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DEC1, a Basic Helix-Loop-Helix Transcription Factor and a Novel Target Gene of the p53 Family, Mediates p53-dependent Premature Senescence*

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Cellular senescence plays an important role in tumor suppression. p53 tumor suppressor has been reported to be crucial in cellular senescence. However, the underlying mechanism is poorly understood. In this regard, a cDNA microarray assay was performed to identify p53 targets involved in senescence. Among the many candidates is DEC1, a basic helix-loop-helix transcription factor that has been recently shown to be up-regulated in K-*ras*-induced premature senescence. However, it is not clear whether DEC1 is capable of inducing senescence. Here, we found that *DEC1* is a novel target gene of the p53 family and mediates p53-dependent premature senescence. Specifically, we showed that DEC1 is induced by the p53 family and DNA damage in a p53-dependent manner. We also found that the p53 family proteins bind to, and activate, the promoter of the *DEC1* gene. In addition, we showed that overexpression of DEC1 induces G_1 arrest and promotes senescence. Moreover, we found that targeting endogenous DEC1 attenuates p53-mediated premature senescence in response to DNA damage. Furthermore, overexpression of DEC1 induces cellular senescence in p53-knockdown cells, albeit to a lesser extent. Finally, we showed that DEC1-induced senescence is p21-independent. Taken together, our data provided strong evidence that DEC1 is one of the effectors downstream of p53 to promote premature senescence.

The p53 protein has emerged as a key tumor suppressor at the crossroads of cellular stress-response pathways. In response to a stress signal, such as DNA damage, hypoxia, or activated oncogenes, p53 is activated and functions as a sequence-specific transcription factor regulating a plethora of downstream target genes, which mediate various p53 functions, such as cell cycle arrest, apoptosis, and senescence (1, 2). However, although many target genes have been identified, those involved in p53-dependent cellular senescence are still poorly understood (3). Thus, identification of novel p53 targets involved in senescence is of great interest because cellular senescence may be as important as apoptosis in mediating p53-dependent tumor suppression (4).

Cellular senescence was first described as "replicative senescence" because of a limited life span of human diploid fibroblasts *in vitro* (5), which is triggered by DNA damage signals originating from progressive telomere shortening during cell divisions (6). Senescent cells are characterized by enlarged cell size, flattened morphology, inability to synthesize DNA, and expression of the biomarker, senescence-associated (SA)[2] β-galactosidase (7). Recent studies have shown that various stress signals, such as aberrant oncogene activity (8) and cancer chemotherapeutic drugs (9, 10), are able to initiate senescence-like phenotypes ("premature senescence"). It has been shown that cellular senescence utilizes both p53 and p16 pathways in human cells (8, 11). p53 up-regulates p21, a pleiotropic inhibitor of cyclin/cyclin-dependent kinases, which initiates growth arrest by preventing pRb phosphorylation by cyclin-dependent kinases. In contrast, p16 specifically inhibits cyclin-dependent kinase 4/6 to prevent pRb phosphorylation (12). In addition, a recent report showed that p53 selectively cooperates with p130, a member of the pRb family, to induce premature senescence when the p16/pRb pathway is disrupted (13). Moreover, DNA damage promotes cancer cell senescence primarily through p130 (14). Interestingly, lack of p53 or p21 diminishes but does not abrogate DNA damage-induced premature senescence in tumor cells (15), which suggests that senescence can occur through a p53-independent mechanism or an unknown p53 target gene.

DEC1 (differentiated embryo-chondrocyte expressed gene 1), also called STRA13 (stimulated with retinoic acid 13) in mouse and SHARP2 (enhancer of split and hairy related protein 2) in rat, along with DEC2, belongs to a new subfamily of basic helix-loop-helix (bHLH) transcription factors (16). DEC1 functions as a transcription repressor by directly binding to class B E-boxes (17) by interacting with components of the basal transcription machinery, such as TFIIB, TBP, and TFIID (18, 19), or by recruiting a histone deacetylase corepressor com-

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[2] The abbreviations used are: SA, senescence-associated; HA, hemagglutinin; siRNA, small interfering RNA; nt, nucleotide; ChIP, chromatin immunoprecipitation; p53-RE, p53-responsive element; bHLH, basic helix-loop-helix; BrdUrd, bromodeoxyuridine; PI, propidium iodide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; KD, knockdown; pRb, retinoblastom protein.

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plex (20). Interestingly, DEC1 is implicated in cell cycle regulation, differentiation, and apoptosis in response to various extracellular stimuli, including hypoxia, serum starvation, and retinoid acid (16). Indeed, overexpression of DEC1 inhibits cell proliferation in multiple cell types, such as NIH3T3 (20), HEK-293T (21), and HaCat cells (18). However, the mechanism by which DEC1 regulates cell proliferation is not clear. Furthermore, a recent report shows that premature senescence induced by oncogene K-*ras*V12 correlates with DEC1 up-regulation (22), but it is not clear whether DEC1 is capable of inducing senescence.

In this study, we identified *DEC1* as a direct target of the p53 family. We found that DEC1 is induced by p53 family proteins and DNA damage in a p53-dependent manner. In addition, we identified a potential p53-binding site in the promoter of the *DEC1* gene. Moreover, we found that overexpression of DEC1 alone elicits premature senescence, and knockdown of DEC1 attenuates DNA damage-induced premature senescence. Furthermore, we found that overexpression of DEC1 is able to initiate cellular senescence in p53-knockdown cells albeit to a lesser extent, and DEC1-induced senescence is p21-independent. Taken together, our data strongly indicate that DEC1 is one of the mediators downstream of p53 to promote premature senescence.

EXPERIMENTAL PROCEDURES

Plasmids—FLAG-tagged wild-type DEC1 and untagged mutant DEC1 cDNAs in pCMV and pcDNA4 expression vectors were described previously (17, 21). To generate untagged wild-type DEC1 in pcDNA4 for tetracycline-inducible expression (Invitrogen), the cDNA fragment was amplified from FLAG-tagged wild-type DEC1 cDNA (17) with forward primer, 5′-AGGAATTCACCATGGAGCGGATCCCCAGCG-3′, and reverse primer, 5′-AGTCTAGAAGGAAGGAAAGCAAAGCAG-3′. To generate a construct for the inducible expression of DEC1 siRNA, two oligonucleotides, 5′-GATCCCC**GCACTAACAAACCTAATTG**TTCAAGAGA**CAATTAGGTTTGTTAGTGC**TTTTTGGAAA-3′ and 5′-AGCTTTTCCAAAAA**GCACTAACAAACCTAATTG**TCTCTTGAA**CAATTAGGTTTGTTAGTGC**GGG-3′, were designed to target the *DEC1* fourth exon (in boldface). The oligonucleotides were annealed and cloned into pBabe-H1, a pol III promoter-driven short hairpin RNA expression vector with a tetracycline operator sequence inserted before the transcriptional starting site (23). The resulting vector was designated pBabe-H1-siDEC1. To generate a construct that stably expresses p21 siRNA, one pair of oligonucleotides with the siRNA targeting region as shown in boldface, sense, 5′-TCGAGGTCC**GCCTCCTCATCCCGTGTTC**TTCAAGAGA**GAACACGGGATGAGGAGGC**TTTTTG-3′, and antisense, 5′-GATCCAAAAA**GCCTCCTCATCCCGTGTTC**TCTCTTGAA**GAACACGGGATGAGGAGGC**GGACC-3′, were annealed and cloned into pBabe-U6 at BamHI and XhoI sites, a pol III promoter-driven vector as described previously (24). The resulting vector was named pBabe-U6-sip21. The construct expressing p53 siRNA was described previously (25).

To generate a luciferase reporter under the control of the *DEC1* promoter (nt −4468 to +170), two genomic DNA fragments were amplified from MCF7 cells and ligated together through a common EcoRV site. The first pair of primers are as follows: forward primer, DEC1-KpnI-4468 (5′-ATGGTACCCAGGCTGGAGTACAGTGGCATGATC-3′), and reverse primer, DEC1-EcoRV-As (5′-ACGCCCACAACTTGCTTGCTCAGATATCAC-3′). The second pair of primers are as follows: forward primer, DEC1-EcoRV-S (5′-AGTGATATCTGAGCAAGCAAGTTGTGGGCATG-3′), and reverse primer, DEC1-XhoI (5′-AACTCGAGCCGCAGATGTTCCTCTGAGTCTGAG-3′). To generate a *DEC1* promoter lacking the potential p53-RE, a fragment from nt −2343 to +170 was amplified with forward primer, DEC1-KpnI-2343 (5′-TTGGTACCCACACAATGAAGCAGGTCGCCC-3′), and reverse primer, DEC1-XhoI as shown above.

Cell Lines—MCF7, RKO, MCF7-p53-KD, and RKO-p53-KD were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C with 5% CO_2. H1299 cell lines that inducibly express p53 family proteins were described previously (26–28). MCF7-p53-KD and RKO-p53-KD are derivatives of MCF7 and RKO, respectively, in which p53 was stably knocked down by RNA interference. MCF7-TR-7, which expresses the tetracycline repressor, was generated in our laboratory. To generate cell lines that inducibly express wild-type or various mutant DEC1 proteins, MCF7-TR-7 cells were transfected with pcDNA4-DEC1, pcDNA4-DEC1-M, or pcDNA4-DEC1-R58P and selected with medium containing 200 μg/ml Zeocin. To generate cell lines in which DEC1 is inducibly knocked down, MCF7-TR-7 cells were transfected with pBabe-H1-siDEC1 and selected with 0.5 μg/ml puromycin. MCF7 cell lines, in which p53 or p21 was stably knocked down and DEC1 is inducibly expressed, were generated by transfecting pBabe-U6-sip53 or pBabe-U6-sip21 into M7-DEC1-16 as generated above, and cells were selected with 0.5 μg/ml puromycin.

Affymetrix GeneChip Assay and Northern Blot Analysis—Total RNAs were isolated by using TRIzol reagent (Invitrogen). The U133-plus GeneChip was purchased from Affymetrix. GeneChip analysis was performed according to the manufacturer's instruction. Northern blot analysis and preparation of p21 and GAPDH probes were described previously (29). Wild-type DEC1 cDNA was used as probe and amplified as described above.

Luciferase Reporter Assay—The dual luciferase assay was performed in triplicate according to the manufacturer's instruction (Promega). Briefly, 0.25 μg of a luciferase reporter, 0.25 μg of empty pcDNA3, or pcDNA3 that expresses p53 or p53(R249S) and 9 ng of an internal control *Renilla* luciferase assay vector pRL-CMV (Promega) were transfected into p53-null H1299 cells by using the ESCORT V transfection reagent according to the manufacturer's instruction (Sigma). Cells were seeded at 2×10^4 per well in 24-well plates 24 h before transfection. 18 h post-transfection, luciferase activity was measured with the dual luciferase kit and Turner Designs luminometer. The fold change in relative luciferase activity is a product of the luciferase activity induced by a p53 family protein divided by that induced by an empty pcDNA3 vector.

Chromatin Immunoprecipitation (ChIP) Assay—ChIP assay was performed as described previously (24). The binding of a

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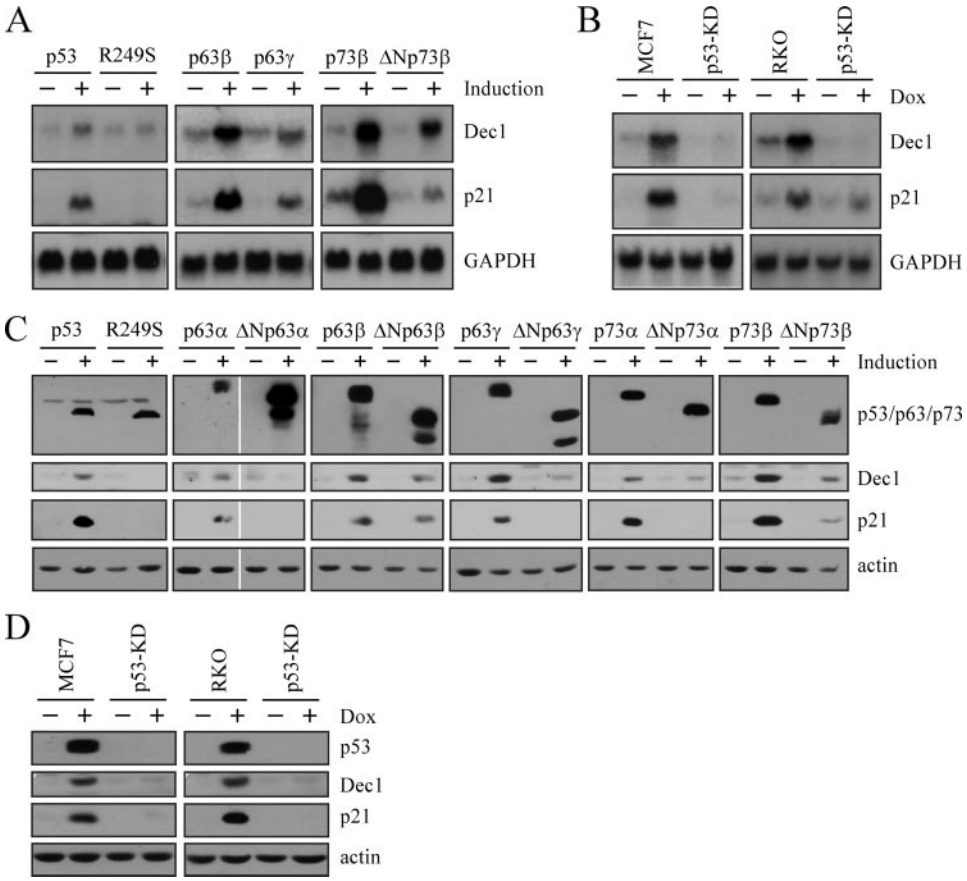

FIGURE 1. **DEC1 is up-regulated by the p53 family.** *A,* DEC1 is induced by p53, p63β, p63γ, p73β, and ΔNp73β but not mutant p53(R249S). Northern blots were prepared with RNAs purified from H1299 cells that were uninduced (−) or induced (+) to express various p53 family proteins as shown at the *top* of the figure. The blots were probed with cDNAs derived from the *DEC1*, *p21*, and *GAPDH* genes, respectively. GAPDH was measured as a loading control. *B,* DEC1 is induced upon DNA damage in a p53-dependent manner. Northern blots were prepared with RNAs purified from MCF7, MCF7-p53-KD, RKO, and RKO-p53-KD cells that were untreated (−) or treated (+) with 0.35 μg/ml doxorubicin (*Dox*) for 24 h. The blots were analyzed as in *A*. *C,* DEC1 expression is up-regulated by p53 family proteins. Western blots were prepared with extracts from H1299 cells that were uninduced (−) or induced (+) to express various p53 family proteins as shown at the *top* of the figure. p53 and mutant p53(R249S) were detected by anti-p53. The Myc-tagged p63 proteins were detected by anti-Myc epitope, and the HA-tagged p73 proteins were detected by anti-HA epitope. DEC1, p21, and actin were detected by their respective antibodies. The level of actin was measured as a loading control. *D,* DEC1 expression is up-regulated by DNA damage in a p53-dependent manner. Western blots were prepared with extracts from MCF7, MCF7-p53-KD, RKO, and RKO-p53-KD cells that were untreated (−) or treated (+) with 0.35 μg/ml doxorubicin for 24 h. The blots were analyzed as in *C*.

p53 family protein to the *DEC1* promoter was detected with forward primer, 5′-GGTTCAAGCGATTCTCCTGCCTC-3′, and reverse primer, 5′-CAGTGGCTCACGCCTGTAATCCT-3′. Primers that were used to amplify the p53-responsive element 1 within the *p21* promoter were described previously (24). Primers for the amplification of the *GAPDH* promoter were used as described previously (30).

Growth Rate and Colony Formation Assay—For growth rate analysis, cells were seeded at 1×10^4 per well in 6-well plates with or without doxycycline (an analog of tetracycline) in triplicate. Attached cells were counted at the indicated times. For colony formation assay, cells were seeded at 500 per well in 6-well plates with or without doxycycline in triplicate. Colonies were fixed with methanol:glacial acetic acid (7:1), washed in H_2O, and stained with 0.02% crystal violet.

DNA Histogram Analysis—Cells were seeded at 5×10^4 per 100-mm plate with or without doxycycline in triplicate. Cells were incubated with 20 μM BrdUrd (Sigma) at 37 °C, 5% CO_2 for 15 min. The harvested living cells were fixed in precooled (−20 °C) ethanol (70%) overnight followed by BrdUrd/PI staining. Briefly, after centrifugation, the cells were treated with 1 ml of 2 N HCl/Triton X-100 for 30 min at room temperature, centrifuged, resuspended in 1 ml of 0.1 M $Na_2B_4O_7$ (pH 8.5) to neutralize the sample, and incubated with fluorescein isothiocyanate-labeled anti-BrdUrd antibody (BD Biosciences) for 30 min at room temperature followed by addition of 300 μl of phosphate-buffered saline/PI (50 μg/ml). Samples were analyzed by fluorescence-activated cell sorting (BD Biosciences).

Western Blot Analysis—Whole cell extracts were prepared with 2× SDS sample buffer and boiled for 5 min at 95 °C. The antibody against DEC1 was generated in rabbit (21). Antibodies against p53, p21, p130, and HA epitope were purchased from Santa Cruz Biotechnology. Anti-actin, and mouse IgG, and rabbit IgG were purchased from Sigma. Anti-Myc epitope was purchased from Abcam. Anti-Rb (clone XZ-77) was used as described (31).

SA-β-Galactosidase Staining Assay—This assay was performed as described previously (7). Cells were washed with 1× phosphate-buffered saline and fixed with 2% formaldehyde, 0.2% glutaraldehyde for 10–15 min at room temperature. Cells were then washed twice with 1× phosphate-buffered saline and stained with fresh SA-β-galactosidase staining solution at 37 °C without CO_2. The SA-β-galactosidase staining solution contains 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$.

RESULTS

Identification of DEC1 as a Novel Target Gene of the p53 Family—To identify novel genes regulated by p53, an Affymetrix GeneChip assay was performed with U133 plus Chips and RNAs purified from MCF7 cells uninduced or induced to express p53. Many known p53 target genes, such as *MDM2*, *p21*, and *PIG3*, and several novel targets, such as DNA polymerase η (pol H) (32) and myosin VI (33), were found to be highly induced by p53. We also found that DEC1 was induced by p53. To confirm this, Northern blot analysis was performed. We showed that DEC1 was induced by p53 but not mutant p53(R249S) in H1299 cells (Fig. 1*A*, *DEC1 panel*). Similarly,

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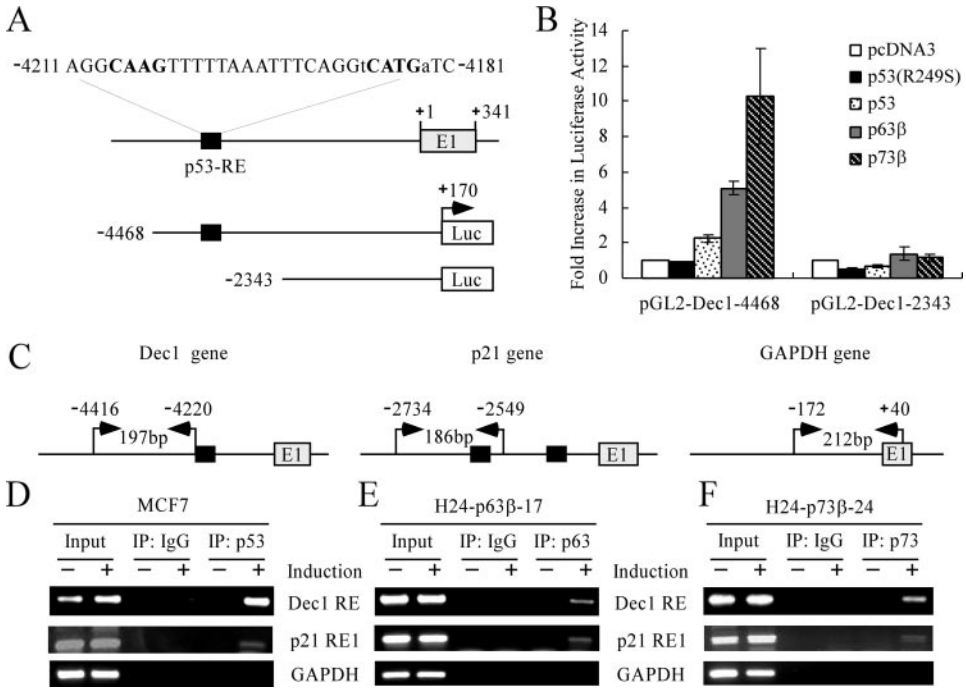

FIGURE 2. **Identification of a potential p53-responsive element (*p53-RE*) in the *DEC1* gene.** *A,* schematic presentation of the *DEC1* genomic structure and luciferase (*Luc*) reporter constructs along with a potential p53-RE located at nt −4211 to −4181 in the promoter of the *DEC1* gene. *B,* potential p53-RE in the *DEC1* gene is responsive to p53, p63*β*, and p73*β* but not to mutant p53(R249S). The luciferase assay was performed as described under "Experimental Procedures." *C,* schematic presentation of the *DEC1*, *p21*, and *GAPDH* promoters with the location of the potential p53-REs and PCR primers used for ChIP assays. *D–F*, p53, p63*β*, and p73*β* bind to the p53-RE *in vivo*. See details in the text.

p21, a well characterized p53 target, was up-regulated by p53 but not mutant p53(R249S) (Fig. 1*A*, *p21 panel*). Because the p53 family proteins, p63 and p73, have been shown to activate some p53-responsive genes, including *p21* (34), we examined whether DEC1 is induced by p63 and p73. We found that both DEC1 and p21 were induced in H1299 cells by p63*β*, p63*γ*, p73*β*, and ΔNp73*β* (Fig. 1*A*, *DEC1* and *p21 panels*).

DNA damage stabilizes and activates p53, leading to induction of p53 target genes (35). If *DEC1* is a true p53 target, it would be induced by DNA damage in cells that contain an endogenous wild-type *p53* gene. To this end, MCF7, MCF7-p53-KD, RKO, and RKO-p53-KD cells were untreated or treated with doxorubicin, an inhibitor of topoisomerase II that can induce DNA double strand breaks (36). We found that DEC1 was induced by doxorubicin in MCF7 and RKO cells (Fig. 1*B*, *DEC1 panel*). Similarly, p21 was induced (Fig. 1*B*, *p21 panel*). In contrast, little if any DEC1 or p21 was detected in p53-knockdown MCF7 and RKO cells (Fig. 1*B*, *DEC1* and *p21 panels*).

Next, we examined whether an increase in DEC1 transcript correlates with an increase in DEC1 protein. We found that DEC1 was up-regulated in H1299 cells by p53, p63*α*, p63*β*, ΔNp63*β*, p63*γ*, ΔNp63*γ*, p73*α*, ΔNp73*α*, p73*β*, and ΔNp73*β* but not mutant p53(R249S) and ΔNp63*α* (Fig. 1*C*, *DEC1 panel*). The expression of p21 was measured as a positive control and found to be induced by p53, p63*α*, p63*β*, ΔNp63*β*, p63*γ*, p73*α*, p73*β*, and ΔNp73*β* but not mutant p53(R249S), ΔNp63*α*, ΔNp63*γ*, and ΔNp73*α* (Fig. 1*C*, *p21 panel*). In addition, we showed that like p21, DEC1 was induced by DNA damage in p53-proficient (MCF7 and RKO) but not p53-knockdown (MCF7-p53-KD and RKO-p53-KD) cells (Fig. 1*D*, *DEC1* and *p21 panels*).

As a transcription factor, p53 regulates gene expression by directly binding to a p53-responsive element (p53-RE) in the target gene. The consensus p53-RE is composed of two half-sites (RRRC(A/T)(A/T)GYYY, where R represents purine and Y pyrimidine) separated by up to 13 nt (37). Thus, if *DEC1* is a direct p53 target, one or more p53-REs should exist in the *DEC1* gene. To test this, we analyzed the genomic locus of the *DEC1* gene and found one potential p53-binding site located between nucleotides −4211 to −4181, with the sequence of AGGCAAGTTTTTAAATTTC-AGGTCATGATC (Fig. 2*A*). Upon alignment with the consensus sequence, this p53-RE contains two mismatches at noncritical positions (Fig. 2*A*, mismatches in *lowercase* and core sequences in *boldface*).

To determine whether this p53-RE is responsive to a p53 family protein, two DNA fragments from the *DEC1* promoter, in which the p53-RE is retained (−4468/+170) or deleted (−2343/+170), were cloned into pGL2-basic luciferase reporter. The resulting vectors were designated pGL2-DEC1-4468 and pGL2-DEC1-2343, respectively (Fig. 2*A*). Next, luciferase reporter assay was performed and showed that p53, p63*β*, and p73*β* were able to increase the luciferase activity for pGL2-DEC1-4468 but not pGL2-DEC1-2343 (Fig. 2*B*). In contrast, mutant p53(R249S) was inert (Fig. 2*B*). As a positive control, the *p21* promoter was highly increased by p53 but not p53(R249S) (data not shown). These data suggest that the p53-RE in the *DEC1* gene is responsive to p53.

To further examine whether a p53 family protein can bind to the p53-RE in the *DEC1* gene *in vivo*, ChIP assay was performed with primers shown in Fig. 2*C* (*left panel*). The binding of the p53 family proteins to the p53-RE in the *p21* promoter was determined as a positive control (Fig. 2*C*, *middle panel*). Additionally, a region within the promoter of the *GAPDH* gene was amplified as a control for nonspecific binding (Fig. 2*C*, *right panel*). To test the binding of p53 to the *DEC1* promoter, MCF7 cells were untreated or treated with doxorubicin to activate p53, and the p53-DNA complexes were immunoprecipitated with anti-p53 antibody or mouse IgG as a control. We found that the captured fragments containing the p53-RE were significantly increased upon induction of p53 by DNA damage (Fig. 2*D*, *DEC1 panel*). Similarly, p53 bound to the p53-RE1 in the *p21* gene in response to DNA damage (Fig. 2*E*, *p21 panel*). However, no fragments were enriched by control IgG (Fig. 2*D*, *DEC1* and *p21 panels*). Furthermore, the *GAPDH* promoter

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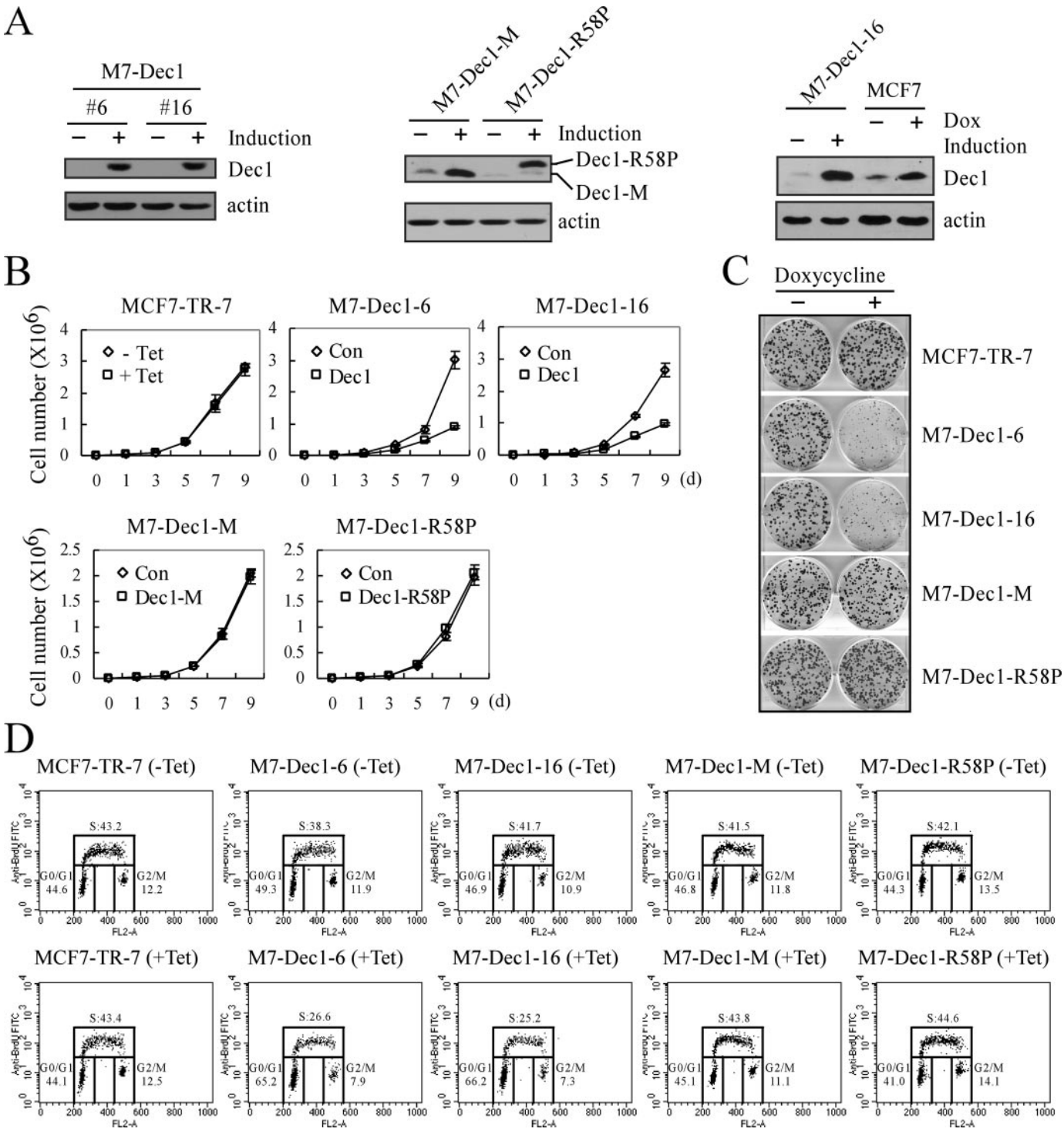

FIGURE 3. **Overexpression of DEC1, but not mutant DEC1-M and DEC1-R58P, inhibits cell proliferation and induces cell cycle arrest in G_1.** *A, left* and *middle panels,* generation of MCF7 cell lines that inducibly express DEC1, mutant DEC1-M, or DEC1-R58P. The levels of DEC1, DEC1-M, and DEC1-R58P were quantified with anti-DEC1. *Right panel,* level of DNA damage-induced DEC1 is comparable with that of ectopic-expressed DEC1. Western blots were prepared with extracts from M7-DEC1-16 cells that were uninduced (−) or induced (+) with doxycycline for 24 h and MCF7 cells that were untreated (−) and treated (+) with 0.35 μg/ml doxorubicin for 24 h. *B,* DEC1, but not DEC1-M and DEC1-R58P, inhibits cell proliferation. The growth rate of MCF7 cells that were uninduced or induced to express DEC1, DEC1-M, and DEC1-R58P was measured over a 9-day period. MCF7 cells (*MCF7-TR-7*) that were treated with or without doxycycline (an analog of tetracycline) were used as a control. *C,* DEC1, but not DEC1-M and DEC1-R58P, inhibits colony formation. Colony formation assay was performed with MCF7 cells that were uninduced or induced to express DEC1, DEC1-M, or DEC1-R58P for 14 days. *D,* DEC1, but not DEC1-M and DEC1-R58P, induces G_1 arrest. MCF7 cells were uninduced or induced to express DEC1, DEC1-M, or DEC1-R58P for 4 days and then used for BrdUrd/PI dual parameter analysis as described under "Experimental Procedures."

was not recognized by p53 (Fig. 2*D*, *GAPDH panel*). To analyze the binding of p63 or p73, H1299 cells were uninduced or induced to express Myc-tagged p63β or HA-tagged p73β and then used for ChIP assay. The p63-DNA complexes were immunoprecipitated with anti-Myc antibody or rabbit IgG as a control (Fig. 2*E*). The p73-DNA complexes were immunopre-

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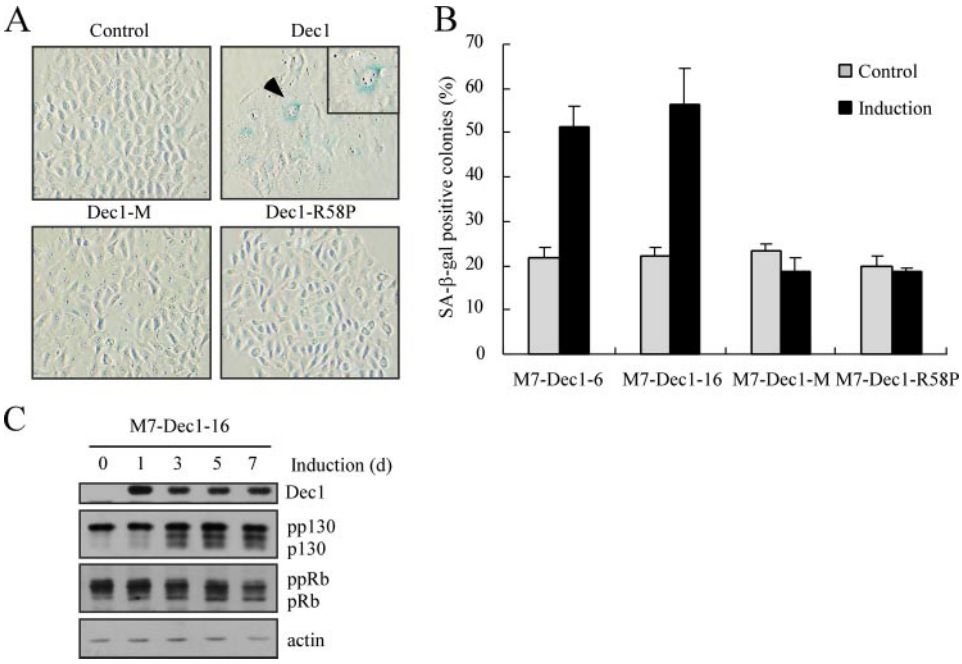

FIGURE 4. **Overexpression of DEC1, but not mutant DEC1-M and DEC1-R58P, induces premature senescence.** *A*, DEC1, but not mutant DEC1-M and DEC1-R58P, is capable of inducing premature senescence. MCF7 cells, which were uninduced or induced to express DEC1, DEC1-M, or DEC1-R58P for 8 days, were analyzed by SA-β-galactosidase staining assay as described under "Experimental Procedures." *B*, quantification of the percentage of SA-β-galactosidase-positive colonies shown in *A*. See details in text. *C*, DEC1-induced senescence results in up-regulation of hypophosphorylated p130. Western blots were prepared using extracts from MCF7 cells that were uninduced (−) or induced (+) to express DEC1 for 0, 1, 3, 5, or 7 days.

cipitated with anti-HA antibody or mouse IgG as a control (Fig. 2*F*). We found that both p63β and p73β bound to the p53-RE in the *DEC1* gene as well as to the one in the *p21* gene (Fig. 2, *E* and *F*, *DEC1* and *p21 panels*). In contrast, the *GAPDH* promoter was not recognized by p63β and p73β (Fig. 2, *E* and *F*, *GAPDH panels*). In sum, these data indicate that *DEC1* is a direct target gene of the p53 family.

DEC1 Induces G_1 Arrest and Initiates Cellular Senescence—To test whether DEC1 is a downstream effector of p53 to mediate senescence, MCF7 cell line was chosen because it has a functional p53 pathway but lacks p16 (38). In addition, MCF7 cells undergo premature senescence upon treatment with doxorubicin (9, 10). Because p53 and p16 are the two major signaling pathways leading to cellular senescence (8, 12), the MCF7 cell line is an ideal system to address how p53 regulates senescence. To analyze the biological activity of DEC1, multiple MCF7 cell lines, which inducibly express DEC1 and mutant DEC1 proteins, DEC1-M and DEC1-R58P, under the control of a tetracycline-inducible promoter, were generated. DEC1-M lacks residues 53–65 in the DNA binding domain and thus is transcriptionally inactive (21). Because of the deletion, DEC1-M has a lower molecular mass than its wild-type counterpart. DEC1-R58P has a point mutation at codon 58 (arginine to proline) within the DNA binding domain, which diminishes its DNA binding activity (21). Four representative cell lines were selected for further studies (Fig. 3*A*, *left* and *middle panels*) as follows: M7-DEC1 (clone 6 and 16) in which wild-type DEC1 can be inducibly expressed, M7-DEC1-M (clone 11) in which DEC1-M can be inducibly expressed, and M7-DEC1-R58P (clone 2) in which DEC1-R58P can be inducibly expressed. To determine whether cellular senescence induced by overexpressed DEC1 is physiologically relevant, Western blot analysis was performed to compare the levels of DNA damage-induced DEC1 and ectopic-expressed DEC1 in MCF7 cells. We showed that the level of DNA damage-induced DEC1 was comparable with that of ectopic-expressed DEC1 (Fig. 3*A*, *right panel*).

Because cells that end at senescence must initially undergo cell cycle arrest, growth rate analysis and colony formation assay were performed to examine whether overexpression of DEC1 affects cell proliferation. We found that overexpression of DEC1 inhibited the proliferation of MCF7 cells over a 9-day period in both DEC1-expressing cell lines (Fig. 3*B*). As controls, doxycycline, DEC1-M, or DEC1-R58P had no effect on cell proliferation (Fig. 3*B*). Consistently, overexpression of DEC1, but not doxycycline, DEC1-M, or DEC1-R58P, inhibited the size and/or number of colonies (Fig. 3*C*). Next, BrdUrd/PI dual parameter analysis was performed to characterize the cell cycle profile and showed that overexpression of DEC1 increased the percentage of cells in G_1 phase, concomitantly with a decrease in the percentage of cells in S (BrdUrd positive cells) and G_2 phases (Fig. 3*D*). In contrast, doxycycline, DEC1-M, and DEC1-R58P had no effect on BrdUrd incorporation (Fig. 3*D*). Taken together, we concluded that the effect of DEC1 on cell proliferation is specific, and the DNA binding activity of DEC1 is required for inducing cell cycle arrest.

To test whether DEC1 is capable of inducing senescence, SA-β-galactosidase staining assay was performed. Microscopic analysis showed that the number of SA-β-galactosidase-positive colonies was increased in DEC1-expressing cells compared with that in control and mutant DEC1-expressing cells. These SA-β-galactosidase-positive colonies exhibited senescence-like phenotypes, such as enlarged cell size, flattened morphology, and perinuclear blue (Fig. 4*A*). To quantify the extent of DEC1-induced senescence, 150–200 colonies were counted and colonies containing ≥50% SA-β-galactosidase-positive cells were defined as senescent colonies. We found that overexpression of DEC1 markedly increased the percentage of senescent colonies in both M7-DEC1-6 and M7-DEC1-16 cell lines (Fig. 4*B*), whereas overexpression of DEC1-M or DEC1-R58P had no effect (Fig. 4, *A* and *B*). We would like to note that a small number of MCF7 cells underwent spontaneous senescence under normal cell culture conditions as reported previously (10). Similarly, overexpression of DEC1 was able to inhibit

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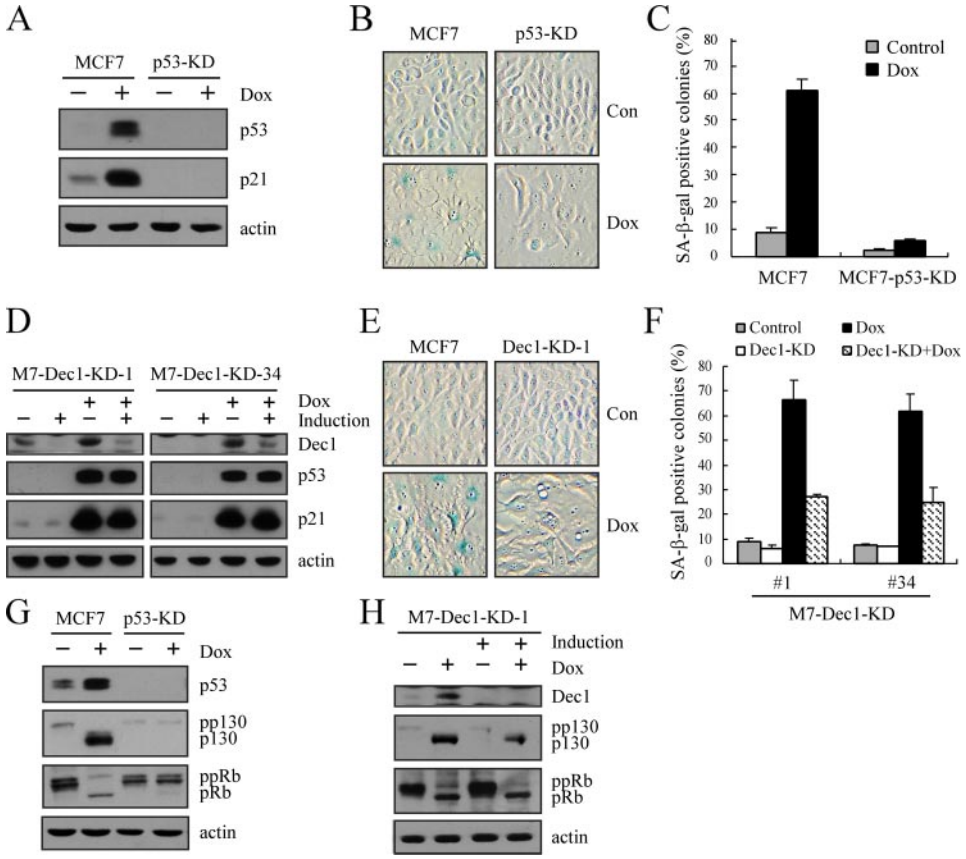

FIGURE 5. **DEC1 is required for DNA damage-induced premature senescence.** *A,* characterization of p53-knockdown MCF7 cell lines. Western blots were prepared with extracts from MCF7 and MCF7-p53-KD cells that were untreated (−) or treated (+) with 0.35 μg/ml doxorubicin for 24 h. *B,* knockdown of p53 diminishes DNA damage-induced premature senescence. MCF7 or MCF7-p53-KD cells, which were cultured for 3 days and then untreated (−) or treated (+) with 0.03 μg/ml doxorubicin for 2 days, were analyzed by SA-β-galactosidase staining assay. *C,* quantification of SA-β-galactosidase-positive colonies shown in *B*. *D,* generation of MCF7 cell lines in which DEC1 is inducibly knocked down. Western blots were prepared with extracts from MCF7 cells that were uninduced (−) or induced (+) to express DEC1 siRNA for 3 days, followed by treatment with (+) or without (−) 0.35 μg/ml doxorubicin for 24 h. *E,* knockdown of DEC1 attenuates DNA damage-induced premature senescence. MCF7 cells, which were uninduced (−) or induced (+) to express DEC1 siRNA for 3 days and then untreated (−) or treated (+) with 0.03 μg/ml doxorubicin for 2 days, were analyzed by SA-β-galactosidase staining assay. *F,* quantification of SA-β-galactosidase-positive colonies shown in *E*. *G,* knockdown of p53 diminishes DNA damage-induced up-regulation of hypophosphorylated p130 and pRb. Western blots were prepared with extracts from MCF7 and MCF7-p53-KD cells that were cultured for 3 days and then untreated (−) or treated (+) with 0.03 μg/ml doxorubicin for 2 days. *H,* knockdown of DEC1 selectively diminishes up-regulation of hypophosphorylated p130 upon DNA damage. Western blots were prepared with extracts from MCF7-DEC1-KD-1 cells that were uninduced (−) or induced (+) to express DEC1 siRNA for 3 days and then untreated (−) or treated (+) with 0.03 μg/ml doxorubicin for 2 days.

cell proliferation and initiate premature senescence in U2OS osteosarcoma cells (data not shown).

A recent study showed that p53 cooperates selectively with p130 to induce cellular senescence when the p16/pRb pathway is disrupted (13). We speculated that DEC1 induces premature senescence through p130 because MCF7 cells are deficient in p16. To test this, the phosphorylation status of p130 and pRb was examined, and it showed that the level of the active, hypophosphorylated p130 was significantly increased by DEC1 (Fig. 4*C*, *p130 panel*), but hypophosphorylated pRb was only slightly increased by DEC1 (Fig. 4*C*, *pRb panel*). Thus, we concluded that overexpression of DEC1 alone is sufficient to promote premature senescence.

DEC1 Is Required for DNA Damage-induced Premature Senescence—Previous studies have shown that p53 plays a key role in premature senescence upon DNA damage (39, 40). To confirm that p53 is essential for DNA damage-induced premature senescence in MCF7 cells, p53 stable knockdown cell line, MCF7-p53-KD, was utilized. As expected, we found that p53 was stabilized by treatment with doxorubicin in MCF7 but not MCF7-p53-KD cells (Fig. 5*A*, *p53 panel*). Similarly, p21 was induced by DNA damage in MCF7 but not MCF7-p53-KD cells (Fig. 5*A*, *p21 panel*). Next, SA-β-galactosidase staining assay was performed and showed that senescence-like phenotypes were induced upon doxorubicin treatment in MCF7 cells but not MCF7-p53-KD cells (Fig. 5*B*). Here, we would like to note that the morphological change induced by DNA damage in MCF7-p53-KD cells is likely due to cell cycle arrest via a p53-independent mechanism (15). By quantifying SA-β-galactosidase-positive colonies, we found that the percentage of senescent cells was markedly reduced upon p53-knockdown (Fig. 5*C*).

Because DEC1 is induced by DNA damage in a p53-dependent manner and overexpression of DEC1 alone promotes senescence, we examined whether DEC1 is required for DNA damage-induced premature senescence. To test this, MCF7 cell lines, in which endogenous DEC1 is inducibly knocked down via siRNA, were generated. Two representative cell lines (M7-DEC1-KD-1 and -34) were selected for further studies. DEC1 was efficiently knocked down upon induction of siRNA regardless of DNA damage (Fig. 5*D*, *DEC1 panel*). The levels of p53 and p21 were measured as positive indicators of DNA damage (Fig. 5*D*, *p53* and *p21 panels*). It has been shown that short hairpin vectors, which can trigger an interferon response, would lead to up-regulation of 2′,5′-oligoadenylate synthetase (OAS1), a classic interferon target gene (41). To rule out the possibility that DEC1 siRNA elicits an interferon response, RT-PCR was performed to measure the induction of OAS1 and showed that OAS1 was not induced upon siRNA expression (data not shown). In addition, growth curve analysis and colony formation assay showed that DEC1-knockdown alone had no effect on cell proliferation in MCF7 cells (data not shown). Next, SA-β-galactosidase staining assay was performed and showed that senescence-like phenotypes were induced by treatment with doxorubicin (Fig. 5, *E* and *F*), but the number of SA-β-galac-

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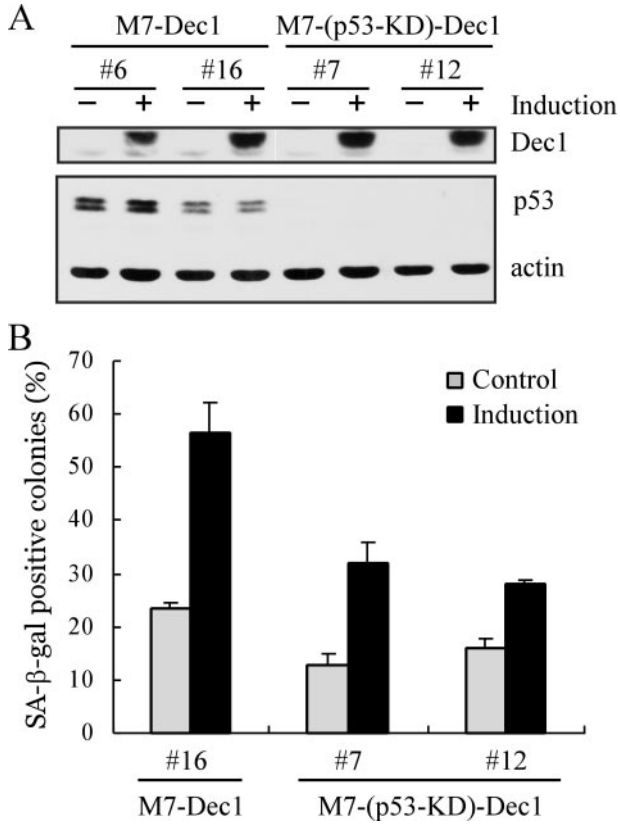

FIGURE 6. **p53 modulates, but is not required for, DEC1-induced premature senescence.** *A,* generation of MCF7 cell lines in which p53 was stably knocked down and DEC1 is inducibly expressed. *B,* overexpression of DEC1 is capable of inducing premature senescence in p53-knockdown MCF7 cells. MCF7 and MCF7-p53-KD cells, which were uninduced or induced to express DEC1 for 8 days, were used for SA-β-galactosidase staining assay. The percentage of SA-β-galactosidase-positive colonies was analyzed as in Fig. 4*B*.

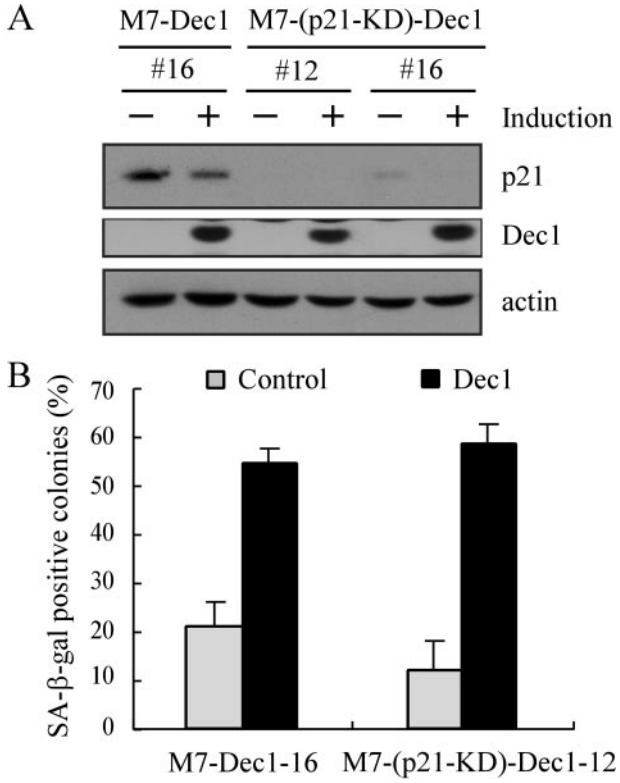

FIGURE 7. **p21 is not required for DEC1-induced premature senescence.** *A,* generation of MCF7 cell lines in which p21 was stably knocked down and DEC1 is inducibly expressed. *B,* overexpression of DEC1 is capable of inducing premature senescence in p21-knockdown MCF7 cells. SA-β-galactosidase staining analysis was performed as in Fig. 6*B*.

tosidase-positive cells was substantially reduced by DEC1-knockdown (Fig. 5, *E* and *F*).

To further analyze the effect of p53- and DEC1-knockdown on DNA damage-induced senescence, we examined the phosphorylation status of p130. We found that the level of hypophosphorylated p130 was significantly increased by treatment with doxorubicin in MCF7 but not in MCF7-p53-KD cells (Fig. 5*G*, *p130 panel*). Likewise, the level of hypophosphorylated p130 was reduced by DEC1-knockdown (Fig. 5*H*, *p130 panel*). Interestingly, we found that hypophosphorylated pRb was altered upon p53-knockdown, but it was not affected by DEC1-knockdown (Fig. 5, *G* and *H*, *pRb panels*). These data indicate that DEC1 is one of the effectors downstream of p53 in DNA damage-induced senescence.

p53 Modulates, but Is Not Required for, DEC1-induced Premature Senescence—Given the importance of p53 in premature senescence upon DNA damage (39, 40), it is likely that p53 plays a role in DEC1-induced premature senescence. To test this, we generated multiple MCF7 cell lines in which p53 was stably knocked down and DEC1 is inducibly expressed. Two representative cell lines (M7-(p53-KD)-DEC1-7 and -12) were selected for future studies (Fig. 6*A*). Western blot analysis showed that comparable levels of DEC1 were inducibly expressed in p53-proficient and -knockdown cell lines (Fig. 6*A*, *DEC1 panel*). However, unlike in M7-DEC1-6 and M7-DEC1-16 cell lines, no basal levels of p53 were detected in M7-(p53-KD)-DEC1-7 and M7-(p53-KD)-DEC1-12 cell lines (Fig. 6*A*, *p53 panel*). Next, we examined the activity of DEC1 in the absence of p53. We found that the ability of DEC1 to inhibit cell proliferation, as measured by growth rate, colony formation, and cell cycle profile, was not significantly affected by p53-knockdown (data not shown). We also found that overexpression of DEC1 was still capable of inducing senescence in p53-knockdown MCF7 cells, although this effect was much weaker than that in p53-proficient MCF7 cells (Fig. 6*B*). These data suggest that DEC1 functions downstream of p53 to initiate cellular senescence and p53 mediates, but is not necessarily required for, DEC1-induced premature senescence.

p21 Is Not Required for DEC1-induced Premature Senescence—p21 was first identified as an overexpressed gene in senescent cells (42). It has been shown that p21 is capable of inducing premature senescence in p53-null H1299 cells (43). To examine whether p21 plays a role in DEC1-induced senescence, multiple MCF7 cell lines, in which p21 was stably knocked down and DEC1 is inducibly expressed, were generated. Two representative cell lines, M7-(p21-KD)-DEC1-12 and M7-(p21-KD)-DEC1-16, are shown in Fig. 7*A*. Compared with p21-proficient MCF7 cells (M7-DEC1-16), p21 was efficiently knocked down in these two cell lines (Fig. 7*A*, *p21 panel*). In addition, a comparable level of DEC1 was expressed in both p21-proficient and -knockdown MCF7 cells (Fig. 7*A*, *DEC1 panel*). Next, growth rate and cell cycle profile analyses were performed and showed that cell proliferation was inhibited by DEC1 regardless of p21

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status (data not shown). Furthermore, the efficiency of DEC1 to promote premature senescence was not affected upon p21-knockdown (Fig. 7*B*). Therefore, we concluded that p21 is not required for the proper function of DEC1 to initiate senescence.

DISCUSSION

DEC1 belongs to the bHLH family of transcription factors and is able to suppress cell proliferation in multiple cell lines (18, 20, 21). Interestingly, a recent study has showed that oncogene K-*ras*V12-induced senescence is correlated with DEC1 up-regulation (22), but whether DEC1 is required for senescence has not been determined. Here we found that DEC1 is induced by p53 and DNA damage in a p53-dependent manner. We also showed that p53 binds to the promoter of the *DEC1* gene and transcriptionally regulates DEC1 through a potential p53-responsive element found in the *DEC1* promoter. Moreover, we showed that overexpression of DEC1 alone initiates G_1 arrest and senescence, and knockdown of DEC1 attenuates DNA damage-induced premature senescence. Furthermore, the phosphorylation status of p130 is altered during DEC1-mediated senescence, consistent with previous studies that p53-mediated and DNA damage-induced senescence is primarily through p130 (13, 14). Taken together, we concluded that DEC1 is one of the effectors downstream of p53 to promote premature senescence.

It has been shown that p53 and p16 are the two major signaling pathways leading to cellular senescence, thus targeting p53 and p16 would circumvent oncogenic *ras*-induced senescence (8). However, downstream effectors of p53 that may promote cellular senescence are little known. The expression of promyelocytic leukemia protein is found to be regulated by p53 (44). In turn, promyelocytic leukemia is capable of inducing premature senescence by stabilizing p53 via promoting p53 acetylation (45). In contrast, deacetylation of p53 antagonizes promyelocytic leukemia-induced premature senescence (46). These data indicate that p53 plays an important role downstream of its target during senescence. Interestingly, here we found that overexpression of DEC1 is able to induce premature senescence in p53-knockdown cells albeit to a less extent (Fig. 6*B*). This suggests that p53 modulates, but is not required for, DEC1-induced cellular senescence. In addition, a well studied p53 target, p21, is capable of initiating premature senescence in p53-null H1299 cells (43). However, the efficiency of DEC1 to promote premature senescence was not affected upon p21-knockdown (Fig. 7*B*). Taken together, it is possible that DEC1 and p21 may independently elicit cellular senescence downstream of p53.

As a transcription factor, DEC1 may directly regulate some targets involved in cell cycle arrest and senescence. To uncover these potential targets of DEC1, an Affymetrix GeneChip assay was performed by using M7-DEC1-16, which was uninduced or induced to express DEC1. Several potential target genes were identified, including epithelium-specific ETS gene-2 (*ELF5/ESE2*) and -3 (*EHF/ESE3*). ELF5 and EHF belong to the Ets family of transcription factors and may be involved in regulating cell proliferation, differentiation, and tumorigenesis (47, 48). Moreover, it has been reported that Ets family proteins, Ets1 and Ets2, can activate the *p16* promoter, and an increase in Ets1 was observed in senescent human diploid fibroblasts (49). Therefore, it is possible that *ELF5* and *EHF* are downstream targets of DEC1 to induce cell cycle arrest and/or cellular senescence. Future studies to identify and confirm potential DEC1 targets involved in senescence would provide an insight into the mechanism by which DEC1 mediates senescence.

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DEC1, a Basic Helix-Loop-Helix Transcription Factor and a Novel Target Gene of the p53 Family, Mediates p53-dependent Premature Senescence

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