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

Yingjuan Qian, Jin Zhang, Bingfang Yan, and Xinbin Chen

From the Center for Comparative Oncology, University of California, Davis, California 95616, the Department of Cell Biology, University of Alabama, Birmingham, Alabama 35294, and the Department of Biomedical Sciences, University of Rhode Island, Kingston, Rhode Island 02881

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 G1 arrest and promotes senescence. Moreover, we showed that DEC1-induced senescence is p21-independent. 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 embryoid-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 TFIIIB, TBP, and TFIID (18, 19), or by recruiting a histone deacetylase corepressor complex.
plex (20). Interestingly, DEC1 is implicated in cell cycle regulation, differentiation, and apoptosis in response to various extracellular stimuli, including hypoxia, serum starvation, and retinoic 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-rasV12 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′-AGGAATTCCATGGAGCGGATC CCCAGG-3′, and reverse primer, 5′-AGCTCTAGAAGGAAGGAAAGCAAAGCAG-3′. To generate a construct for the inducible expression of DEC1 siRNA, two oligonucleotides, 5′-GATCCCCGACTACAAACA CTAATTGTTGAGAGCAGAATTTGATTAGTTGTTAGTGCTTT TGGAAA-3′ and 5′-AGCCTTACAAAACTA ATTGTTGAGAGCAGAATTTGATTAGTTGTTAGTG CCGG-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, 5′-TCGAGGGCTCGCCCTCCTCATCCC GTTTCTTCTAAGAGAAACCGGGATGAGAGG GCTTTTTG-3′, and antisense, 5′-GATCCAAAAAGGCCTC CTATCCCTGTTTCTCTTTTGAGAACACGGAGAAT GAGAGGGCGGCC-3′, were annealed and cloned into pBabe-U6 at BamHI and Xhol 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′-ATGGTACCCAGGCTTGAGTACGTGCGATGC-3′), and reverse primer, DEC1-EcoRV-As (5′-ACGCCCCAACTTGGCTGTCGATATACCC-3′). The resulting vector was designated pBabe-H1-siDEC1.

**Cells**—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% CO₂. 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 GST-RNA 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 GST-RNA were isolated by using TRIzol reagent (Invitrogen). The U133-plus GeneChip was purchased from Affymetrix.
**DECI Is a Mediator of p53-dependent Premature Senescence**

**A**

| p53  | R249S | p63β | p63γ | p73β | ΔNp73β | Induction | p21 | DECI |
|------|-------|------|------|------|--------|----------|-----|------|
| -    | +     | -    | -    | -    |        | Induced  | -   | -    |
| +    | -     | -    | -    | -    |        | Induced  | -   | -    |

**B**

| MCF7 | p53-KD | RKO  | p53-KD |
|------|--------|------|--------|
| -    | +      | -    | +      |

**C**

| p53  | R249S | p63α | ΔNp63α | p63β | ΔNp63β | Induction | p21 | p73α | ΔNp73α | p73γ | ΔNp73γ | actin |
|------|-------|------|--------|------|--------|-----------|-----|------|---------|------|---------|-------|
| -    | +     | -    | -      | -    | -      | Induced   | -   | -    |         | -    |         | -     |
| +    | -     | -    | -      | -    | -      | Induced   | -   | -    |         | -    |         | -     |

**D**

| MCF7 | p53-KD | RKO  | p53-KD |
|------|--------|------|--------|
| -    | +      | -    | +      |

**FIGURE 1. DECI is up-regulated by the p53 family.** A, DECI 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 DECI, p21, and GAPDH genes, respectively. GAPDH was measured as a loading control. B, DECI 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, DECI 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. Western blots were prepared with extracts from H1299 cells that were uninduced (−) or induced (+) 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, DECI expression is up-regulated by DNA damage in a p53-dependent manner. Western blots were prepared with extracts from H1299 cells that were uninduced (−) or treated (+) with 0.35 μg/ml doxorubicin for 24 h. The blots were analyzed as in C.

**RESULTS**

**Identification of DECI 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 DECI was induced by p53. To confirm this, Northern blot analysis was performed. We showed that DECI was induced by p53 but not mutant p53(R249S) in H1299 cells (Fig. 1A, DECI panel). Similarly,
p21, a well characterized p53 target, was up-regulated by p53 but not mutant p53(R249S) (Fig. 1A, 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, p63β, and p73β but not to mutant p53(R249S). The luciferase assay was performed as described under “Experimental Procedures.”

To determine whether this p53-RE is responsive to a p53 family member, 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. 2A). Next, luciferase reporter assay was performed and showed that p53, p63β, and p73β were able to increase the luciferase activity for pGL2-DEC1-4468 but not pGL2-DEC1-2343 (Fig. 2B). In contrast, mutant p53(R249S) was inactive (Fig. 2B). 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. 2C (left panel). The binding of the p53 family proteins to the p53-RE in the p21 promoter was determined as a positive control (Fig. 2C, middle panel). Additionally, a region within the promoter of the GAPDH gene was amplified as a control for nonspecific binding (Fig. 2C, 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. 2D, DEC1 panel). Similarly, p53 bound to the p53-RE1 in the p21 gene in response to DNA damage (Fig. 2E, p21 panel). However, no fragments were enriched by control IgG (Fig. 2D, DEC1 and p21 panels). Furthermore, the GAPDH promoter
was not recognized by p53 (Fig. 2D, GAPDH panel). To analyze the binding of p63 or p73, H1299 cells were uninduced or induced to express Myc-tagged p63/H9252 or HA-tagged p73/H9252 and then used for ChIP assay. The p63-DNA complexes were immunoprecipitated with anti-Myc antibody or rabbit IgG as a control (Fig. 2E). The p73-DNA complexes were immuno-
DEC1 Is a Mediator of p53-dependent Premature 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). Similarly, overexpression of DEC1 was able to inhibit proliferation of MCF7 cells over a 9-day period in both DEC1-expressing cell lines (Fig. 3B). As controls, doxycycline, DEC1-M, or DEC1-R58P had no effect on cell proliferation (Fig. 3B). Consistently, overexpression of DEC1, but not doxycycline, DEC1-M, or DEC1-R58P, inhibited the size and/or number of colonies (Fig. 3C).

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 G1 phase, concomitantly with a decrease in the percentage of cells in S (BrdUrd positive cells) and G2 phases (Fig. 3D). In contrast, doxycycline, DEC1-M, and DEC1-R58P had no effect on BrdUrd incorporation (Fig. 3D). 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. 4A). 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. 4B), whereas overexpression of DEC1-M or DEC1-R58P had no effect (Fig. 4A and B).

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.
DEC1 Is a Mediator of p53-dependent Premature Senescence

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 p130, which was uninduced upon DEC1 knockdown MCF7 cell lines. Western blots were prepared with extracts from MCF7 and MCF7-p53-KD cells that were untreated or treated with 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 cell lines 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.

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 cell lines 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.

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. 5A, p53 panel). Similarly, p21 was induced by DNA damage in MCF7 but not MCF7-p53-KD cells (Fig. 5A, 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. 5B). 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. 5C).

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. 5D, DEC1 panel). The levels of p53 and p21 were measured as positive indicators of DNA damage (Fig. 5D, 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-
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. 5G, p130 panel). Likewise, the level of hypophosphorylated p130 was reduced by DEC1-knockdown (Fig. 5H, 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. 6A). Western blot analysis showed that comparable levels of DEC1 were inducibly expressed in p53-proficient and -knockdown cell lines (Fig. 6A, 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. 6A, 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. 6B). 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. 7A. Compared with p21-proficient MCF7 cells (M7-DEC1-16), p21 was efficiently knocked down in these two cell lines (Fig. 7A, p21 panel). In addition, a comparable level of DEC1 was expressed in both p21-proficient and -knockdown MCF7 cells (Fig. 7A, DEC1 panel). Next, growth rate and cell cycle profile analyses were performed and showed that cell proliferation was inhibited by DEC1 regardless of p21
**DEC1 Is a Mediator of p53-dependent Premature Senescence**

status (data not shown). Furthermore, the efficiency of DEC1 to promote premature senescence was not affected upon p21-knockdown (Fig. 7B). 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-rasV12-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 G1 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. 6B). 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. 7B). 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 (ELFS/ESE2) and -3 (EHF/ESE3). ELFS 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 ELFS 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.

**Acknowledgments**—We thank A. Chen for technical assistance; K. Harms for the M7-TR-7 cell line; W. Yan for the pBabe-U6-sip53 and pBabe-U6-sip21 constructs; A. Scoumanne for the OAS1 primers; and S. Helton, L. Shu, Y. Xu, and G. Liu for suggestions.

**REFERENCES**

1. Prives, C., and Hall, P. A. (1999) J. Pathol. 187, 112–126
2. Ko, L. J., and Prives, C. (1996) Genes Dev. 10, 1054–1072
3. Levine, A. J., Hui, W., and Feng, Z. (2006) Cell Death Differ. 13, 1027–1036
4. Smith, J. R., and Pereira-Smith, O. M. (1996) Science 273, 63–67
5. Haylick, L. (1965) Exp. Cell Res. 37, 614–636
6. d’Adda di Fagagna, F., Teo, S. H., and Jackson, S. P. (2004) Genes Dev. 18, 1781–1799
7. Dimri, G. P., Lee, X., Basilie, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E. E., Linksens, M., Rubelj, I., Pereira-Smith, O., Peacocke, M., and Campisi, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9363–9367
8. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., and Lowe, S. W. (1997) Cell 88, 593–602
9. Chang, B. D., Broude, E. V., Dokmanovic, M., Zhu, H., Ruth, A., Xuan, Y., Kandel, E. S., Lausch, E., Christov, K., and Roninson, I. B. (1999) Cancer Res. 59, 3761–3767
10. te Poel, R. H., Okorokov, A. L., Jardine, L., Cummings, J., and Joel, S. P. (2002) Cancer Res. 62, 1876–1883
11. Campisi, J. (2005) Science 309, 886–887
12. Ben-Porath, I., and Weinberg, R. A. (2005) Int. J. Biochem. Cell Biol. 37, 961–976
13. Kapic, A., Helmbold, H., Reimer, R., Klotzsche, O., Deppert, W., and Bohn, W. (2006) Cell Death Differ. 13, 324–334
14. Jackson, J. G., and Pereira-Smith, O. M. (2006) Mol. Cell. Biol. 26, 2501–2510
15. Schmitt, C. A. (2007) Biochim. Biophys. Acta 1775, 5–20
16. Yamada, K., and Miyamoto, K. (2005) Front. Biosci. 10, 3151–3171
17. Li, Y., Xie, M., Song, X., Gragen, S., Sachdeva, K., Wan, Y., and Yan, B. (2003) J. Biol. Chem. 278, 16899–16907
18. Zawel, L., Yu, J., Torrance, C. J., Markowitz, S., Kinzler, K. W., Vogelstein, B., and Zhou, S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 2984–2985
19. Shen, M., Yoshida, E., Yan, W., Kawamoto, T., Suardita, K., Koyano, Y., Fujimoto, K., Noshiro, M., and Kato, Y. (2002) J. Biol. Chem. 277, 50112–50120
20. Sun, H., and Taneja, R. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4058–4063
21. Li, Y., Zhang, H., Xie, M., Hu, M., Ge, S., Yang, D., Wan, Y., and Yan, B. (2002) Biochem. J. 367, 413–422
22. Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A. J., Barradas, M., Benguria, A., Zaballos, A., Flores, J. M., Barbadic, M., Beach, D., and Serrano, M. (2005) Nature 436, 642
23. van de Wetering, M., Oving, I., Muncan, V., Pon Fong, M. T., Brantjes, H., and Clevers, H. (2003) EMBO Rep. 4, 609–615
24. Liu, G., Xia, T., and Chen, X. (2003) J. Biol. Chem. 278, 17557–17565
25. Yan, W., and Chen, X. (2006) J. Biol. Chem. 281, 7856–7862
26. Zhu, J., Zhang, S., Jiang, J., and Chen, X. (2000) J. Biol. Chem. 275, 39927–39934
27. Dohn, M., Zhang, S., and Chen, X. (2001) Oncogene 20, 3193–3205
28. Nozell, S., Wu, Y., McNaughton, K., Liu, G., Willis, A., Paik, J. C., and Chen, X. (2003) Oncogene 22, 4333–4347
29. Chen, X., Bargonetti, J., and Prives, C. (1995) Cancer Res. 55, 4257–4263
30. Liu, G., and Chen, X. (2005) J. Biol. Chem. 280, 20111–20119
DEC1 Is a Mediator of p53-dependent Premature Senescence

31. Hu, Q. J., Bautista, C., Edwards, G. M., Defeo-Jones, D., Jones, R. E., and Harlow, E. (1991) Mol. Cell. Biol. 11, 5792–5799
32. Liu, G., and Chen, X. (2006) Mol. Cell. Biol. 26, 1398–1413
33. Jung, E. J., Liu, G., Zhou, W., and Chen, X. (2006) Mol. Cell. Biol. 26, 2175–2186
34. Harms, K., Nozell, S., and Chen, X. (2004) Cell. Mol. Life Sci. 61, 822–842
35. Jin, S., and Levine, A. J. (2001) J. Cell Sci. 114, 4139–4140
36. Nelson, W. G., and Kastan, M. B. (1994) Mol. Cell. Biol. 14, 1815–1823
37. el-Deiry, W. S., Kern, S. E., Pietenpol, J. A., Kinzler, K. W., and Vogelstein, B. (1992) Nat. Genet. 1, 45–49
38. Parry, D., Bates, S., Mann, D. J., and Peters, G. (1995) EMBO J. 14, 503–511
39. Elmore, L. W., Rehder, C. W., Di, X., McChesney, P. A., Jackson-Cook, C. K., Gewirtz, D. A., and Holt, S. E. (2002) J. Biol. Chem. 277, 35509–35515
40. Di Leonardo, A., Linke, S. P., Clarkin, K., and Wahl, G. M. (1994) Genes Dev. 8, 2540–2551
41. Bridge, A. J., Pebernard, S., Ducraux, A., Nicoulaz, A. L., and Iggo, R. (2003) Nat. Genet. 34, 263–264
42. Noda, A., Ning, Y., Venable, S. F., Pereira-Smith, O. M., and Smith, J. R. (1994) Exp. Cell Res. 211, 90–98
43. Wang, Y., Blandino, G., and Givol, D. (1999) Oncogene 18, 2643–2649
44. de Stanchina, E., Querido, E., Narita, M., Davuluri, R. V., Pandolfi, P. P., Ferbeyre, G., and Lowe, S. W. (2004) Mol. Cell 13, 523–535
45. Pearson, M., Carbone, R., Sebastiani, C., Ciocca, M., Fagioli, M., Saito, S., Higashimoto, Y., Appella, E., Minucci, S., Pandolfi, P. P., and Pelicci, P. G. (2000) Nature 406, 207–210
46. Langley, E., Pearson, M., Faretta, M., Bauer, U. M., Frye, R. A., Minucci, S., Pelicci, P. G., and Kouzarides, T. (2002) EMBO J. 21, 2383–2396
47. Kas, K., Finger, E., Grall, F., Gu, X., Akbarali, Y., Boltax, J., Weiss, A., Oettgen, P., Kapeller, R., and Libermann, T. A. (2000) J. Biol. Chem. 275, 2986–2998
48. Zhou, J., Ng, A. Y., Tymms, M. J., Jermiin, L. S., Seth, A. K., Thomas, R. S., and Kola, I. (1998) Oncogene 17, 2719–2732
49. Ohtani, N., Zebedee, Z., Huot, T. J., Stinson, J. A., Sugimoto, M., Ohashi, Y., Sharrocks, A. D., Peters, G., and Hara, E. (2001) Nature 409, 1067–1070