YPEL3, a p53-Regulated Gene that Induces Cellular Senescence

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Abstract

Cellular senescence, the limited ability of cultured normal cells to divide, can result from cellular damage triggered through oncogene activation (premature senescence) or the loss of telomeres following successive rounds of DNA replication (replicative senescence). Although both processes require a functional p53 signaling pathway, relevant downstream p53 targets have been difficult to identify. Discovery of senescence activators is important because induction of tumor cell senescence may represent a therapeutic approach for the treatment of cancer. In microarray studies in which p53 was reactivated in MCF7 cells, we discovered that Yippee-like-3 (YPEL3), a member of a recently discovered family of putative zinc finger motif coding genes consisting of YPEL1-5, is a p53-regulated gene. YPEL3 expression induced by DNA damage leads to p53 recruitment to a cis-acting DNA response element located near the human YPEL3 promoter. Physiologic induction of YPEL3 results in a substantial decrease in cell viability associated with an increase in cellular senescence. Through the use of RNAi and H-ras induction of cellular senescence, we show that YPEL3 activates cellular senescence downstream of p53. Consistent with its growth suppressive activity, YPEL3 gene expression is repressed in ovarian tumor samples. One mechanism of YPEL3 downregulation in ovarian tumor cell lines seems to be hypermethylation of a CpG island upstream of the YPEL3 promoter. We believe these findings point to YPEL3 being a novel tumor suppressor, which upon induction triggers a permanent growth arrest in human tumor and normal cells. Cancer Res; 70(9); 3566–75. ©2010 AACR.

Introduction

Biochemical and genetic data show that in response to a variety of cellular stresses, the p53 tumor suppressor protein plays a critical role in the inhibition of cell proliferation. Although p53 can elicit transcription-independent apoptosis (1), it also functions as a transcription factor inducing genes and miRNAs to mediate its complex biological functions (2). Although p53 transcriptional and nontranscriptional mechanisms to induce apoptosis have been linked to tumor inhibition (3), recent in vivo studies have shown that p53-dependent cellular senescence represents an important mechanism to block tumor development (4, 5). These findings suggest that studies focused on understanding signaling pathways in senescence may lead to new targets.

It is known that p53 and Rb signaling pathways are at the nexus of senescence resulting from oncogene activation (premature senescence) or telomere shorting (replicative senescence; ref. 6). However, unlike apoptosis and transient cell cycle arrest, less is known about the p53 targets critical to cellular senescence. Although it is accepted that cyclin-dependent kinase inhibitor and p53 target gene p21 is induced as cells undergo senescence, in some cell systems, the loss of p21 leads to senescence bypass (7); yet in other cell systems, p21-null cells can still undergo a ras-mediated senescence (8). In contrast to p21, plasminogen activator inhibitor 1 represents a critical p53-regulated gene in cellular senescence of both murine and human cells (9).

In studies in which we triggered a p53-dependent cell cycle arrest in MCF7 cells through the RNAi targeting of HdmX or Hdm2, both p53-negative regulators, we uncovered several novel p53-regulated genes, one of which was YPEL3 (10). Located on the short arm of chromosome 16, YPEL3 is part of a five-member family of closely related paralogues—YPEL1-5—that are named in reference to their Drosophila orthologue (11). The Drosophila Yippee protein was identified as a putative zinc finger motif containing protein with a high degree of conservation among the cysteines and histidines that form the motif (12).

Murine YPEL3 has been shown to have an association with the induction of cell growth inhibition through apoptosis. Originally named SUAP for small unstable apoptotic protein, murine YPEL3 was linked to apoptosis in a screen for genes involved in the programmed cell death of murine myeloid precursor cells during differentiation (13).

In the present study, we set out to examine whether the human YPEL3 gene was a novel p53 transcriptional gene
target. Using DNA damage, chromatin immunoprecipitation, and luciferase reporter assays, we show that YPEL3 is regulated by p53. In assessing the biological activity of YPEL3, we show that it encodes a protein that induces cellular senescence in human tumor and normal cells. Consistent with this growth-suppressive activity, YPEL3 is seen downregulated in ovarian tumor samples. Using ovarian tumor cell lines, we observed that CpG hypermethylation seems to be one mechanism by which YPEL3 gene expression is suppressed. Overall, these studies identify YPEL3 as a p53-dependent, tumor suppressor gene.

Materials and Methods

YPEL3 expression plasmids. The tetracycline-inducible (tet-on) lentiviral YPEL3-V5–tagged expression construct was designed by cloning the YPEL3 open reading frame into pENTR/SD/D-TOPO and then into the pLenti4-T/O-V5 DEST vector through the Gateway cloning system (Invitrogen).

Reverse transcription-PCR. Total RNA was isolated using the e.Z.N.A. Total RNA kit (Omega Bio-Tek) according to the manufacturer’s instructions. RNA quality and quantity was assessed on a RNA nanochip (Agilent Technologies). One microgram of total RNA was used as a template for cDNA synthesis using the reverse transcriptase core reagent kit (Applied Biosystems). Taqman-based PCR was performed in triplicate using Assay on Demand probe sets (Applied Biosystems) and an Applied Biosystems 7900 Sequence Detection System. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. All Assay on Demand probes used in these experiments (YPEL3, p21, Bax, and GAPDH) were validated gene targets.

Cell lines. H1299 cells are derived from non–small cell lung carcinoma devoid of p53. U2OS osteosarcoma cells, HepG2 hepatocarcinoma, and MCF7 breast carcinoma cells all express wild-type p53. HCT116 +/+ and −/− p53 colon carcinoma cell lines are isogenic with the exception of p53 status. IMR90 cells are primary human diploid fibroblasts. All of these cell lines were purchased from the American Type Culture Collection. 76ntert immortalized mammary epithelial cells also express wild-type p53 and were a generous gift from Dr. Vimla Band (University of Nebraska, Omaha, NE). A2780, Cp70, and C30 cell lines were obtained from Dr. Hamilton (Fox Chase, Philadelphia, PA). A2780 cells express wild-type p53, whereas Cp70 and C30 cells are p53 null or mutated, respectively. All cell line passages in these experiments were grown for no longer than 3 mo.

DNA damage experiments. Twenty-four hours before treatment, 76ntert-immortalized mammary epithelial cells were seeded at a density of 150 k-cells/6-cm plate. The following day, the plates were treated with increasing doses of bleomycin or doxorubicin. Twenty-four hours later, the cells were harvested. Total RNA was extracted from the harvested cells and gene expression was analyzed by reverse transcription-PCR (RT-PCR).

Reporter assays. The YPEL3-luc reporter plasmid contains –1386 to +120 of the YPEL3 promoter region (+1 representing the transcriptional start site) cloned in front of the luciferase gene in pGL3-Basic. The YPEL3(−485+120) reporter plasmid contains −485 to +120 of the YPEL3 promoter cloned into pGL3-Basic. A 50-bp region of the YPEL3 promoter (Fig. 2D), a triple mutant (containing mutations to the critical nucleotides in each half site), and a randomly generated scramble sequence were cloned into pGL3-Basic. H1299 cells were plated at 60 k cells per well in 12-well plates 24 h before cotransfection with the indicated expression and luciferase vectors. The following day, the cells were lysed on the plate using 1× passive lysis buffer (Promega) and were analyzed by the Dual Luciferase Assay kit (Promega) according to the manufacturer’s instructions. The experimental reporter’s luciferase activity was normalized to Renilla luciferase activity measured in relative light units. All experiments for each treatment were performed in triplicate. Error bars show variation as the SD of each set of biological triplicates. Statistical significance was determined by using the paired student’s t test (Sigma Plot, P <0.05).

Immunoblotting. Whole-cell extracts and Western blotting were performed as previously described with the following modifications (14). Cell pellets were lysed in three freeze-thaw cycles using single lysis buffer [50 mmol/L Tris (pH 8.0), 150 mmol/L NaCl, 1% NP40] containing a protease inhibitor cocktail (Sigma). For endogenous YPEL3 and p53 Westerns, 10% to 20% gradient Tricine gels were used to separate 100 μg of protein extract. Proteins were transferred to polyvinylidene difluoride using a semidry transfer apparatus (Fisher Biotech). Antibodies were obtained from Proteintech (YPEL3) and Calbiochem (p53, Ab-4, and Ab-6).

Chromatin immunoprecipitation. Chromatin immunoprecipitation of the YPEL3 promoter region was performed using the ChIP-It Express Chromatin Immunoprecipitation kit (Active Motif) according to the manufacturer’s instructions. Briefly, six 15-cm culture plates of HCT116 cells expressing wild-type p53 were grown to 70% confluence after which doxorubicin (0.3 μg/mL) was applied to three plates. Following a 20-h incubation, the cells were fixed in formaldehyde and sonicated to average chromatin fragments of 300 to 500 bp. Overnight incubation of chromatin-protein cross-linked complexes with either 2 μg p53 DO-1 antibody or an equivalent amount of normal mouse IgG (Santa Cruz) and 50 μL of magnetic bead slurry (Active Motif) was carried out for each sample. PCR of enriched chromatin was performed using GoTaq master mix (Promega). PCR cycling parameters used were as follows: an initial denaturation step at 95°C for 2 min followed by 36 to 40 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 40 s with a final extension time of 5 min. Primers used will be provided upon request.

Lentiviral production and colony formation assay. Lentivirus was produced by the cotransfection of 293FT cells with a pLenti vector and lentiviral packaging mix (Invitrogen) according to the manufacturer’s instructions. Lentivirus-containing supernatant was harvested at 48 h posttransfection, purified by centrifugation, and stored at −80°C. MCF7 and U2OS cells containing tetracycline repressor (100,000 cells per well) were transduced with Lenti-YPEL3 or untreated followed by incubation in complete medium for 24 h. Infections...
were carried out overnight in the presence of 4 μg/mL Polybrene (Sigma). Following transductions, cells were selected with 750 and 500 μg/mL Zeocin, respectively. Tetracycline was used at a concentration of 1 μg/mL for induction of YPEL3-V5.

After 15 d, the cells were stained with 1% crystal violet in 70% methanol for 5 min. Plates were washed with PBS for 5 min and colonies were counted and then photographed. Each well was destained with 1 mL 10% acetic acid for 5 min and the absorbance recorded at 595 nm.

Cell viability assay. U2OS-TetR cells were transduced with Lenti-YPEL3 or Lenti-LacZ, selected with Zeocin, and then pooled populations of parental, YPEL3-infected or LacZ-infected U2OS-TetR cells were plated onto 96-well plates (1,000 cells per well). The next day, indicated cells were treated with 1 μg/mL tetracycline for an additional 24 h, after which the cells were refed with the medium containing 10% Cell Quanti-Blue reagent. Cells were incubated for 30 min, after which the cell medium was transferred to a black 96-well dish and fluorescence was detected using a Safire microplate reader following the manufacturer’s recommendation (BioAssay Systems). Each treatment condition was assessed in quadruplicate after the subtraction of fluorescence values from samples containing only the medium with the 10% Cell Quanti-Blue reagent.

Senescence-associated β-galactosidase staining. Cells were processed with the Senescence β-galactosidase Staining kit (Cell Signaling Technology) according to the manufacturer’s instructions and were visualized on an Olympus 1 × 70 fluorescence microscope.

Senescence-associated heterochromatic foci analysis. To examine senescence-associated heterochromatic foci (SAHF) formation, cells that were previously subjected to β-galactosidase staining were washed twice with Dulbecco’ phosphate-buffered saline (DPBS), permeabilized with 0.2% TritonX-100/ PBS for 10 min, stained with 25 μg/mL Hoechst dye for 5 min, and then washed twice with DPBS. Cells were examined under an Olympus 1 × 70 fluorescence microscope.

TissueScan tumor screen. TissueScan Ovarian Cancer Tissue qPCR Array HORT101 was purchased from OriGene. cDNA was rehydrated per manufacturer’s instructions and RT-PCR was performed as described above except that the GAPDH Assay on Demand was multiplexed with the YPEL3 Assay on Demand (Applied Biosystems, validated targets).

5-azadeoxycytidine treatment. A2780, C70, and C30 cell lines were plated at 15,000 per 10-cm plate. After 24 h, the medium was replaced and the cells were treated with 5-azadeoxycytidine at 0, 2.5, 5, and 10 μmol/L concentrations. Cells were refed with the appropriate dose of 5-azadeoxycytidine every 24 h for 5 d. Following 5 d of treatment, RNA was isolated as described above and quantified using the Nanodrop with 260/280 ratios ranging from 2 to 2.2. RT-PCR was then performed as described above. Error bars represent 95% confidence interval obtained from an individual experiment. These experiments were repeated in triplicate with similar results obtained. For CpG methylation, sodium bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation kit following manufacturer’s protocol (Zymo Research). Retrogen performed DNA sequencing of Topo-cloned PCR products. Primers for CpG island PCR fragments A through D will be made available on request.

Results

YPEL3 is a p53-regulated gene. YPEL3 possessed a gene expression profile in MCF7 cells transfected with various combinations of small interfering RNAs targeting Hdm2,

![Figure 1. YPEL3 increases in response to DNA damage.](image-url)

A, Hct116+/+p53 cells were exposed to increasing doses of doxorubicin (0–0.5 μg/mL) or (B) 76NTert mammary epithelial cells exposed to bleomycin (0–0.1 U/mL) for 24 h. YPEL3 and p21 mRNA levels were assayed by RT-PCR and normalized to GAPDH mRNA levels. Bars, 95% confidence intervals.

C, endogenous YPEL3 (left) and p53 (right) protein levels increase in Hct116+/+p53 cells exposed to 0.4 μg/mL doxorubicin for 40 h (compare untreated (-) to doxorubicin treated (+)). shYPEL3 represents Hct116+/+p53 cells transduced with a shYPEL3-expressing lentivirus.

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HdmX, and p53 consistent with it being a p53 target gene (10). To confirm that YPEL3 was in fact a p53-regulated gene, we first examined how endogenous YPEL3 mRNA and protein was affected by DNA damage. Increases in YPEL3 mRNA levels were seen in doxorubicin-treated Hct116 colon carcinoma cells (Fig. 1A) and 76Ntert-immortalized mammary epithelial cells exposed to the radiomimetic bleomycin (Fig. 1B). The DNA damage induction of YPEL3 was also observed at the protein level as an ∼15-kDa protein was increased 2-fold in doxorubicin-treated Hct116 cells (Fig. 1C). We believe this represents endogenous YPEL3 as Hct116 cells transduced with a shYPEL3 retrovirus results in the loss of the protein (Fig. 1C, shYPEL3). Additionally, the level of YPEL3 mRNA induction was significantly reduced in doxorubicin-treated Hct116 cells lacking p53 (Supplementary Fig. S1B). These findings are consistent with the p53 activation of YPEL3 in human tumor cells possessing wild-type p53.

Figure 2. YPEL3 is a p53-regulated gene. A, a 1.5-Kbp region of the YPEL3 promoter spanning −1386 to +120 relative to the start site of transcription was cloned upstream of a firefly luciferase reporter gene (YPEL3-luc). *, putative p53 half sites as determined by p53MH algorithm (15). Bottom, location of the three p53 half sites with the percentage of conservation to the p53 consensus response half site. B, YPEL3-luc activity was increased in Hct116−/− p53 (left) or H1299 (right) when cotransfected with a human p53 expression vector. YPEL3-luc luciferase activity was normalized by cotransfection with pCMV-Renilla-Luc and then by performing dual luciferase assay for both firefly and Renilla luciferase. Each assay was performed in triplicate (columns, mean; bars, SEM). Next, a truncation of region of the YPEL3 promoter spanning −486 to +120 was subcloned into the luciferase reporter vector used in A. The reporter was transfected into Hct116 cells harboring wild-type p53 and then subjected to DNA damage by doxorubicin treatment for 24 h before firefly luciferase analysis. Normalization was performed by cotransfection with a pSV40-β-galactosidase plasmid and then by performing β-galactosidase assays. C, a 50-bp region encompassing the region of the three putative p53 half sites near the YPEL3 promoter (black columns), a triple mutant where each half site harbored mutations at the critical C and G residues (gray columns), and a scrambled sequence (white columns) were cotransfected with increasing amounts of p53 plasmid. Bottom, the presence of p53 protein levels in all three sets of transfections. Relative luciferase activity represents firefly luciferase over Renilla luciferase reading normalized to no p53 transfections. D, chromatin immunoprecipitation assays were performed using undamaged or doxorubicin-treated Hct116−/+ p53 cells. Cross-linked chromatin was immunoprecipitated with p53 or IgG-negative control antibodies. In, input chromatin that was reverse cross-linked. The PCRs were performed using primers specific to regions A, B, and C. PCR product A contains the putative upstream p53 binding site showing in A. These primer sequences will be made available upon request.
firefly luciferase reporter vector. Based on a bioinformatic screen (15), two putative p53 binding sites were identified, one 1.3-Kbp 5′ of the YPEL3 promoter and a second immediately upstream of the YPEL3 promoter (Fig. 2A). Cotransfection of the YPEL3-luciferase reporter with wild-type p53 in either Hct116−/−p53 or H1299 cells led to an increase in YPEL3 promoter activity (Fig. 2B). Next, a deletion of the YPEL3-luciferase reporter was created, effectively removing the far upstream p53 binding site. When this reporter YPEL3(−485: +120)Luc was transfected into Hct116+/+ p53 cells, the addition of doxorubicin resulted in an increase in luciferase activity (Fig. 2B). Finally, when a 50-bp region encoding the p53 binding sites was cloned into a luciferase reporter construct, p53 was able to activate the reporter in contrast to a scrambled 50-mer (open columns) or a 50-mer in which critical p53 binding site nucleotides were mutated (Fig. 2C, gray columns).

Having established that the YPEL3 promoter could be activated by p53 when cloned into a plasmid reporter construct, we next set out to test whether p53 protein associated with the YPEL3 promoter in vivo. p53 chromatin immunoprecipitation assays were performed with Hct116 cells undamaged (nontreated) or damaged with doxorubicin. As expected, p53 was shown to bind in vivo to the YPEL3 promoter in DNA-damaged Hct116 cells (Fig. 2D).

YPEL3 induces growth inhibition by triggering cellular senescence. Murine YPEL3 (SUAP) has been found to inhibit cell growth and to induce programmed cell death in murine myeloid precursor cells (13). To test the biological effect of human YPEL3, we cloned the YPEL3 cDNA into a tetracycline-inducible expression vector. MCF7 and U2OS cells expressing the TetR repressor protein were infected with lentivirus carrying the YPEL3 construct and were placed under Zeocin selection. We added tetracycline to

Figure 3. Induction of YPEL3 leads to growth suppression. A, representative images of colony formation assays using MCF7TetR and U20STetR cells transduced with lentivirus containing a tet-inducible YPEL3-V5 cDNA, selected with Zeocin, and treated with and without 1 μg/mL Tetracycline (Tet). B, histograms of average colony numbers from colony formation assays were repeated in duplicate (U20STetR) or triplicate (MCF7TetR). Columns, mean; bars, SEM. C, top, Western blot showing YPEL3-V5 and LacZ protein expression in the indicated cell lines. Bottom, Q-RTPCR analysis of YPEL3 mRNA expression in MCF7TetR cells following treatment with tetracycline. D, MTT assay of U20STetR cells transduced with the indicated lentivirus and then treated for 24 h with or without tetracycline. Columns, mean of conditions performed in quadruplicate; bars, SEM.
induce YPEL3 expression to a level comparable with the increase in endogenous YPEL3 mRNA that was observed following DNA damage (Fig. 3C). Using a colony formation assay, both cell lines showed considerably fewer colonies when expressing YPEL3 compared with cells not induced to express YPEL3 (Fig. 3A and B). Growth suppression was also observed after shorter time periods of YPEL3 induction. When pooled populations of U2OS-TetR cells transduced with no construct (parental), LacZ, or YPEL3 were induced for 24 hours with tetracycline, only the YPEL3 tetracycline treated U2OS cells showed a significant decrease in cell viability (Fig. 3D).

Due to difficulties in the long-term growth of YPEL3-expressing cells, the inability to detect apoptosis, and observed morphologic changes, we examined the possibility that induction of YPEL3 would trigger cellular senescence. Two hallmarks of cellular senescence in human cells are the detection of increased acidic β-galactosidase activity (16) and the appearance of foci within the nuclei of senescent cells (SAHF; refs. 17, 18). Parental-, LacZ-, and YPEL3-V5–expressing U2OS-TetR cells were grown for 6 to 7 days in the presence or absence of tetracycline and then assayed for β-galactosidase activity or the presence of SAHF nuclei. A clear increase (~38%) in cellular senescence was observed in U2OS/YPEL3-TetR cells exposed to tetracycline (Fig. 4A and B). No increase in basal cellular senescence (~5%) was observed in parental or LacZ-transduced U2OS cells exposed to tetracycline. Similar levels of senescence were observed based on SAHF analysis (Fig. 4C and D). Induction of cellular

Figure 4. YPEL3 induction triggers cellular senescence. A and C, representative images of U2OS-TetR parental, U2OS-TetR/LacZ, and U2OS-TetR/YPEL3 cells treated with 1 μg/mL Tetracycline for 6 d and subjected to SA-β-galactosidase staining (A; ×100 magnification) or subjected to Hoechst dye staining (C; ×400 magnification). SA-β-galactosidase staining (B) or SAHF analysis (D) was quantified for three independent experiments in which a minimum of 100 cells were counted per treatment condition. Columns, mean percentage of positive cells; bars, SEM.
senescence was also observed in MCF7/YPEL3-TetR cells (Supplementary Fig. S2A) or when YPEL3 was transduced into IMR90 human diploid fibroblasts (Supplementary Fig. S2B and C). These findings, together with the observation that YPEL3 gene is activated by p53, suggest that YPEL3 protein may represent a novel p53-senescent activator.

YPEL3 functions downstream of p53 in ras-mediated premature senescence. IMR90 cells were transduced with (a) H-ras, (b) GFP, (c) H-ras + GFP, (d) YPEL3, (e) H-ras + YPEL3, (f) H-ras + shp53, (g) H-ras + shp53 + YPEL3, (h) H-ras + shYPEL3, and (i) H-ras + shLacZ and incubated under appropriate selection for 7 d. Cells were then subjected to SA-β-galactosidase staining (A) or SAHF analysis by Hoechst dye staining (B). SA-β-galactosidase images were captured at ×100 magnification. SAHF images were captured at ×400 magnification. Representative images from each treatment condition are shown. Histograms below images represent the average percentage of β-galactosidase (A) or SAHF-positive (B) cells from three biological replicate assays in which a minimum of 100 cells were counted per treatment condition. Columns, mean; bars, SEM.

In experiments to confirm YPEL3 overexpression and knockdown, we observed that H-ras induction did not trigger the expected increase in YPEL3 mRNA levels at day 7 post-transduction (Supplementary Fig. S4A, transductions A and C). Seven days after transduction, senescent cells were identified by β-galactosidase activity (Fig. 5A) or by SAHF (Fig. 5B). As expected, H-ras (Fig. 5a and c) and YPEL3 (Fig. 5d) triggered senescence in 80% of the transduced cells. The combination of H-ras and YPEL3 (Fig. 5e) did not result in an increase in senescent cells, suggesting that the two proteins may function in the same pathway. The decrease in cellular senescence when H-ras was coinfectected with a short hairpin RNA–targeting YPEL3 suggests that YPEL3 is a critical target in ras-mediated senescence (Fig. 5h). Finally, the shp53 block of H-ras-mediated premature senescence could be reversed by the addition of YPEL3 (Fig. 5f and g). This finding suggests that YPEL3 functions downstream of p53 signaling and leads us to propose that YPEL3 represents a novel p53-senescent activator. Similar experiments were performed using U2OS cells. Interestingly, although H-ras only induces ~20% of the U2OS cells to undergo senescence, YPEL3 is very potent in activating premature senescence in this tumor cell line (Supplementary Fig. S3).

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of p21 mirrors that reported by Mason and colleagues (19) for several known senescence inducible genes suggesting that molecular targets for senescence, such as YPEL3 and p21, are transiently induced as cells reprogram to a permanently arrested state.

**YPEL3 gene expression is reduced in human tumors.** Because **YPEL3** is a p53-regulated gene with the ability to trigger cellular senescence in normal and tumor cells, we reasoned that its expression should be downregulated in human tumors. In a screen of eight different human tumor
types, a significant decrease in YPEL3 mRNA expression was observed in lung, colon, and ovarian tumor samples relative to normal controls. In a larger screen of 30 ovarian tumors relative to 6 normal ovarian samples, we observed a significant decrease in YPEL3 in the tumor samples (P < 0.012; Fig. 6A). Analysis of the YPEL3 promoter uncovered a 950-bp CpG island, suggesting that YPEL3 downregulation may be occurring through CpG hypermethylation. Consistent with this model, the treatment of human ovarian cell lines harboring wild-type p53 (A2780), null for p53 (Cp70), or p53 mutant (C30) with increasing doses of 5-azadeoxycytidine, a DNA methyltransferase inhibitor, produced a significant increase in YPEL3 gene expression (Fig. 6B). Bisulfite treatment of genomic DNA from Cp70 cells treated with 5-azadeoxycytidine enabled us to identify a significant decrease in the hypermethylation of the YPEL3 CpG island (Fig. 6C). The decreased expression of YPEL3 in both human tumors and human tumor cell lines provide further support that YPEL3 possesses tumor suppressor–type activity.

**Discussion**

The *YPEL3* gene was first identified as SUAP, a small unstable apoptotic protein that seemed to be induced upon interleukin-3 removal in a myeloid precursor cell line (13). Later SUAP received its current identification as *YPEL3* due to it being one of five human genes with homology to the *Drosophila* Yippee protein (11). Our interest in *YPEL3* originated from a microarray experiment in which we reactivated p53 in MCF7 cells by using RNAi approaches targeting Hdm2 and HdmX, negative regulators of p53, and observed an induction of *YPEL3* gene expression (10). In the present study, we provide data supporting *YPEL3* as a novel p53-regulated gene. These findings are supported by the observation that *YPEL3* seems to be a putative p53-regulated gene based on computational and microarray analyses (20). Using a variety of biochemical and molecular approaches, we have shown that *YPEL3* is a p53-inducible gene (Figs. 1 and 2). It is important to note that in the course of these studies, we uncovered that *YPEL3* is also induced by 1A isoforms of p63 and p73.5 Given that *YPEL3* activation leads to growth arrest (Fig. 3) and is expressed in epithelial tissue (11), a tissue in which p63 has important functional attributes, we are currently exploring the possibility that *YPEL3* may play a role in p63-mediated differentiation of epithelial cells.

As mentioned previously, we anticipated that induction of human YPEL3 may trigger apoptosis but have been unable to show any consistent apoptotic response using sub-G1 or poly ADP ribose polymerase cleavage indicators of programmed cell death. In contrast, our data strongly supports that the decrease in cell growth observed by colony formation or MTT-based assays (Fig. 3) results from the induction of cellular senescence (Supplementary Figs. S3 and S4; Figs. 4 and 5). We were able to trigger premature cellular senescence in primary cells and two tumor cell lines (U2OS and MCF7), using overexpression by viral transduction and controlled tet-regulated expression to induce a physiologically relevant expression. Because recent results indicate that Nutlin, a small-molecule inhibitor of Hdm2:p53 association, can trigger reversible β-galactosidase activity (21), cellular senescence was confirmed in these studies using both β-galactosidase activity and the detection of senescent-activated heterochromatin foci.

Several important findings regarding the signaling of senescence by *YPEL3* were uncovered in this study. First, induction of *YPEL3* alone is sufficient to trigger cellular senescence in all three cell lines studied (IMR90, U2OS, and MCF7). Experiments are currently ongoing to determine whether other known senescence-related proteins within the p53 signaling pathway are required to be present during this *YPEL3*-initiated cellular senescence. Our data with IMR90 and U2OS cells are also consistent with previous reports showing that the loss of p53 can block H-ras–mediated cellular senescence (18). This has allowed us to show another critical finding, namely that YPEL3 could rescue cellular senescence when cotransduced with H-ras + shp53–expressing lentivirus. Taken with the fact that shYPEL3 could block H-ras–mediated premature senescence (Fig. 5h), we conclude that *YPEL3* functions downstream of p53 signaling consistent with our demonstration that *YPEL3* is a p53-regulated gene.

Finally, consistent with our model that *YPEL3* represents a novel tumor suppressor gene, we have shown in ovarian (Fig. 6), lung (data not shown), and colon tumors4 that *YPEL3* gene expression is reduced in tumor samples relative to normal tissue. In Cp70 ovarian cells, the molecular basis of this downregulation seems to be the hypermethylation of a CpG island immediately upstream of the *YPEL3* promoter (Fig. 6C). The induction of *YPEL3* mRNA expression in ovarian cell lines treated with the DNA methylation inhibitor 5-azadeoxycytidine confirms that DNA hypermethylation can affect *YPEL3* expression. Thus, the combination of low doses of demethylating agents with standard chemotherapy treatments may represent an effective approach toward the reactivation of *YPEL3* in human tumors and subsequent induction of the tumor cell senescence pathway.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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A. Todd, personal communication.

R. Tuttle, manuscript in preparation.

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4 R. Tuttle, manuscript in preparation.
References

8. Pantoja C, Serrano M. Murine fibroblasts lacking p21 undergo senescence and are resistant to transformation by oncogenic Ras. Oncogene 1999;18:4974–82.
YPEL3, a p53-Regulated Gene that Induces Cellular Senescence

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