Oncogenes and Tumor Suppressors

p53 Maintains Baseline Expression of Multiple Tumor Suppressor Genes

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Abstract

TP53 is the most commonly mutated tumor suppressor gene and its mutation drives tumorigenesis. Using ChIP-seq for p53 in the absence of acute cell stress, we found that wild-type but not mutant p53 binds and activates numerous tumor suppressor genes, including PTEN, STK11(LKB1), mir-34a, KDM6A(UTX), FOXO1, PHD3A, and TNFRSF10B through consensus binding sites in enhancers and promoters. Depletion of p53 reduced expression of these target genes, and analysis across 18 tumor types showed that mutation of TP53 associated with reduced expression of many of these genes. Regarding PTEN, p53 activated expression of a luciferase reporter gene containing the p53-consensus site in the PTEN enhancer, and homozygous deletion of this region in cells decreased PTEN expression and increased growth and transformation. These findings show that p53 maintains expression of a team of tumor suppressor genes that may together with the stress-induced targets mediate the ability of p53 to suppress cancer development. p53 mutations selected during tumor initiation and progression, thus, inactivate multiple tumor suppressor genes in parallel, which could account for the high frequency of p53 mutations in cancer.

Implications: In this study, we investigate the activities of p53 under normal low-stress conditions and discover that p53 is capable of maintaining the expression of a group of important tumor suppressor genes at baseline, many of which are haploinsufficient, which could contribute to p53-mediated tumor suppression. Mol Cancer Res; 1–12. ©2017 AACR.

Introduction

TP53 germline mutation is associated with Li-Fraumeni cancer susceptibility syndrome, and its somatic mutation occurs in about half of all cancers (1). p53-null or mutant mice develop spontaneous tumors in multiple organs (2). p53 is a transcription factor that binds DNA in a sequence-specific manner (3), and the majority of missense mutations that inactivate p53 in cancer are clustered within the DNA-binding domain (1). p53 is well established to mediate acute stress response in the cell by activating known targets including CDKN1A(p21), GADD45a, BBC3 (PUMA), and NOXA (4). Interestingly, p53 stress response targets that regulate apoptosis, cell-cycle arrest, and senescence are not required for p53-mediated tumor suppression in genetically engineered model systems (5–7). p53 is expressed at measurable levels in various tissues of the body in the absence of acute stress (8). Basally expressed p53, which has been attributed to various physiologic stresses (9, 10), activates the constitutive expression of CDKN1A(p21), MDM2, and mir-34a through consensus binding sites in cis elements (11–15).

Inactivation of both alleles of a tumor suppressor can be necessary for tumor development; but for haploinsufficient tumor suppressors, inactivation of just one allele is sufficient to cause tumor development alone or in cooperation with other gene mutations (16). Here, we show that basally expressed p53 acts as a transcriptional activator of multiple tumor suppressors, several of which are dose responsive in mice (17–20), a finding that suggests that they may play a role in p53-mediated tumor suppression.

Materials and Methods

Cell culture

MCF10A, MCF7, and HCC38 cell lines were purchased from the ATCC. The U2OS cell line was a gift from Dr. James Manfredi (purchased from the ATCC). Cell lines were obtained between 2010 and 2015. The ATCC authenticates cell lines using several
methods, including DNA fingerprinting. Cell lines were clear of Mycoplasma as determined by the Lonza Kit (L107-418) within 6 months of their use. Cell lines were further authenticated in 2015 by LabCorp using a short tandem repeat method. All cells were cultured at 37°C and 5% CO₂. See the Supplementary Materials and Methods for cell line-specific culturing conditions.

Transient knockdown of p53
Cells were transfected using the Lipofectamine 2000 reagent (Thermo 11668019) in Opti-MEM media (Invitrogen 11058021). ON-TARGET plus Human TP53 siRNA (SMARTpool and individual) and MISSION siRNA Universal Negative Control (Sigma SIC001) were used. The cells were harvested 48–96 hours posttransfection, as indicated. Additional detail is provided in the Supplementary Materials and Methods, including the specific siRNA oligos used.

Luciferase reporter assay
H1299 cells were seeded at 2 × 10⁵ cells per well of Falcon 6-well dishes. The transfections were carried out the following day using Lipofectamine (18324-020) and Plus (11514-015) reagents according to the manufacturer’s instructions. The cells were harvested 24 hours later using reagents supplied by the Luciferase Assay System (Promega E1910). The total protein concentration was determined using the Bio-Rad Protein Assay reagent (Bio-Rad Protein Assay System (Promega E1910)). The total protein concentration was used to normalize luciferase expression. The cells were harvested in 1X PBS using Lipofectamine (18324-020) and Plus (11514-015) reagents according to the manufacturer’s instructions and were quantitated using a TD-20e Luminometer (Turner). Luciferase expression is normalized to protein and expressed as Luciferase units/μg protein. Plasmids for the luciferase reporter assay are shown in the Supplementary Materials and Methods.

CRISPR of Hu PTEN-eP53RE
Cloning of the custom CRISPR guides targeting Hu PTEN-eP53RE was performed according to the LentiCRISPRv2 vector cloning protocol in the Lentiviral CRISPR toolbox from the Zhang laboratory that uses single guide RNAs (21, 22). The custom targeting oligos used are shown in the Supplementary Materials and Methods. The LentiCRISPRv2 (Addgene #52961) plasmid backbone with the custom guide targeting Hu PTEN-eP53RE was named p53-PTEN-LentiCRISPRv2. Lentivirus was produced in HEK-293T cells as previously described (23). Limiting dilution was used to isolate single colonies, DNA from which was amplified and sequenced by Genewiz using the primers listed in the Supplementary Materials and Methods.

Chromatin immunoprecipitation (ChIP-qPCR and ChIP-seq)
Chromatin immunoprecipitation (ChIP) assays were performed as previously described (24). Cells were cross-linked in formaldehyde for 10 minutes and sonicated for 20 minutes (30s on, 30s off) on the Diagonen Bioruptor Twin (UCD-400) sonicator at 4°C. For ChIP-seq, cells were sonicated similarly for 40 minutes. Lysates were precleared for 1 hour with the appropriate beads (For Chip-qPCR: Protein A Agarose/Salmon Sperm DNA beads EMD Millipore 16-157, and for ChIP-seq: Magna ChIP Protein A-G Magnetic Beads EMD Millipore 16-663). Precleared lysates were then incubated with 7 μg of antibody overnight at 4°C (3 million cells per antibody). Samples were then incubated with appropriate beads (same as preclear) for at least 2 hours at 4°C and beads were repeatedly washed. The Protein-DNA complexes were eluted, crosslinks were reversed, and DNA was purified using phenol/chloroform extraction followed by sodium acetate/ethanol precipitation. For ChIP-seq, DNA was purified again using the Qiaquick PCR Purification Kit (Qiagen 28104). Purified DNA was then subject to qPCR (ChIP-qPCR) or high throughput sequencing (ChIP-seq). For ChIP-qPCR, % input was calculated and normalized as a fold change from IgG. Antibodies used were p53 (DO1-X, sc-126X), H3K27Ac (Millipore 07-360), H3 (ab 1791), and IgG (sc-2025).

ChIP-qPCR primers and details regarding the ChIP-seq data analysis pipeline are located in the Supplementary Materials and Methods.

Three-dimensional cell culture assay
Three-dimensional culture assays were performed as previously described (PMID: 12798140) in the 8-well chamber slides (BD Falcon 08-774). Assay medium (DMEM/F12 supplemented with 2% horse serum, 10 μg/mL insulin, 1 ng/mL cholaeca toxin, 100 μg/mL hydrocortisone, 50 units/mL penicillin, and 50 μg/mL streptomycin) containing 5 ng/mL epidermal growth factor and 2% growth factor–reduced Matrigel (BD Biosciences 356221). Media were replaced every 4 days. Cells were collected using Cell Recovery Solution (BD Biosciences 354253) to remove Matrigel.

Immunofluorescence on 3D cultures
Cultures were fixed with 4% paraformaldehyde/PBS, permeabilized with 0.5% Triton X-100/PBS, and washed 3 times with PBS buffer (137 mmol/L NaCl, 2.7 mmol/L KCl, 4.3 mmol/L Na₂HPO₄, 1.47 mmol/L KH₂PO₄. Adjust to a final pH of 7.4). Primary antibodies were suspended in IF buffer (3% BSA, 0.1% NP-40 in PBS) and incubated overnight. The cultures were washed with 0.1% NP-40/PBS 6 times and incubated with Alexa fluorophore–conjugated rabbit or mouse secondary antibodies (Life Technologies) in IF buffer. The slides were washed with 0.1% NP-40/PBS 6 times, counterstained with 4′, 6-diamidino-2-phenylindole (DAPI), and mounted with antifade solution (Life Technologies). Confocal microscopy was done using the Zeiss LSM 780 laser scanning confocal microscope (Carl Zeiss AG), p- AKT(S473) (4060) and cleaved caspase-3 (9664) antibodies were from Cell Signaling Technology. Rabbit polyclonal antibody against Ki67 (ab833) was from Abcam, and Mouse anti-laminin-5 (MAB1947) was from EMD Millipore. Alexa Fluor 488 anti-rabbit (A11008) and Alexa Fluor 594 anti-mouse (A21201) secondary antibodies, and Prolong Gold antifade reagent with DAPI (P36931) were obtained from Life Technologies.

Results
p53 targets a group of tumor suppressor genes in the absence of acute stress
Breast cancers are known to harbor abnormalities in the tumor suppressors p53 and PTEN; hence, we examined their relationship in primary breast tumors. We observed that mutation of TP53 was associated with decreased PTEN transcript (P < 0.05) and protein levels (P < 0.01), and enrichment of the previously published PTEN-loss signature (ref. 25; Supplementary Fig. S1A). This result was confirmed by qRT-PCR in tumors from the same cohort (P < 0.05, Supplementary Fig. S1B). The idea that p53 expressed in breast tissues could regulate baseline expression of PTEN is
interesting because PTEN is a dose-dependent tumor suppressor whereby only a 20% deficit in its expression can lead to tumor development in mice (17).

We sought to explore the possibility that basal wild-type p53 could have targets such as PTEN that are important for tumor suppression. In the absence of acute stress, we performed ChIP-seq for basal p53 in nontransformed human mammary epithelial MCF10A cells (wild-type for p53) and ranked the peaks by significance. Examination of the ranked list of peaks revealed that eleven of the first 200 peaks \((q < 10^{-5})\) were within or adjacent to ten known tumor suppressor genes, including PTEN. The number of tumor suppressor genes associated with peaks increased slightly to fourteen when the top 800 peaks \((q < 10^{-5})\) were queried (Fig. 1A left, Supplementary Spreadsheet S1). These tumor suppressor genes were also among the top basal p53 peaks when ranked by sequence read pileup before processing for significance (Supplementary Fig. S1C). The basal p53-binding sites at known tumor suppressors were STK11 (LKB1), miR-34a, CDKN1A (p21) both 5' and 3' sites, PHLD3, DLEU1, FAT1, KDM6A (UTX), TNFRSF10B, FOXO1, PTEN, WT1, SMARCA2 (BRM), and BMPRIA (Fig. 1A; Supplementary Fig. S2). These tumor suppressors are highly validated using evidence from mouse models, inherited cancer predisposition syndromes, and somatic mutations in human cancer (Supplementary Table S1). We also observed that basal p53 binds near other genes, including known basal p53 target MDM2 (ref; 14; Supplementary Fig. S2). Others have performed basal wild-type p53 ChIP-seq on U2OS cells as a control for comparison to the pattern of peaks detected after DNA damage or p53 stabilization (26). We used their control p53 ChIP-seq data to determine whether targets of basal p53 in MCF10A cells were bound in another low-stress context. When comparing the two ranked lists, all of the tumor

Figure 1.
Basally expressed p53 binds to multiple other important tumor suppressors. A, Basal p53 ChIP-seq peaks ordered by significance in MCF10A (left) and U2OS cells (right). \(r\) is Spearman coefficient, and corresponding \(P\) value is reported. B, GSEA of basal MCF10A p53 ChIP-seq list (ordered by significance) for TSGs and Oncogenes as classified in the Cancer Gene Census from COSMIC. Enrichment scores and \(P\) values are indicated. C, ChIP-seq traces show fold enrichment over input for genes of interest in MCF10A (left) and U2OS (right) cells. Location of peak is indicated by triangle. Scales are indicated and start at zero. D, ChiP-qPCR of p53 binding to PTEN (left) and 5' CDKN1A(p21; right) loci in MCF10A and U2OS cells transfected with control or p53-targeting siRNA (pool of 4 siRNAs). Relative DNA Binding is the percentage of input (normalized to IgG). Error bars, mean \(\pm\) SD of representative experiment (performed twice), triplicate measurements. Significance: two-way ANOVA, Sidak correction. E, ChiP-qPCR for basal p53 (left) and H3K27Ac (right) on the STK11(LKB1), PTEN, and 5' CDKN1A (p2) loci in PBMCs from a healthy donor. Error bars: mean \(\pm\) SD, triplicate measurements. Significance (over IgG): paired \(t\) tests. (***, \(P < 0.0001\); ***, \(P < 0.001\); ***, \(P < 0.01\); *, \(P < 0.05\)).
p53 maintains expression of a group of tumor suppressor genes through consensus p53-binding sites in enhancers and promoters

p53 ChIP-seq studies have been published to investigate properties of wild-type p53 after its induction or baseline mutant p53, but the baseline wild-type peaks were not analyzed (26, 28–30). We examined these baseline p53 ChIP-seq datasets from three nontumorigenic normal cell lines containing wild-type p53 (MCF10A, IMR90, and HFKs), three cancer cell lines containing wild-type p53 (U2OS, MDA-MB-175-VII, and MCF7) and three cancer cell lines containing mutant p53 (BT-549, HCC-70, and MDA-MB-468) and found that both normal and cancer cell lines containing wild-type p53 had significantly more basal p53-binding peaks near TSGs than the p53-mutant group, and the p53-mutant group did not have any basal p53 peaks near TSGs (Table 1, C, P < 0.05; refs. 26, 28–30). We also determined that the R273L mutation abrogated p53 binding to PTEN or 5′-CDKN1A sites in HCC38 cells (Supplementary Fig. S4A).

We next explored the possibility that p53 mutation is associated with lower target gene expression in cancer. Using data from The Cancer Genome Atlas (TCGA) from 4899 cases across 18 types of cancer, we found that TP53 mutation significantly increased the odds of transcriptional downregulation (by ≥ 1 SD) of tumor suppressor targets of basal p53 (PHLDA3, TNFRSF10B, and PTEN, Fig. 2) in cases with normal copy number, and most of the tumor suppressor targets showed this trend to some degree. For example, STK11 had a weaker association (Supplementary Fig. S4B). This association was also detected in
many cancer types when this analysis was performed without correcting for copy number change (Supplementary Fig. S4C).

To determine whether basal p53 regulates the mRNA expression of the identified binding targets, we performed a siRNA knockdown of p53 in MCF10A cells and measured transcript levels of select targets. With the exception of DLEU1, WT1, and SMARCA2, knockdown of p53 in MCF10A cells decreased mRNA of all of the identified binding targets and lowered protein levels for the three candidates that were tested (Fig. 3A and B, validated with individual siRNAs in Supplementary Fig. S4D). Knockdown of p53 in primary human mammary epithelial cells (hMEC; ref. 31) also decreased mRNA for six out of seven of the tested TSGs (Supplementary Fig. S4E). Analysis of eleven of the top 400 q<0.001; **, P < 0.01; *, P < 0.05). To examine the epigenetic landscape near the basal p53-binding sites, we used previously published ChIP-seq data from hMEC cells (33, 34). Chromatin marks histone 3 lysine 4 monomethylation (H3K4me1), histone 3 lysine 4 trimethylation (H3K4me3), and histone 3 lysine 27 acetylation (H3K27Ac) in combination with the distance from the TSS were used to classify each basal p53-binding region as a promoter or an enhancer. We found that 9 of 14 of the tumor suppressor binding sites were located in enhancers (7 intergenic, 2 intragenic; 4 of 14 were in promoters; CDKN1A(p21) had both promoter and enhancer peaks, and BMPRIA did not fit either category (Supplementary Table S3). The intergenic enhancer elements were located between 2 and 450 kb from the TSS of TSGs. These results show that basal p53 binds to DNA elements that activate transcription of tumor suppressors. For example, the basal p53-binding sites near PTEN, STK11, and KDM6A are all located in active enhancers (Fig. 3D).

Using JASPAR software, which predicts transcription factor DNA-binding sites, we found that the basal p53 ChIP-seq peak for all of the tumor suppressors contains a predicted p53 response element (Fig. 3C; Supplementary Table S2). These sites have no spacer DNA and obey the classic p53 consensus site (ref. 3; RRRCWWGYYY-RRRCWWGYYY, where R = purine, Y = pyrimidine, W = adenine or thymine), suggesting that binding is mediated by the DNA sequence motif. Furthermore, we examined previously published Hi-C (32) and chromatin state segmentation using the ChromHMM method (33) and determined that physical interaction exists between basal p53-binding sites distal to TSGs (20 kb or more from the transcriptional start site, TSS) and the gene promoter/TSS of the TSGs (Supplementary Fig. S5). Collectively, our results suggest that these tumor suppressors are direct targets of p53 in a basal, low-stress context.

To determine whether basal p53 regulates the mRNA expression of the identified binding targets, we performed a siRNA knockdown of p53 in MCF10A cells and measured transcript levels of select targets. With the exception of DLEU1, WT1, and SMARCA2, knockdown of p53 in MCF10A cells decreased mRNA of all of the identified binding targets and lowered protein levels for the three candidates that were tested (Fig. 3A and B, validated with individual siRNAs in Supplementary Fig. S4D). Knockdown of p53 in primary human mammary epithelial cells (hMEC; ref. 31) also decreased mRNA for six out of seven of the tested TSGs (Supplementary Fig. S4E). Analysis of eleven of the top 400 q<0.001; **, P < 0.01; *, P < 0.05). To examine the epigenetic landscape near the basal p53-binding sites, we used previously published ChIP-seq data from hMEC cells (33, 34). Chromatin marks histone 3 lysine 4 monomethylation (H3K4me1), histone 3 lysine 4 trimethylation (H3K4me3), and histone 3 lysine 27 acetylation (H3K27Ac) in combination with the distance from the TSS were used to classify each basal p53-binding region as a promoter or an enhancer. We found that 9 of 14 of the tumor suppressor binding sites were located in enhancers (7 intergenic, 2 intragenic; 4 of 14 were in promoters; CDKN1A(p21) had both promoter and enhancer peaks, and BMPRIA did not fit either category (Supplementary Table S3). The intergenic enhancer elements were located between 2 and 450 kb from the TSS of TSGs. These results show that basal p53 binds to DNA elements that activate transcription of tumor suppressors. For example, the basal p53-binding sites near PTEN, STK11, and KDM6A are all located in active enhancers (Fig. 3D).

A p53-dependent enhancer maintains expression of PTEN

The basal p53-binding site upstream of PTEN is representative because the predicted binding site has no spacer DNA, it was classified as an enhancer, and it is distal (~20 kb upstream) to the TSS of its target gene. In fact, H3K27Ac and H3K4me1 peaks occur with this site across many cell types (Supplementary Fig. S6). Because the p53-binding site on PTEN is in an enhancer based on histone marks, we named this site PTEN-pP53RE and a previously characterized p53 response element in the PTEN promoter PTEN-pP53RE (refs. 35, 36; Supplementary Fig. S7A). p53 is known to bind to PTEN-pP53RE in response to stress, resulting in a subtle increase in PTEN levels in certain contexts (35, 36). The PTEN gene locus is also in proximity with the ATAD1, CFL1P1, and KLLN loci (Supplementary Fig. S7A), and as shown in Supplementary Fig. S5, physical interaction occurs between the basal p53 binding site...
Figure 3.
p53 maintains baseline expression of its tumor suppressor target genes. A, MCF10A cells were transfected with control or p53-targeting siRNAs (pool of 4 siRNAs), transcript levels were measured by qRT-PCR. Error bars, mean ± SD of representative experiment (performed twice), triplicate measurements. Significance: two-way ANOVA, Sidak correction (**, P < 0.0001). B, Western blots measuring KDM6A(UTX), STK11(LKB1), p53, and PTEN at 48, 72, and 96 hours post-transfection. β-Actin is loading control. Blot is quantified below (signal normalized to β-Actin, shown as fold change from siCtrl 48 hours). C, Alignment of p53 consensus DNA-binding sites. Includes gene, location of p53 peak, and p53 consensus binding site sequence. Deviations from the known p53 consensus are highlighted in red. D, p53-bound DNA near PTEN, STK11, and KDM6A has H3K27Ac and H3K4me1 enhancer marks. For each gene, top: basal p53 ChIP-seq in MCF10A cells (fold enrichment over input), and bottom (2 tracks): published data of enrichment of H3K4me1 and H3K27Ac ChIP-seq in hMEC cells (33,34). p53 peaks are indicated (red triangle). Sequence of p53 consensus site is shown.
and these genes in lymphoblastoid cells. We found that p53 knockdown in MCF10A and U2OS cells led to decreased transcript levels of ATAD1 and KLLN, while CFL1P1, a pseudogene, was not expressed (Supplementary Fig. S7B). Therefore, the p53-bound enhancer for PTEN also regulates other genes in the locus, as is typical for enhancers. In freshly isolated PBMCs, p53 is strongly bound to PTEN-eP53RE but not PTEN-pP53RE (Supplementary Fig. S7C). Furthermore, PTEN-eP53RE is highly conserved in many mammals (Supplementary Fig. S8A–S8B). Interestingly, p53 knockdown led to a decreased H3K27Ac signal at PTEN-eP53RE in U2OS and MCF10A cells, suggesting loss of p53-dependent enhancer activity (Supplementary Fig. S8C–S8D). To investigate whether the sequence of PTEN-eP53RE can activate transcription, we overexpressed empty vector or p53 along with luciferase reporter plasmids containing 5’CDKN1A-eP53RE (a known p53 response element located in an intergenic enhancer), PTEN-eP53RE, or PTEN-pP53RE. 5’CDKN1A-eP53RE and PTEN-eP53RE were able to strongly activate transcription, whereas PTEN-pP53RE activates transcription to a lesser extent (Fig. 4A).

We then investigated the dynamics of regulation of the basal p53 tumor suppressor targets using the p53-stabilizing drug Nutlin-3 in wild-type p53 MCF10A, U2OS and MCF7 cells. MCF7 cells, which had low basal p53 binding to tumor suppressor targets (Table 1), displayed significantly increased expression of PTEN, KDM6A, PHLDA3, TNFRSF10B, and CDKN1A after Nutlin-3 treatment (Fig. 4B). However, induction was limited to CDKN1A in MCF10A and U2OS cell lines with higher basal binding to tumor suppressor targets, possibly due to saturation of the targets at baseline (Table 1; Fig. 4C and D). MCF7 cells harbor a SNP in MDM2 that leads to increased transcriptional activation of MDM2 (37). This SNP could cause reduced basal p53 levels and binding to tumor suppressors (37), allowing for restoration of tumor suppressor expression upon Nutlin-3 treatment.

We discovered two cancer cases from TCGA that harbor somatic focal deletions of the enhancer containing PTEN-eP53RE (called as deep deletions, likely homozygous), both of which had down-regulated PTEN transcript levels by RNA-seq (Supplementary

Figure 4.
Transcriptional activation of tumor suppressor targets of p53 at baseline and after p53 induction. A, Luciferase reporter assay showing luciferase/Renilla activity (fold change/empty vector) in H1299 (p53-null) cells overexpressing p53 with a luciferase reporter vector containing 5’CDKN1A-eP53RE, PTEN-eP53RE, or PTEN-pP53RE (constructs shown); error bars, mean ± SEM; n = 3 experiments. Significance: two-tailed Student t test. B, MCF7 cells treated with Nutlin-3 for 24 hours; error bars, mean ± SD, repeated twice. Transcripts measured by qRT-PCR. Significance: two-tailed Student t test. C, MCF10A cells treated with 10 μmol/L Nutlin-3 for 6, 10, and 24 hours; error bars, mean ± SD, triplicate measurements. D, U2OS cells treated with 10 μmol/L Nutlin-3 for 24 hours; error bars, mean ± SEM, n = 4 experiments, triplicate measurements. Significance: one-way ANOVA, Dunnnett correction. (***, P ≤ 0.0001; ***, P ≤ 0.001; **, P ≤ 0.05; n.s., P > 0.05).
Loss of PTEN-eP53RE results in downregulation of PTEN and increased tumor cell phenotypes. Given that the region of chromatin containing PTEN-eP53RE is an active enhancer for PTEN, we wanted to characterize its effect on growth properties of the cells. MCF10A clones showed no differences in proliferation under low serum conditions and did not grow in soft agar (Supplementary Fig. S10A). In U2OS cells, we also observed increased pAkt(Thr308) in Clone 1 (PTEN-eP53RE/−−; Fig. 5D). In U2OS Clone 1 (PTEN-eP53RE/−−), because the deletion includes only half of PTEN-eP53RE, we used ChIP-qPCR to test whether this prevents p53 binding. As expected, p53 no longer binds at PTEN-eP53RE in Clone 1 (Fig. 5E). To confirm that the decrease in PTEN expression in the PTEN-eP53RE/−− clones is dependent on PTEN-eP53RE, we performed a p53 knockout in U2OS Empty Vector cells and Clone 1 (PTEN-eP53RE/−−) and discovered that a decrease in PTEN expression only occurred when PTEN-eP53RE was present (Supplementary Fig. S10A–S10B).

Deletions in the p53-dependent enhancer for PTEN induce cell transformation phenotypes

Given that the region of chromatin containing PTEN-eP53RE is an active enhancer for PTEN, we wanted to characterize its effect on growth properties of the cells. MCF10A clones showed no differences in proliferation under low serum conditions and did not grow in soft agar (Supplementary Fig. S10C); hence, we no differences in proliferation under low serum conditions and decreased (Fig. 5I) compared with Empty Vector. Similar results for pAKT(S473), Ki67, and cleaved Caspase-3 revealed that pAKT (S473) and Ki67 levels were increased in acini from clones 5 (PTEN-eP53RE/−−; ref. Fig. 5C). In U2OS cells, we also observed increased pAkt(Thr308) in Clone 1 (PTEN-eP53RE/−−; Fig. 5D). In U2OS Clone 1 (PTEN-eP53RE/−−), because the deletion includes only half of PTEN-eP53RE, we performed ChIP-qPCR to test whether this prevents p53 binding. As expected, p53 no longer binds at PTEN-eP53RE in Clone 1 (Fig. 5E). To confirm that the decrease in PTEN expression in the PTEN-eP53RE/−− clones is dependent on PTEN-eP53RE, we performed a p53 knockout in U2OS Empty Vector cells and Clone 1 (PTEN-eP53RE/−−) and discovered that a decrease in PTEN expression only occurred when PTEN-eP53RE was present (Supplementary Fig. S10A–S10B).

Figure 5.
Loss of PTEN-eP53RE results in downregulation of PTEN and increased tumor cell phenotypes. A and B, qRT-PCR of CRISPR/Cas9 clones in (A) MCF10A and (B) U2OS cells (PTEN-eP53RE/−− clones in red). Error bars: mean ± s.d., triplicate measurements. Significance: one-way ANOVA, Sidak correction. C and D, Western blots of MCF10A (C) and U2OS clones (D). Vinculin and total-AKT were loading controls. E, ChIP-qPCR for p53 in U2OS clones on PTEN-eP53RE. Relative DNA binding is the percentage input (normalized to IgG). Error bars: mean ± SD of representativ expression (performed twice), triplicate measurements. Significance: two-way ANOVA, Sidak correction. F, MCF10A clones grown in 3D culture for 20 days, representative of data shown, acinar size is quantified. Error bars: mean ± SD of representative expression, triplicate measurements. Significance: one-way ANOVA, Dunnett correction. Representative immunofluorescence staining for Laminin V (red, all rows), pAkt(Thr473) (green), Ki67 (H), and cleaved Caspase-3 (red), DAPI (blue, all rows), and Merge (right, all rows) scale bars, 100 μm. Quantifications on right. Error bars: mean ± SD of representative expression, triplicate measurements. Significance: two-tailed t test. J, Proliferation assay of U2OS clones in low serum showing the percentage of confluence over time (days). Triplicate readings taken every 6 hours; error bars, mean ± SD. K, Soft agar colony formation assay of U2OS clones showing colonies/well after 3 weeks (triplicate measurements); error bars, mean ± SD of representative expression (performed twice), significance: one-way ANOVA, Sidak correction. (⁎⁎⁎, P ≤ 0.0001; *, P ≤ 0.001; †††, P ≤ 0.01; ‡‡‡, P ≤ 0.05).
development of methodology: Authors’ Contributions

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure 6.
The basal targets of p53 may contribute to p53-mediated tumor suppression. The low levels of p53 present under normal physiologic stress activate a set of basal targets. Mutation of p53 could cause spontaneous tumorigenesis through the loss of expression of tumor suppressor targets of basal p53, combined with the inability of p53 to appropriately activate stress-responsive targets.
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