSFs expressing either WT-p53 or p53C 30, a p53 mutant lacking the C-terminal 30 amino acids,³³ and then we investigated their binding to p16. In addition, HFSN1 cells and empty vector transfected MSFs were employed as positive and negative controls, respectively (Fig. 3E). A clear decrease in the levels of p21 (the major p53 target) was noted in p53C 30-expressing MSFs as compared to WT-p53 expressing cells (Fig. 3E).

Next, reciprocal immunoprecipitation was performed with anti-p53 and anti-p16 antibodies, and the presence of p16 and p53 was assessed by immunoblotting (Fig. 3F). While no protein was pulled-down from control cells (empty vector), p16 was pulled-down with anti-p53 antibody from *p53*^{-/-} MSFs expressing WT-p53 but not p53 C30 cells. Similar band was obtained from HFSN1 cells (Fig. 3F). Likewise, p53 was pulled-down with anti-p16 antibody only from *p53*^{-/-} MSFs containing WT-p53, but not from those expressing the p53 C30. Thus, the C-terminal domain of the p53 protein is crucial for its association with p16.

3.5 | p16 enhances the ability of p53 to bind its cognate DNA binding site

Next, we investigated the possible implication of p16 in the binding of p53 to its cognate site on the *CDKN1A* promoter using EMSA. To this end, biotin-labeled oligonucleotides were incubated with nuclear extracts prepared from EH1 cells used as positive control or U2OS cells expressing either the control plasmid, or plasmids expressing either WT-p16 or 2 of its mutated forms p16 AR4 and p16S^{140C}. EH1 cells are U2OS derivative that express ectopic p16 under the control of an IPTG inducible promoter. However, there is low p16 expression in these cells even in absence of IPTG.³⁹ Figure 4A (lane 1) shows that upon electrophoresis, nuclear extracts from control cells enabled the shift of a single low intensity

DNA-protein complex. This band disappeared in absence of NE, or following pre-incubation with 50-fold excess of cold probe used as competitor (Fig. 4A, lanes 6 and 7, respectively), which indicates specific binding to this sequence. Interestingly, the intensity of this band was markedly higher in the presence of WT-p16 reaching a level similar to that obtained with EH1 nuclear extract (Fig. 4A, lanes 2 and 5, respectively). However, the expression of p16^{AR4} or p16S^{140C} had only slight effect as compared to the control (Fig. 4A, lanes 3 and 4, respectively). These data indicates that p16, through its 4th ankyrin repeat, promotes the binding of p53 to its consensus binding site presents in the *CDKN1A* promoter. To further confirm this, we employed pure proteins for the EMSA (Fig. 4B). While no band shift was observed upon adding p16 (1 µg) alone, adding p53 (1 µg) alone resulted in weak binding and shift of the *CDKN1A* oligomer (Fig. 4B, lanes 2 and 3). Importantly, adding p16 to p53 (1 µg each) caused robust binding as compared to p53 protein alone (Fig. 4B, lane 4). Thus, p16 enhances the binding of p53 to its binding site. Using NE from EH1 cells yielded band shift similar to the one obtained with p16 and p53 proteins (Fig. 4B, lane 1). Thus, an efficient DNA-binding of p53 occurs in the presence of p16.

To demonstrate the existence of p16 in the complex that binds the p53 cognate site, the EMSA assay was performed using nuclear extracts from EH1 cells and the *CDKN1A* promoter biotin-labeled oligonucleotides. In addition, specific antibodies against p53 and p16 were separately added to the extracts in order to determine their presence in this complex. As expected, incubation with anti-IgG, a negative control, yielded a band shift pattern similar to that obtained with the nuclear extract (Fig. 4C, lanes 1 and 4). By contrast, incubation of the NE with either anti-p53 or anti-p16 antibodies resulted in a supershift of the band, indicating the presence of both proteins in the DNA binding complex (Fig. 4C, lanes 2 and 3).

3.6 | The formation of the p16/p53 heterocomplex is DNA-dependent

Next, we sought to investigate whether the formation of the p16/p53 complex requires the presence of DNA. To achieve this, cytoplasmic(C) and nuclear (N) cell lysates were prepared from HFSN1 cells, and were either sham-treated or exposed to DNase for 30 min, and then the p53/p16/CDK4 complex was pulled-down using anti-p53 or anti-p16 antibodies (mouse IgG was used as control) and immunoblotting was performed using specific antibodies. Figure 5A shows pull-down of both proteins p16 and p53 in the non-treated cells only from the nuclear fractions, but not from the cytoplasmic ones. CDK4 was pulled-down with anti-p53 antibody only from the non-treated nuclear fraction, while it was associated with p16 in both the cytoplasmic and the nuclear fractions, and the association was not affected by the DNase treatment (Fig. 5A). This indicates that while p16/CDK4 association is DNA-independent and occurs in both the cytoplasm and the nucleus, p16 interacts with p53 only in the nucleus in a DNA-dependent manner.

To further demonstrate the DNA-dependent formation of p16/p53 heterocomplex, we investigated the association between p16 and p53 using pure recombinant proteins under non-denaturing conditions in the presence and absence of DNA. To this end, recombinant p16 and p53 proteins (2 µg each) were incubated in the binding buffer either alone or together in the presence or absence of oligonucleotides of the p53 consensus binding site of

the *CDKN1A* promoter for 2 h, and then the proteins and their corresponding complexes were resolved on 6% native PAGE gel. Figure 5B shows that, in absence of DNA, the combination of both proteins did not yield a higher molecular weight band, but only those corresponding to p16 and p53 (Fig. 5B). On the other hand, adding oligonucleotides containing the *CDKN1A* promoter resulted in the disappearance of the p16 protein and shifted the p53 protein (Fig. 5B). On the other hand, p16 associated with CDK4 in absence of DNA, which was required for the formation of the heterocomplex p53/p16/CDK4 (Fig. 5B). Together, these findings indicate that the formation of the p16/p53 heterocomplex is DNA-dependent.

Since, the p53-CTD is involved for both DNA and protein interactions,^{23,40–42} and the p16/p53 association is DNA-dependent, we asked whether p53-CTD can interact with p16 using the EMSA assay as described above. Figure 5C shows that the p53-CTD interacts with a segment of DNA containing the p53 binding site on the *CDKN1A* promoter, and the corresponding band was super shifted in the presence of recombinant p16, which does not bind DNA. This indicates that the p53-CTD associates with both DNA and p16.

3.7 | p53 binds the *CDKN1A* and *BAX* promoters in a p16-dependent manner

Since, p16 is not a DNA binding protein, it is therefore possible that its interaction with p53 is important for efficient binding of p53 to its cognate binding site on the *CDKN1A* promoter. To test this possibility, we first performed the ChIP assay to study the binding of p53 to the *CDKN1A* promoter and the effect of p16 on this binding in vivo. Chromatin was prepared from U2OS cells containing either the control plasmid, or plasmids expressing WT-p16 or different mutated forms of the protein, and the p53-DNA as well as the H3-DNA complexes were pulled-down using anti-p53 antibody, while anti-IgG was used as negative control. Subsequently, the *CDKN1A* promoter was amplified using PCR and qPCR. The *GAPDH* promoter served as unlinked locus control. A strong amplification of the *CDKN1A* promoter was found when ChIPed with anti-p53 and with anti-H3 antibodies in U2OS cells expressing WT-p16 as compared to control cells. Interestingly, such binding was significantly reduced in cells expressing the mutated p16 proteins (Fig. 6A). The control ChIP with anti-IgG did not yield any product, while the *GAPDH* promoter was amplified only when chromatin was immunoprecipitated with anti-H3 antibody in cells expressing WT-p16 (Fig. 6A). Similar results were obtained when the *BAX* promoter was amplified upon the ChIP reactions (Fig. 6B). Thus, efficient binding of p53 to the *CDKN1A* and *BAX* promoters is p16-dependent.

To confirm the presence of p16 at the promoter of both *CDKN1A* and *BAX*, we performed the same ChIP experiments using anti-p16 antibody for immunoprecipitation. A significant amplification of both promoters was obtained when ChIPed with anti-p16 and with anti-H3 antibodies in U2OS cells expressing WT-p16 as compared to control cells. However, such binding was significantly reduced in cells expressing the mutated p16 proteins (Fig. 6C). This shows the presence of p16 at the promoters of the *CDKN1A* and *BAX* genes. To further confirm this and show the association with p53 we performed ChIP/Re-ChIP experiments using the same cells. Therefore, chromatin was immunoprecipitated with anti-p53, and then was re-ChIPed with anti-p16 antibody, and then both promoters were

amplified using the same controls as described above. Figure 6D shows strong amplification of both promoters in U2OS cells expressing WT-p16 as compared to control cells and cells expressing the mutated p16 proteins. This shows the presence of both proteins p16 and p53 at the *CDKN1A* and *BAX* promoters.

3.8 | The expression of the p53 target genes *CDKN2A* and *BAX* is p16-dependent

To further confirm the physical association between p16 and p53, the *CDKN1A* and *BAX* promoter sequences bearing wild-type p53 binding site were inserted independently upstream of a Gaussia Luciferase/Secreted alkaline phosphatase (SeAP) reporter vector (Fig. 7A, upper panel); and were introduced into U2OS cells along with either a control plasmid, or plasmids expressing WT-p16 or the mutated protein p16 AR4. The reporter activity was significantly enhanced in U2OS cells expressing WT-p16 as compared to the control cells. Interestingly, this effect was significantly reduced ($P = 0.000125$) in cells expressing p16 AR4 (Fig. 7A, lower panel). Thus, p16 promotes p53-dependent transactivation of the *CDKN1A* and *BAX* promoters.

We have next investigated the effect of the deletion of the p16 ankyrin repeat four on the expression of the endogenous *CDKN1A* and *BAX* mRNAs using qRT-PCR in U2OS cells expressing either the control plasmid or those expressing either WT-p16 or p16 AR4. While the ectopic expression of WT-p16 strongly increased the level of the *CDKN1A* and *BAX* mRNAs, the presence of the mutated protein p16 AR4 did not affect the level of both mRNAs as compared to control cells (Fig. 7B). Similar results were obtained for the *TP63* and *TP73* mRNAs (Fig. 7B). However, ectopic expression of WT-p16 or the mutated forms of the protein (p16 AR4 or p16S^{140C}) did not affect the level of *PCNA*, *SIAH1*, and *HPRT1* mRNAs as compared to control cells. Therefore, the 4th ankyrin repeat of p16 is necessary for the expression of several p53 target genes. This effect was confirmed at the protein level (Fig. 7C). Indeed, the level of the p21 and Bax proteins was affected by various p16 mutations as compared to WT-p16, while the level of the p53 protein changed only slightly (Fig. 7C). Interestingly, the presence of p16 AR4, p16S^{140C}, p16H^{142R}, or p16A^{132P} mutations abolished p21 expression (Fig. 7C).

3.9 | p16/p53 heterocomplex is important for the induction of p21, Bax, and apoptosis in response to X-rays

p53 plays major roles in the cellular response to ionizing radiation, such as p21 up-regulation and apoptosis.^{43–45} The fact that p53 forms a DNA-dependent heterocomplex with p16 prompted us to investigate the possible implication of p16 in these responses. To this end, MCF10A cells expressing either a control plasmid, or plasmids expressing WT-p16, the mutated protein p16 AR4, p16S^{140C}, or p16 AR3 were either mock-treated or challenged with X-rays (50 Gy) and re-incubated for 72 h. Subsequently, cells were collected and cell death was assessed using the annexin V/PI- flow cytometric analysis. Figure 8A shows that mainly apoptosis was induced, and that the proportion of apoptosis reached 29.5% in cells expressing WT-p16, while it was only marginal in cells expressing the control plasmid or p16 AR4 and p16S^{140C} mutated proteins. Interestingly, the proportion of X-ray-induced apoptosis in cells expressing p16 AR3, which interacts with p53, was similar (25.8%) to the positive control cells (Fig. 8A). This indicates that the

formation of the p16/p53 heterocomplex is important for the induction of apoptosis in response to ionizing radiation.

Since, we have shown that the expression of p53 targets, p21, and Bax, is p16-dependent, we sought to investigate the implication of p16 in their up-regulation in response to X-rays. Therefore, MCF10A cells transformed with the plasmids described above were either mock-treated or challenged with X-rays (5 Gy) and re-incubated for 3 and 6 h. Subsequently, cells were collected and the expression levels of the p21 and Bax proteins were assessed by immunoblotting. Figure 8B shows that treatment with x-rays increased the level of the p21 and Bax proteins in WT-p16- and p16^{AR3}-expressing cells but not in control cells, p16^{AR4}- and p16S^{140C}-expressing cells. On the other hand, the Bcl2 level changed only slightly in all treated cells (Fig. 8B). This resulted in fourfold and threefold increase in the Bax/Bcl-2 ratio as compared to control cells at 6 h post-treatment in WT-p16- and p16^{AR3}-expressing cells, respectively (Fig. 8C). On the other hand, the level of the Bax/Bcl-2 ratio changed only marginally in p16^{AR4}- and p16S^{140C}-expressing cells (Fig. 8C). This indicates that X-rays up-regulate p21 and Bax only in cells wherein p16 and p53 formed a heterocomplex.

4 | DISCUSSION

p16 and p53 are two master tumor suppressor proteins that belong to two major age/cancer-related pathways: p16/pRB/E2F1 and ARF/ p53/p21, which interact at several points and cross-regulate each other, but can also act independently.^{46,47} The present findings show another layer of cross-talk between these two aging and tumor suppressor genes and pathways. Indeed, p16 and p53 coregulate the expression of several genes. We have first shown that the mRNA levels of several well-known p53 target genes are also modulated in a p16-dependent manner in both human and mouse cells. Most of these genes are regulators of cell cycle such as *CDKN1A*, *E2F1* and *RBI*, and apoptosis (*BAX* and *BCL-2*). Interestingly, although most of these genes (71.4%) were similarly affected by p16 down-regulation in both human and mouse cells, some of them such as PTEN, were affected only in human cells at both the mRNA and protein levels. This coregulation of many genes by p16 and p53 could be mediated through independent effectors and/or via direct interaction between both proteins. Thereby, we investigated the second possibility, and have found that p16 and p53 form a heterocomplex that also contains CDK4, both in vitro using recombinant proteins and in vivo. Interestingly, like the monomers, the level of the heteromer p16/p53 increased in aging human fibroblasts and also in various mouse organs as well as in response to H₂O₂. This association between p16 and p53 was further confirmed by showing that both proteins colocalize in the nucleus of both fibroblast and luminal cells. This colocalization was also present following genotoxic stress, which generated different p16/p53 foci. However, no colocalization was observed in the presence of mutated p16, which is defective in the association with p53.

The association between p16 and p53 is mediated through the fourth ankyrin repeat of the p16 protein and the C-terminal region of the p53 protein. The C-terminal domain of p53 includes the tetramerization domain and plays a key role in regulating the specific interactions of p53 with target binding sites. Indeed, a number of viral and cellular proteins

have been shown to bind to the CTD of p53, including p18, p52, YB1, and BRCA1.²³ These cofactors selectively modify the transcriptional program of p53 and its interaction with its cognate sites. Indeed, p18 binds to p53 and enhances the transcription of *NOXA*, but not *BAX*, *CDKN1A*, or *PUMA*, leading to an increase in the apoptotic response.⁴⁸ On the other hand, by interacting with the CTD, BRCA1 selectively coactivates p53 toward genes that direct DNA repair and cell cycle arrest but not toward those involved in apoptosis.⁴⁹ Similarly, p16 controls the expression of several p53 targets but not all. Most of the cancer-related p16 mutations occur in the 4th ankyrin repeat,³⁷ which is also important for the association of p16 with Sp1¹⁵ and GRIM-19.³¹

Importantly, the p16/p53 heteromeric complex is formed only in the presence of DNA, both in vitro using recombinant pure proteins and in the nucleus but not in the cytoplasm of human cells. However, the heterocomplex p16/CDK4 is formed in absence of DNA.

Notably, the p53-CTD is also involved in site-specific DNA binding and promotes structural changes within the central DNA binding domain.^{23,40–42} Therefore, we made use of synthesized p53-CTD, and have shown that this polymer of 30 amino acids binds DNA and is sufficient to associate with p16. Interestingly, like p53, the p53 CTD interacted with p16 only in the presence of DNA, which clearly shows that this p53 domain is indeed critical for the binding of p53 to both DNA and p16.

We have also shown that p16/p53 interaction is required for efficient p53 binding to the *CDKN1A* and *BAX* promoters and the transactivation of these genes as well as their up-regulation upon cellular exposure to ionizing radiation. Indeed, while X-rays up-regulated the *CDKN1A* and *BAX* genes in cells expressing WT-p16 or p16-AR3, which interact with p53, DNA damage did not up-regulate both p53 target genes in cells expressing p16-AR4 or p16-S140C, which do not associate with p53. This suggests the importance of p16 in X-ray-dependent induction of the *CDKN2A* and *BAX* genes. Consequently, the p16/p53 association was also important for X-ray-induced apoptosis through increase in the BAX/Bcl-2 ratio.

The formation of the p16/p53 heterocomplex concords well with the functional similarities of these two proteins. Indeed, the level of both proteins increases during aging, senescence as well as in response to different cytotoxic agents such as UV light,^{50–53} H₂O₂ (Fig. 2D), as well as natural molecules such as curcumin.^{54,55} Furthermore, we have recently shown that the levels of p16 and p53 as well as their common target p21 are reduced in breast cancer-associated fibroblasts as compared to their adjacent counterparts from histologically normal tissues.^{56,57} Further investigations showed that p16 and p53 have non-cell-autonomous tumor suppressor functions in stromal fibroblasts.^{56,58,59} In another recent study, we have reported that both p16 and p53 are down-regulated in breast stromal fibroblasts when exposed to the cytokine IL-6.⁶⁰ Additionally, both proteins act as barrier to induced pluripotent stem cell reprogramming.^{61,62}

Together, these results clearly show DNA-dependent formation of p16/p53 heterocomplex, which transactivates several genes implicated in various physiological functions. These include apoptosis, aging/senescence, and may also include other p53-related functions such

as cell cycle arrest and DNA repair. This indicates the formation of a potent tumor suppressor complex with various anti-cancer activities, which explains the high frequency of mutations in these two genes in different types of human cancers. However, the present findings do not exclude that p16 and p53 may also act separately in these important processes via activating some of their specific targets by association with other partners.

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Abbreviations:

ATCC	American Type Culture Collection
ChIP	chromatin immunoprecipitation
DMSO	Dimethyl Sulfoxide
EMSA	Electrophoretic mobility shift assay
EMT	epithelial to-mesenchymal transition
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
PBS	phosphate buffered saline
RT-PCR	reverse transcriptase-polymerase chain reaction

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FIGURE 1.

p16 controls the expression of various p53 targets. Total RNA was prepared from the indicated cells, and then was amplified by qRT-PCR utilizing *TP53* targets PCR arrays. (A) Human *TP53* targets. (B) Mouse *TP53* targets. Error bars represent means $\pm$ SD, values of three different experiments. * $P = 0.000963$ of HFSN1.p16sh as compared to HFSN1.Ctl and ** $P = 3.3977 \times 10^{-5}$ of p16^{-/-} as compared to p16^{+/+}. (C) Whole cell lysates were prepared from the indicated cells and used for immunoblotting utilizing antibodies against the indicated proteins

FIGURE 2.

p16 and p53 are physically associated. Whole cell lysates were prepared from the indicated cells (A, B) and from organs obtained from young (Y) and old (O) mice (C) and utilized for immunoprecipitation (IP), and then immunoblotting was performed using antibodies against the indicated proteins. (D, E) Whole cell lysates were prepared from the indicated cells either sham-treated or challenged with H₂O₂ (200 μ M) for 24 h and utilized for immunoblotting (D) and immunoprecipitation (E). (F) Immunofluorescence analysis utilizing specific antibodies against the p16 and p53 proteins, scale bars represent 25 μ m

FIGURE 3.

The p16 fourth ankyrin repeat and the p53 C-terminal domain are required for the association between p16 and p53. (A [upper panel], B, C, F) Whole cell lysates were prepared from cells expressing the indicated constructs, and utilized for immunoprecipitation, and then immunoblotting was performed using antibodies against the indicated proteins. (A [lower panel], E) Whole cell lysates were prepared from the indicated cells expressing the indicated constructs, and then utilized for immunoblotting using specific antibodies against the indicated proteins. (D) Immunofluorescence analysis utilizing specific antibodies against the p16 and p53 proteins, scale bars represent 25 μ m

FIGURE 4. p53 binds the *CDKN1A* promoter in a p16-dependent manner in vitro. (A) Nuclear extracts (NE) from EH1 and U2OS cells expressing the indicated constructs were incubated with biotin-labeled p53 consensus binding site of the *CDKN1A* promoter, and then the EMSA assay was performed. (B) NE from EH1 cells or pure proteins (1 µg) in the indicated combinations were incubated with biotin-labeled oligonucleotides of the p53 consensus binding site of the *CDKN1A* promoter, and then the EMSA assay was performed. (C) NE from EH1 cells were pre-incubated with the indicated antibodies (1 µg) for 1 h before adding the biotin-labeled oligonucleotides of the p53 consensus-binding site of the *CDKN1A* promoter, and then the EMSA assay was performed

FIGURE 5.

The formation of the p16/p53 heterocomplex is DNA-dependent. (A) Cytoplasmic (C) and nuclear (N) cell lysates were prepared from HFSN1 cells, and were either sham-treated (-) or exposed to DNase (+) for 30 min, and then the p53/p16/CDK4 complex was pulled down using anti-p53 or anti-p16 antibodies (mouse IgG was used as control) and immunoblotting was performed using specific antibodies against the indicated proteins. (B) Pure proteins (2 μ g) were incubated at room temperature for 2 h in the presence or absence of oligonucleotides corresponding to the *CDKN1A* promoter that contains the p53 binding site, and then were resolved in 6% non-denaturing polyacrylamide gel. The gel was then stained with SYPRO® Ruby protein gel stain and scanned using a Typhoon scanner. (C) p16 and the p53-CTD (2 μ g) were incubated separately or in combination as indicated with biotin-labeled oligonucleotides of the p53 consensus binding site of the *CDKN1A* promoter, and then the EMSA assay was performed

FIGURE 6.

p53 binds the *CDKN1A* and *BAX* promoters in a p16-dependent manner in vivo. (A-C) ChIP assay: chromatin was purified from the indicated cells, and then immunoprecipitated using the indicated antibodies. Subsequently, *CDKN1A* or *BAX* promoters bearing the p53-binding site were amplified by PCR (A, upper panel) and qPCR (A, lower panel, B and C) using specific primers. The *GAPDH* promoter was used as unlinked locus control, and the abundance of the promoters was plotted relative to *GAPDH* after normalization of each sample by its own input. These experiments were performed three times, error bars represent

means $\pm$ S.D (*, $P=0.000264$). (D) The binding of p53 to the *CDKN1A* or *BAX* promoters was analyzed in the indicated cells by ChIP-Re-ChIP experiments with anti-p53 antibody for the first ChIP and anti-p16 antibody for the second ChIP

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FIGURE 7.

p16 controls the expression of *CDKN1A* and *BAX*. (A) Upper panel, schematic representation of the luciferase reporter vector. Lower panel (histogram), U2OS cells expressing the indicated constructs were stably transfected with a luciferase reporter vector bearing either the *CDKN1A* or *BAX* promoter segment containing the p53 binding site. The reporter activity was assessed at 72 h post-transfection. Data were presented as % relative reporter activity as compared to the negative control cells. Error bars indicate mean $\pm$ SD of three independent experiments (*, $P = 0.000125$). (B) Total RNA was prepared from U2OS cells expressing the indicated constructs, and then the indicated genes were amplified by qRT-PCR using *GAPDH* as internal control. Error bars indicate mean $\pm$ SD of three independent experiments. (C) Whole cell lysates were prepared from U2OS cells expressing the indicated constructs, and utilized for immunoblotting using antibodies against the indicated proteins

FIGURE 8.

Role of p16 in p53-related cellular responses to X-rays. (A) MCF10A cells transfected with the indicated constructs were either mock-treated or challenged with X-rays (50 Gys), and then re-incubated for 72 h. Apoptosis was analyzed by annexin V/PI flow cytometry, and the proportions of early and late apoptosis are shown. (B) MCF10A cells were either mock-treated or exposed to x-rays (5 Gys), and then re-incubated for the indicated periods of time, before being harvested and proteins extracted. 30 μ g of proteins were used for immunoblotting using antibodies against the indicated proteins. (C) Graph showing the levels of Bax/Bcl-2 ratio after treatment with X-rays. Error bars represent standard deviation of at least three different experiments