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Modulation of the Vitamin D3 Response by Cancer-Associated Mutant p53

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SUMMARY

The p53 gene is mutated in many human tumors. Cells of such tumors often contain abundant mutant p53 (mutp53) protein, which may contribute actively to tumor progression via a gain-of-function mechanism. We applied ChIP-on-chip analysis and identified the vitamin D receptor (VDR) response element as overrepresented in promoter sequences bound by mutp53. We report that mutp53 can interact functionally and physically with VDR. Mutp53 is recruited to VDR-regulated genes and modulates their expression, augmenting the transactivation of some genes and relieving the repression of others. Furthermore, mutp53 increases the nuclear accumulation of VDR. Importantly, mutp53 converts vitamin D into an antiapoptotic agent. Thus, p53 status can determine the biological impact of vitamin D on tumor cells.

INTRODUCTION

The p53 tumor suppressor is a major barrier against cancer progression. The p53 pathway is impaired in almost all human cancers (Vogelstein et al., 2000). About 50% of human cancers carry p53 mutations (Soussi and Wiman, 2007), mostly missense mutations resulting in overproduction of mutant p53 (mutp53) protein (Weisz et al., 2007b). This might imply a strong selection for mutp53 expression in carcinogenesis. Indeed, p53 mutations result not only in loss of tumor-suppressing activities by the mutant allele but also in *trans*-dominant inactivation of the remaining wild-type p53 (wtp53) (Shaulian et al., 1992). Importantly, at least some cancer-associated mutp53 variants acquire oncogenic activities, defined as gain of function (GOF) (Weisz et al., 2007a). Specifically, mutp53 can enhance proliferation, survival, and tumorigenicity in mice (Bossi et al., 2006; Weisz et al., 2004). Furthermore, at least for some types of cancer, patients harboring particular missense p53 mutations in their tumors tend to be less responsive to chemotherapy (Soussi and Beroud, 2001).

Mechanistically, mutp53 can exert a dominant-negative effect over the p53 family members p63 and p73 and inhibit their biochemical and biological activities (Irwin et al., 2003; Lang et al., 2004). Moreover, mutp53 can regulate specific sets of target genes independently of p63 and p73 (Lin et al., 1995; Zalcenstein et al., 2003; Weisz et al., 2004; Scian et al., 2004). Accordingly, the transcriptional activation domain of p53 is necessary for gene regulation by mutp53 as well as for its interference with apoptosis (Matas et al., 2001). Most cancer-associated p53 mutations occur in the DNA binding domain (DBD) and abolish the ability of the protein to bind to the specific DNA sequences recognized by wtp53. Hence, the ability of mutp53 to regulate gene expression may require interactions with other proteins that tether it to the DNA, as suggested for NF-Y (Di Agostino et al., 2006) and NF-κB (Weisz et al., 2007a).

In this study, we used chromatin immunoprecipitation (ChIP) coupled with microarray analysis (ChIP-on-chip) to identify DNA regions selectively associated with mutp53.

Significance

Mutation of the p53 tumor suppressor gene is the most common genetic alteration in human cancer. Once mutated, p53 not only loses its tumor suppressor activity but gains oncogenic functions. Indeed, p53 is being examined as an important target for cancer therapy. Likewise, vitamin D and its analogs are being evaluated as potential anti-cancer agents. Our findings provide a mechanism for mutp53 GOF, based on the interaction between p53 and VDR. The results we obtained may have clinical implications and suggest that p53 status should be considered when contemplating vitamin D analogs for cancer therapy.

Table 1. Gene Promoters Preferentially Bound by Mutp53

Spot Name	p Value	Binding Ratio	Refseq	Location	Start	End
VIPR2	1.3E−14	11.26	NM_003382	chr7	158354273	158355212
KIAA1243	8.2E−12	6.42	NM_014048	chr16	14325637	14326400
MAGEH1	1.4E−09	10.23	NM_014061	chrX	54444648	54445501
PRDM5_2,0	3.5E−09	10.23		chr4	122304464	122305398
MLLT2_1,0	1.3E−08	4.24		chr4	88384955	88385854
PGBD1_0,0	7.0E−08	6.23		chr6	28356784	28357645
ECE2	1.0E−07	3.87	NM_014693	chr3	185314038	185314863
TGIF2_0,1	3.2E−06	5.50		chr20	35887837	35888790
FLJ22031	4.4E−06	4.20	NM_025074	chr4	79803252	79803961
HIRA_0,0	1.7E−06	2.37		chr22	17793360	17794375
GHR	2.0E−05	4.90	NM_000163	chr5	42468831	42469760
EMK1	2.1E−05	2.46	NM_004954	chr11	63430695	63431630
ZFP161_0,0	2.3E−05	3.01		chr18	5283120	5284074
CPT1B	3.1E−05	2.69	NM_004377	chr22	49149214	49150151
SKP2	6.0E−05	2.36	NM_032637	chr5	36196967	36197905
NFS1	6.5E−05	2.12	NM_021100	chr20	35002468	35003216
IGFBP2_0,0	6.8E−05	2.07		chr2	217699947	217700698
ECRG4	7.2E−05	2.22	NM_032411	chr2	106302804	106303731
TIEG	7.2E−05	2.36	NM_005655	chr8	103624358	103625159
HARP11	9.2E−05	2.47	NM_018477	chr14	56656150	56656961
LIG1	9.2E−05	2.45	NM_000234	chr19	53365208	53366136
HOXB2_0,2	1.0E−04	2.07		chr17	47094840	47095801
STATH	1.2E−04	2.11	NM_003154	chr4	71177406	71178146
TUBA6	1.2E−04	1.98	NM_032704	chr12	47944440	47945247
FLJ21415	1.4E−04	2.20	NM_024738	chr12	115587568	115588515
MSX1_0,0	1.4E−04	2.4		chr4	4925239	4926223
miR-219_i	3.6E−04	2.07		chr5	33222063	33222888
PCDHA7	4.2E−04	2.7	NM_018910	chr6	122304464	122305398
TGFB1_0,1	5.4E−04	2.1		chr19	46550014	46551047
TMEM2	6.0E−04	2.43		chr9	69840372	69840999

Results of ChIP-on-chip performed on mutp53 in SKBR3 cells. Thirty of the promoter and regulatory sequences exhibiting the highest statistical significance for mutp53 binding.

RESULTS

Identification of Promoters Bound by Mutp53

To elucidate the molecular basis for the ability of mutp53 to modulate specific gene expression, ChIP coupled with promoter microarray hybridization (ChIP-on-chip; see Experimental Procedures) analysis was performed on SKBR3 breast cancer-derived cells, which harbor an endogenous mutant, p53R175H. About 70 promoters were bound with a p value of <0.001. Table 1 lists 30 genes whose promoters scored highest.

Identification of Transcription Factor Motifs Overrepresented in Promoters Bound or Regulated by Mutp53

A bioinformatics analysis was next performed on the ChIP-on-chip data in order to identify transcription factor binding motifs overrepresented in mutp53-bound promoters. Every gene was scanned for binding sites from 1000 bp upstream to 200 bp downstream from its transcription starting site, for overrepresentation of 414 different binding motifs relative to the genes across the whole genome (Tabach et al., 2007). A similar analysis was performed on the putative promoters of mutp53-regulated genes identified in an expression microarray experiment, performed with p53 null H1299 lung adenocarcinoma cells stably transfected with p53R175H (Weisz et al., 2004). Table 2 lists transcription factors exhibiting a statistically significant association with mutp53 in at least one of the two experiments. Remarkably, the vitamin D receptor/retinoid X receptor (VDR/RXR) response element (VDRE; consensus: AGGTCAnnnAGGTCA), which mediates the transcriptional effects of vitamin D, scored positive in both the ChIP-on-chip and the expression microarray analysis. When a similar bioinformatics analysis was applied to ChIP-on-chip data obtained from wtp53-expressing U2OS cells using the same arrays, it identified p53RE as the most significant motif but did not score VDRE (Table S1, available online), thus confirming the validity of the analysis. Two motifs, HEN-1 and MEF-2, were found to be overrepresented in promoters bound by both wtp53 and mutp53.

Table 2. Transcription Factor Motifs Overrepresented in Promoter Sequences Preferentially Bound by Mutp53

TF Name	SKBR3 ChIP-on-Chip	H1299 Expression Array
V$VDR_RXR.01	**1.08E−04**	**8.10E−04**
V$ELF2.01	1	**3.85E−04**
V$PAX8.01	**3.92E−04**	1
V$HEN1.01	2.01E−01	**3.98E−04**
V$PDX1.01	**7.64E−04**	3.36E−01
V$E4BP4.01	1	**7.50E−03**
V$PAX6.02	5.92E−01	**1.25E−03**
V$MYT1.02	1	**1.34E−03**
V$HIVEP1_01	1	**1.54E−03**
V$NFY.02	**1.58E−03**	5.02E−01
V$NFKAPPAB.02	**9.60E−03**	**1.70E−03**
V$MYCMAX.03	1.93E−01	**1.73E−03**
V$BKLF.01	**1.80E−03**	1
V$MUSCLE_INI.	**1.93E−03**	2.08E−01
V$OCT1P.01	**2.15E−03**	1
V$MAZ.01	**2.28E−03**	1E−02

Bold highlighting indicates a statistically significant p value for a TF motif overrepresentation in promoter sequences identified either by ChIP-on-chip (SKBR3) or expression array (H1299).

Mutp53 Is Associated with VDR Response Elements in Promoters of Vitamin D Target Genes

To confirm the interaction of mutp53 with VDR/RXR-containing promoters and to assess the impact of the active form of vitamin D3 [1α25(OH)$_2$D3] on such interaction, a pool of SKBR3 cells stably transfected with control short hairpin RNA (shRNA) and another pool transfected with p53 siRNA (Figure S1A) were subjected to ChIP analysis. Cells were either grown in medium containing charcoal-stripped serum to deplete residual vitamin D3 or treated with vitamin D3. Immunoprecipitation was performed using antibodies specific for either p53 or VDR, or beads only as a non-specific control. The immunoprecipitated DNA was subjected to qPCR analysis with primers specific for the regions encompassing putative VDREs in the promoters of the *HIRA* and *TGF-β1* genes, which scored positive for mutp53 binding in the ChIP-on-chip screen. Significant binding of mutp53 to the VDRE-containing regions of both promoters was clearly detectable (Figure 1A), but was not seen with a ChIP-control genomic sequence (bottom panel). Vitamin D3 treatment markedly increased VDR binding. Remarkably, knockdown of endogenous mutp53 reduced the binding not only of mutp53 but also of VDR. Importantly, vitamin D3 elicited a further increase in mutp53 binding relative to non-treated cells. Thus, mutp53 indeed associates with VDRE-containing regions within specific genes, and this is further enhanced by vitamin D3.

To further validate the binding of mutp53 to VDRE, we performed an in vitro "Southwestern" assay, in which nuclear extracts are incubated with membrane-immobilized synthetic oligonucleotides followed by western blot analysis to detect proteins that bound to the oligonucleotides. As seen in Figure S1B, vitamin D3 treatment augmented the binding of VDR to a wild-type but not mutant VDRE sequence, as expected (IB: VDR). Importantly, mutp53 displayed weak specific binding to the VDRE under basal conditions, which was significantly augmented upon vitamin D3 treatment (IB: p53). Of note, mutp53 did not bind to a wtp53 binding sequence (wtp53RE).

Mutp53 Enhances Vitamin D3-Induced Transcription

To investigate the functional consequences of the recruitment of mutp53 to VDRE-containing promoters, the firefly luciferase gene was placed downstream to three tandem copies of a canonical VDRE or a mutant incapable of VDR/RXR binding (mVDRE), In p53 null H1299 cells, cotransfection of expression plasmids encoding either p53R175H or p53R273H led to a mild increase in the transcriptional activity of the wild-type but not mutant VDRE promoter. Vitamin D3 increased transcription from the wild-type but not mutant VDRE (Figure 1B, top). Both cancer-associated mutp53 isoforms further increased vitamin D3-induced transcription. Importantly, wtp53 failed to augment the transcriptional activity of this promoter. Neither the p53R175H22,23 triple mutant, possessing a defective transactivation domain (TAD), nor p73DD, which has a dominant-negative effect over both p63 and p73, had any effect on transcription (Figure S1C; see also Figure S4E). Hence, the effects of mutp53 in our experimental models are mostly independent of other p53 family members, but require a functional p53 TAD.

Next, the same reporter plasmids were transfected into SKBR3 cells together with siRNA oligonucleotides specific for p53 (p53i) or LacZ as a control (LacZi). As expected, vitamin D3 induced a robust increase in transcription from a promoter containing wild-type but not mutant VDRE (Figure 1B, bottom). Importantly, knockdown of endogenous mutp53 reduced both basal and vitamin D3-induced transcription. Similar results were obtained with SW480 colorectal cancer cells expressing H273R mutp53 (Figure S1D). Hence, mutp53 can cooperate with vitamin D3 to maximize VDRE-dependent transcription, at least from some promoters.

Mutp53 Increases Nuclear VDR Levels

The VDR gene is a transcriptional target of wtp53 (Maruyama et al., 2006) as well as of p63 and p73 (Kommagani et al., 2006, 2007). p63/p73 activity can be quenched by mutp53; indeed, p53R248W can elicit a dominant-negative effect over transfected p73 in the regulation of *VDR* gene transcription (Kommagani et al., 2006, 2007). Knockdown of mutp53 in either SKBR3 or MDA-MB-231 cells caused a 30% to 50% reduction in total VDR protein (Figure 2A, top panel) but had no significant impact on *VDR* mRNA levels (data not shown), which suggests that mutp53 may lead to a mild stabilization of VDR. In H1299R175H-i cells, induction of mutp53 expression by Zn^{2+} led to only a slight (30%) increase in *VDR* mRNA (Figure 2B) and a mild (1.4-fold) increase in total VDR protein (Figure 2C, lanes 1 and 2).

Transcriptional activation by vitamin D3 requires translocation of VDR into the nucleus. We therefore examined the effect of mutp53 on VDR localization 3 hr after addition of vitamin D3. Remarkably, mutp53 had a dramatic effect on the amount of VDR in the nuclear fraction (Figures 2A and 2C). Immunostaining analysis revealed that while in untreated H1299R175H-i cells VDR is largely cytoplasmic, vitamin D3 caused a substantial nuclear translocation of VDR in some cells (Figure 2D, top two

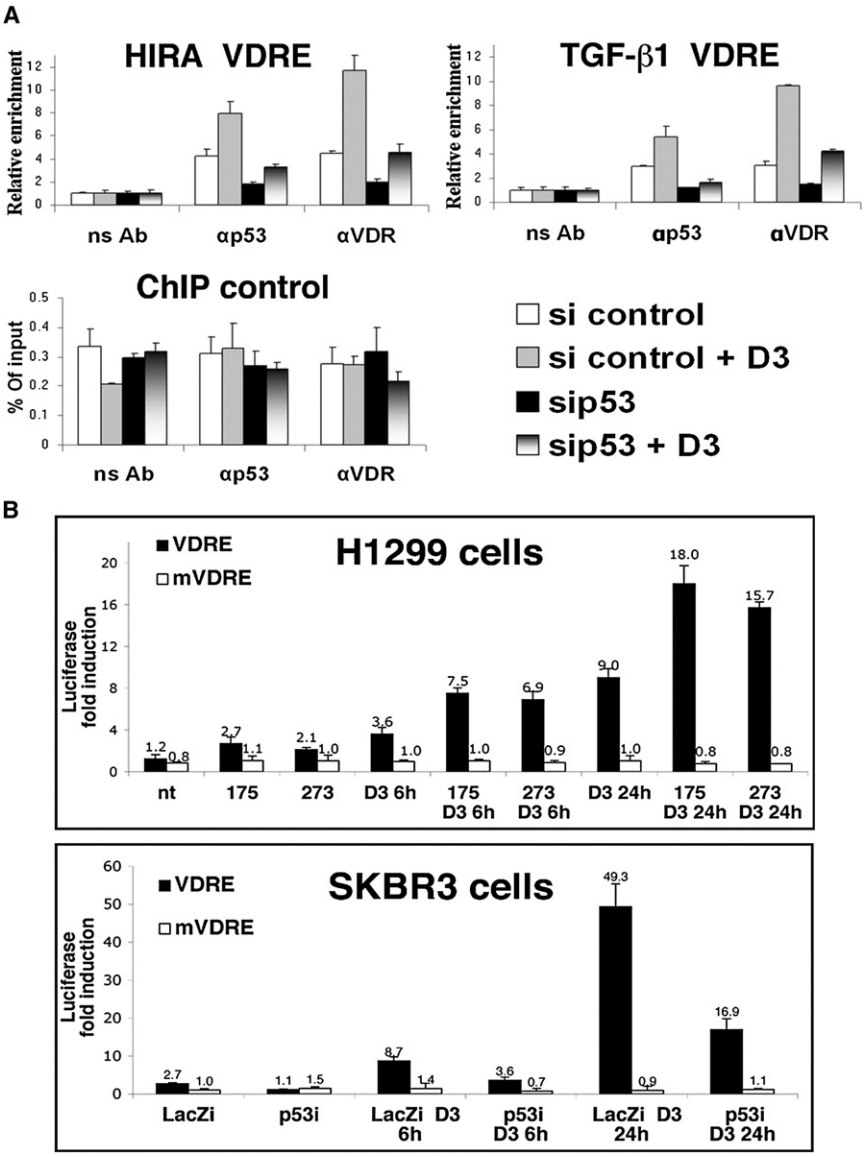

Figure 1. Mutp53 Associates with VDREs and Augments Transcription from Such Elements

(A) SKBR3 cells expressing p53R175H, transfected with either siRNA for p53 (sip53) or control siRNA (sicontrol) and treated with 1α25(OH)$_2$D3 (D3;100nM), were subjected to ChIP with antibodies against p53 or VDR or with a non-specific antibody as a negative control. The precipitated DNA was subjected to real-time PCR amplification using sets of primers specific to either ChIP control (negative control) or regions of the *HIRA* and *TGF-β1* gene promoters spanning putative VDREs. Results are presented relative to the values obtained with an aliquot of the input chromatin corresponding to 1% of the total material. The results of *HIRA* and *TGF-β1* gene promoters are presented as enrichment fold over non-specific antibody and are also normalized to values obtained for the ChIP control results.

(B) Reporter plasmids were either cotransfected together with mutp53 expression plasmids (R175H or R273H) into p53 null H1299 cells (top) or cotransfected with synthetic p53i or LacZi into SKBR3 cells (bottom). Cells were either treated with 1α25(OH)$_2$D3 (D3; 100 nM) for the indicated times or left untreated (nt). Values shown represent fold activation relative to the PGL3-promoter empty vector reading. See also Figures S1C and S1D. All scale bars represent ± SD.

rows). Interestingly, induction of mutp53 by Zn^{2+} caused nuclear translocation of VDR in many cells without vitamin D3 (Figure 2D, third row). Moreover, cells expressing high amounts of mutp53 exhibited more pronounced nuclear VDR staining. Furthermore, combining mutp53 induction with vitamin D3 rendered VDR staining more prominent and almost exclusively nuclear (Figure 2D, bottom row). The ability of mutp53 to enhance the nuclear translocation and accumulation of VDR probably contributes to the augmented vitamin D3-induced transcription.

Mutp53 Physically Interacts with VDR

Mutp53 may be tethered to VDRE elements through a complex with VDR/RXR. To explore the existence of such a complex, coimmunoprecipitation analysis was performed on SKBR3 cells with or without treatment with 100 nM vitamin D3. As a control for wtp53, a similar experiment was performed on MCF7 cells treated with Nutlin-3A to induce p53 accumulation. As seen in Figure 3A (top), pull-down of VDR coprecipitated p53 from both SKBR3 and MCF7 cell extracts, indicative of a complex comprising both proteins. This interaction was significantly increased by treatment with vitamin D3. Reciprocal coimmunoprecipitation yielded a similar picture (Figure 3A, bottom). Interestingly, the coimmunoprecipitation of mutp53 with VDR was further augmented when a wild-type, but not mutant, VDRE consensus oligonucleotide was added to the cell extract during incubation (Figure S2A), indicating that the VDRE sequence can recruit both proteins simultaneously.

The p53 protein is composed of three major domains: an N-terminal TAD, a central DBD, and a C-terminal regulatory domain. VDR was overexpressed in H1299 cells together with either full-length H175Rp53, ΔN-H175Rp53 (aa 97–393), or ΔC-H175Rp53 (aa 1–292) or the DBD of H175Rp53 (aa 97–292). Pull-down of VDR followed by western blot analysis for p53 revealed that full-length H175Rp53 and ΔN-H175Rp53 bound VDR, whereas ΔC-H175Rp53 and DBD-H175Rp53 did not (Figure 3B); hence, the interaction between p53 and VDR is mediated through the p53 C-terminal regulatory domain.

Knockdown of Mutp53 Attenuates the Transcriptional Response to Vitamin D3

Next, we knocked down mutp53 expression in SKBR3 cells, in SW480 colorectal cancer cells that harbor a combination of the p53R273H and p53P309S mutations, and in MCF7 cells that

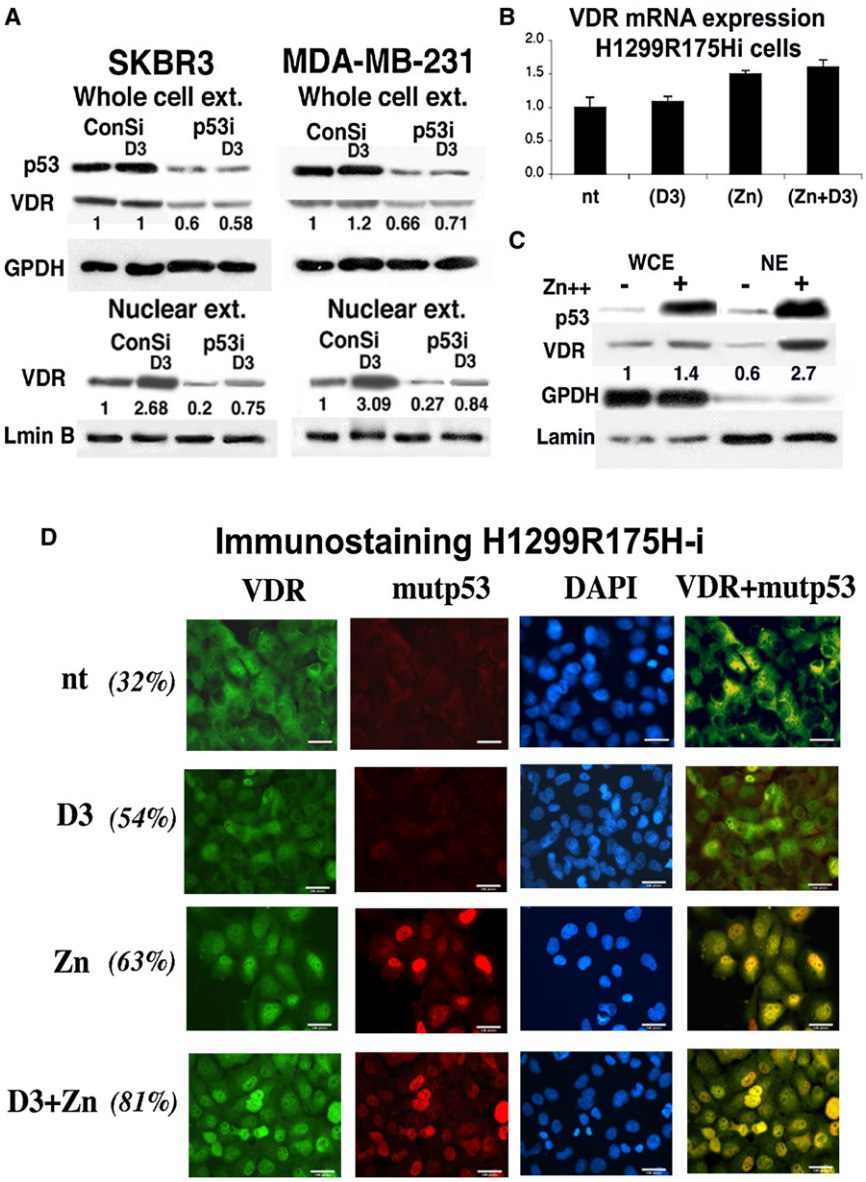

Figure 2. Mutp53 Increases Nuclear VDR Levels

(A) SKBR3 and MDA-MB-231 cells were transfected with p53i or control siRNA (ConSi; Dharmacon) as negative control. Forty eight hours after transfection, cells were treated with 100 nM $1\alpha25(OH)_2D3$ for 3 hr. Nuclear extracts were then prepared, subjected to SDS-PAGE, and followed by western blot analysis with antibodies directed against p53 or VDR.

(B) H1299R175H-i cells were either left untreated (nt) or maintained for 12 hr either with or without 100 μM Zn^{2+}, and then 100 nM 1,25(OH)2D3 (D3) was added for an additional 24 hr where indicated. Cells were harvested, RNA was extracted, and qRT-PCR was performed with primers specific for *VDR* or *GAPDH*. Relative levels of *VDR* mRNA were normalized to *GAPDH*.

(C) H1299R175H-i cells were either induced with 100 μM Zn^{2+} (+) or not induced (−). Eight hours later, 100 nM $1\alpha25(OH)_2D3$ was added for 3 hr to all cultures. Part of each sample was extracted directly (whole cell extract; WCE). Nuclei were prepared from the rest of the sample and extracted (nuclear extract; NE). Extracts were subjected to SDS-PAGE, followed by western blot analysis with antibodies directed against p53 or VDR. Relative intensities of the VDR bands are indicated below each band. The bottom panel shows Ponceau staining of the corresponding region of the membrane. All error bars represent ± SD.

(D) H1299R175H-i cells were seeded on coverslips. One day later, 100 nM Zn^{2+} was added for 12 hr where indicated to induce mutp53 expression, and then $1\alpha25(OH)_2D3$ (D3) was added for an additional 5 hr where indicated. Cells were fixed with 4% paraformaldehyde, incubated with polyclonal anti-VDR antibody and monoclonal anti-p53 antibody, and followed by suitable secondary antibodies conjugated to Cy5 or Cy3, respectively. Nuclei were visualized by DAPI staining. Nuclear VDR intensity was quantified using the Volosity software (PerkinElmer, Inc.), and is presented as percentage of total VDR staining. All pictures were taken with the same exposure time. All scale bars represent a size of 27 μm.

express wtp53. RT-qPCR analysis of transcripts of VDR target genes revealed that vitamin D3 caused a dramatic increase in *CYP24A1* mRNA and a 3-fold induction of *IGFBP3* mRNA in both SW480 and SKBR3 cells (Figure 3C), which was attenuated by knockdown of endogenous mutp53. Hence, mutp53 is required for an optimal transcriptional response of at least some genes to vitamin D3. In contrast, knockdown of wtp53 in MCF7 cells had no significant effect on either endogenous or vitamin D3-induced mRNA levels of *CYP24A1* and *IGFBP3*.

Mutp53 Enhances Transcription by Recruiting VDR and p300 to VDRE of Target Genes

p53 can transactivate its target genes by recruiting transcriptional coactivators such as CBP/p300. Furthermore, both wtp53 and mutp53 interact with CBP/p300 through their TADs (Avantaggiati et al., 1997; Gu et al., 1997). We therefore assessed the binding of mutp53, VDR, and p300 to VDRE in the *CYP24A* promoter. As seen in Figure 3D, mutp53, VDR, and p300 showed comparable binding (4- to 8-fold enrichment over control) under basal conditions. As expected, vitamin D3 strongly increased the binding of VDR and p300. Binding of p53R175H to the *CYP24A* promoter also increased significantly, to 20-fold over background. Importantly, mutp53 knockdown significantly reduced both basal and vitamin D3-induced binding of all three proteins. Hence, mutp53 augments the recruitment of both VDR and p300 to VDRE-containing chromatin. To confirm the interaction of VDR, mutp53, and p300 with the same DNA segment, we performed a re-ChIP experiment. As seen in Figure 3E, ChIP for p53 followed by re-ChIP for VDR or p300 enriched for the presence of the CYP24A VDRE when treated with vitamin D3.

Mutp53 Compromises Vitamin D3-Mediated Repression

Vitamin D can also repress many genes, including *CYP27B* (Kim et al., 2007; Murayama et al., 2004), by a mechanism involving

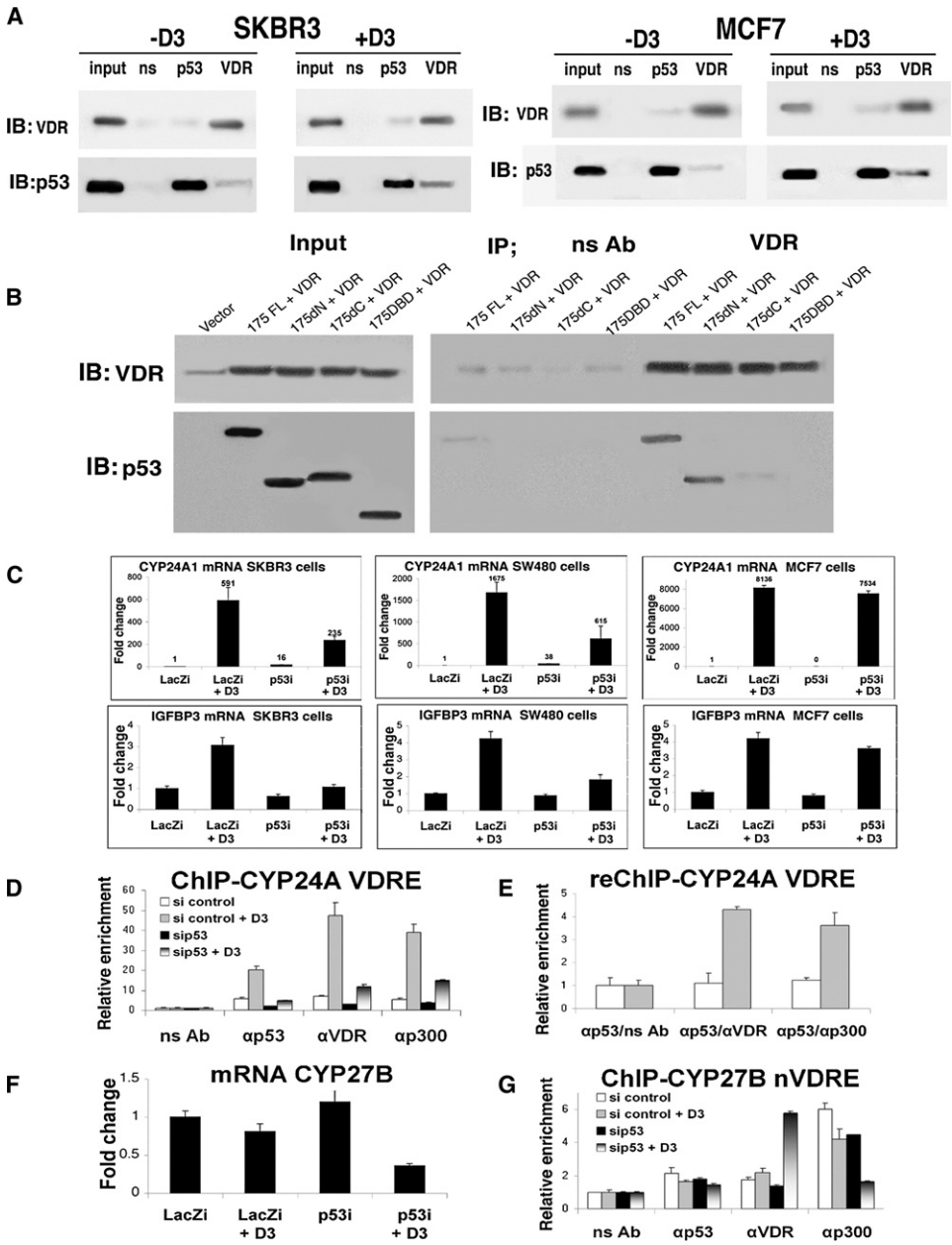

Figure 3. Mutp53 Binds VDR and Augments the Transcriptional Response of Classical VDR Target Genes to Vitamin D3

(A) SKBR3 and MCF7 cells were either grown in charcoal-stripped serum-supplemented medium (−D3) or treated with 1α25(OH)$_2$D3 (+D3) for 3 hr; the MCF7 cells were pretreated with Nutlin (40 μM, 24 hr) to induce wtp53 levels. Cell extracts were subjected to immunoprecipitation with either mutp53-specific antibody PAb240, anti-VDR antibody C-20 (Santa Cruz Biotechnology), or control antibody (PAb419, directed against SV40 large T-antigen; ns). Immunoprecipitated proteins were subjected to SDS-PAGE, followed by western blot analysis with antibodies against p53 (p53-HRP; R&D Systems; bottom panels) or VDR (top panels). See also Figures S1B and S2A.

(B) H1299 cells were transfected with a VDR expression plasmid together with plasmids expressing either full-length H175Rp53, ΔN-H175Rp53 (aa 97–393), or ΔC-H175Rp53 (aa 1–292) or the DBD of H175Rp53 (DBD; aa 97–292). Total protein input levels are shown in the left panel. Cells were treated with 1α25(OH)$_2$D3 for 3 hr and cell extracts were subjected to immunoprecipitation with either anti-VDR C-20 (Santa Cruz Biotechnology) or PAb419 as a non-specific control (ns Ab). Immunoprecipitated proteins were analyzed as in (A).

(C) SKBR3 and SW480 cells were transfected with p53i or LacZi as negative control. After 24 hr, 1α25(OH)$_2$D3 (D3; 100 nM) was added for 24 hr where indicated. Cells were harvested, RNA was extracted, and qRT-PCR was performed with primers specific for *IGFBP3*, *CYP24A1*, or *GAPDH*. Relative levels of *IGFBP3* and *CYP24A1* mRNA were normalized to *GAPDH* expression. See also Figures S2B and S2C.

(D) SKBR3 cells were transfected with either p53 siRNA (sip53) or control siRNA (si control) and treated with 1α25(OH)$_2$D3 (D3;100 nM, 2 hr) or left untreated. Cells were subjected to ChIP with antibodies directed against either p53, VDR, p300, or non-specific antibody (ns Ab). The precipitated DNA was subjected to real-time qPCR amplification using sets of primers specific to a region of the *CYP24A* promoter spanning the VDRE. Results are presented as fold over non-specific antibody.

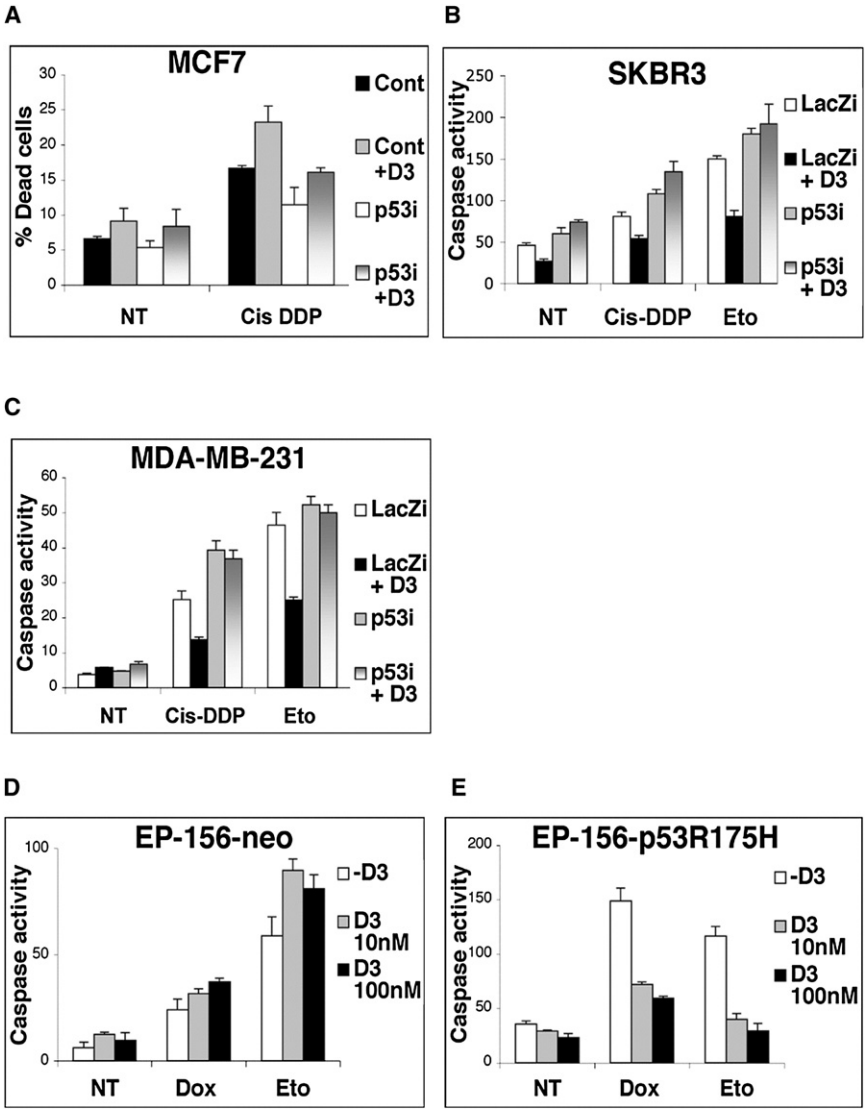

Figure 4. Mutp53 Converts Vitamin D3 from a Proapoptotic into an Antiapoptotic Effector

(A) MCF7 cells stably transfected with empty vector (Cont) or with p53 shRNA expression plasmid to knockdown endogenous wtp53 (p53i) were seeded in a 96 well plate at 5000 cells/well. After 24 hr, 1α25(OH)$_2$D3 (D3; 100 nM) was added for 48 hr where indicated. Cisplatin (Cis-DDP; 5 μg/ml) was then added for 24 hr, and cells were harvested and monitored under the microscope for trypan blue dye exclusion. At least 150 cells/assay were counted; the percentage of dead (trypan blue-positive) cells is shown. NT, not treated.

(B) SKBR3 cells were seeded in a 96 well plate at 8000 cells/well. Cells were transfected with p53i or LacZi as control. Twenty-four hours later, 100 nM 1α25(OH)$_2$D3 (D3) was added for 48 hr every 24 hr, followed by treatment with cisplatin (Cis-DDP; 2 μg/ml) or etoposide (Eto; 5 μM) for 24 hr. Caspase activity was measured using the caspase 3/7 activity assay (Promega). Caspase activity readings were normalized for the corresponding WST-1 cell proliferation assay (Roche) readings of the same samples. NT, not treated. See also Figures S3C, S4D, and S3G for Annexin V and PI assays.

(C) MDA-MB231 cells were seeded and treated and the results were analyzed as in (B). See also Figures S3D–S3H for Annexin V and PI assays.

(D) EP-156-neo cells were seeded as in (A). Twenty-four hours later 100 nM 1,25(OH)2D3 (D3) was added for 72 hr, followed by treatment with doxorubicin (Dox; 1.5 μg/ml) or etoposide (Eto; 9 μM). Caspase activity was quantified as in (B) and normalized to cell number.

(E) EP-156-p53R175H cells were analyzed as in (D). All scale bars represent ± SD.

a negative vitamin D response element (nVDRE). As seen in Figure 3F, vitamin D3 caused a significant repression of *CYP27B* only in mutp53-depleted (p53i+D3) but not mutp53-proficient (LacZi+D3) SKBR3 cells, arguing that mutp53 prevents VDR-mediated transrepression. Mutp53 was not associated with the region of the *CYP27B* gene harboring the nVDRE (Figure 3G). Remarkably, while in cells depleted of mutp53 vitamin D3 strongly stimulated the binding of VDR, presumably to the nVDRE, this was attenuated by mutp53. Furthermore, while vitamin D3 caused a dissociation of p300 from this region in the absence of mutp53, p300 remained associated when mutp53 was present. These results imply that mutp53 interferes with the binding of VDR to nVDRE, enabling the relevant target genes to remain transcriptionally active.

Vitamin D3 Exerts Mutp53-Dependent Antiapoptotic Effects

Vitamin D has been reported to promote tumor cell death (Deeb et al., 2007; Duque et al., 2004). Indeed, vitamin D3 slightly augmented the death of wtp53-positive MCF7 breast cancer cells (Figure 4A). This effect increased modestly when vitamin D3 was combined with cisplatin treatment (Figure 4A, Cis-DDP) and was somewhat attenuated by endogenous wtp53 knockdown (p53i).

(E) MDA-MB-231 cells grown in medium containing charcoal-stripped serum, or treated with 1α25(OH)2D3 (gray bars; D3; 100 nM, 2 hr) or left untreated (white bars), and then subjected to ChIP with antibodies directed against p53. The immunoprecipitated chromatin was eluted and then a second immunoprecipitation was performed with antibodies against VDR, p300, or non-specific antibody (ns Ab) as a negative control. Real-time qPCR was performed as in (D).

(F) RNA was prepared from the experiment shown in (C) and subjected to qRT-PCR analysis with primers specific for *CYP27B*.

(G) Immunoprecipitated DNA from experiment shown in (D) was subjected to real-time PCR amplification with primers specific to a region of the *CYP27B* promoter spanning the nVDRE. Results are presented as fold over non-specific antibody. All scale bars represent ± SD.

Surprisingly, vitamin D3 significantly reduced cisplatin- and etoposide-induced apoptosis of SKBR3 cells, as measured by caspase activity (Figure 4B and Figure S3B) or Annexin V or PI assays (Figures S3C and S3F). This protective effect was strictly mutp53 dependent, since it was abolished upon knockdown of endogenous p53R175H. Thus, while vitamin D often promotes apoptosis in cells lacking mutp53, mutp53 can convert vitamin D3 into a cytoprotective agent. A similar pattern was seen in an SKBR3-derived cell line expressing inducible p53 shRNA (Figure S3B).

Mutp53-dependent antiapoptotic activity of vitamin D3 was also observed in two other breast cancer cell lines, MDA-MB-231 that harbors endogenous p53R280K (Figure 4D and Figure S3D) and MDA-MB-468 that expresses endogenous p53R273H (Figure S3E), and a colorectal cancer cell line, SW480 that expresses p53R273H and p53P309S (Figure S3F). Furthermore, vitamin D3 led to a mild but consistent increase in colony formation of H1299 cells transfected with various mutp53 isoforms but not with GFP control plasmid (Figure S3I). However, in the breast cancer cell line BT474, which expresses p53E285K, vitamin D3 protection was not significant (data not shown). Hence, even though the observed effects are common to many mutp53-expressing tumor cells, the scope of the effect is cell context dependent.

To compare the impact of wtp53 and mutp53 on the biological response to vitamin D in a genetically defined experimental model, we used primary prostate epithelial cells immortalized by the introduction of hTERT and then transfected with either an empty vector (EP-156-neo cells) or a mutp53 expression plasmid (EP-156-p53R175H cells) (Kogan et al., 2006). EP-156-neo cells express endogenous wtp53 whereas EP-156-p53R175H cells overexpress p53R175H on the background of wtp53. As seen in Figure 4E, treatment of EP-156-neo cells with vitamin D3, either alone or in combination with genotoxic agents, had a mild proapoptotic effect. However, mutp53 altered the response dramatically: instead of enhancing apoptosis, vitamin D3 now conferred strong protection (Figures 4D and 4E). Hence, p53 mutations convert vitamin D3 from death promoting into protective.

Mutp53 Modifies the Vitamin D Transcriptional Program

Although mutp53 exerts a general stimulatory effect on VDR transcriptional activity, this by itself cannot explain how mutp53 reverses the impact of vitamin D on apoptosis. Rather, the answer might lie in differential effects on the expression of specific genes. Therefore, expression microarray analysis was performed on SKBR3 cells without or with endogenous mutp53 knockdown, with or without vitamin D3 treatment. The expression level of each individual gene in cells transfected with p53 siRNA but not treated with $1\alpha25(OH)_2D3$ (equals no mutp53, no vitamin D), averaged from two biological repeats, was taken as baseline. Relative expression levels of each gene in the other biological samples were calculated as fold change over baseline. Genes were then sorted according to their fold induction by vitamin D3, mutp53, or the combination of both. When we analyzed the genes most highly induced by mutp53 (Table S2), vitamin D3 association was strongly significant (31 out of 38 genes, excluding the two p53 probes; $p = 4 \times 10^{-18}$, Student's t test). Hence, in SKBR3 cells, most mutp53-upregulated genes are also VDR targets, implicating the interaction with VDR as a major regulatory mechanism by mutp53 in these cells.

Figure 5A illustrates the effects of mutp53 and vitamin D on gene expression. The x axis shows the effect of vitamin D3 alone on each gene, calculated from the ratio between expression levels with and without vitamin D3 (50 hr) in mutp53 knocked down cells. The y axis shows the combined effect of vitamin D3 and mutp53, calculated from the ratio between expression levels with vitamin D3 in control cells and without vitamin D3 in mutp53 knocked down cells. Most genes are along the diagonal, near the center of the plot, indicating lack of regulation by either vitamin D3 or mutp53. A few genes lie further up or down on the diagonal: these are regulated by vitamin D3, but the extent of regulation is not affected by mutp53. On that background, several gene clusters exhibit distinct response patterns that diverge from the behavior of the bulk transcriptome.

Cluster 1 (blue) contains genes that are highly induced by the combination of mutp53 and vitamin D3, a behavior consistent with the luciferase experiments. Many classical VDR target genes populate this cluster. Of note, the majority of those genes are also dependent on mutp53 for optimal basal level expression, as revealed by comparison of non-treated samples without (Figure 5B, lanes 1 and 2) and with (lanes 6 and 7) p53 siRNA. This suggests that a functional interaction between VDR and mutp53 may exist even without vitamin D3 or at low residual levels of vitamin D3 produced by SKBR3 cells.

Cluster 2 (red) comprises genes repressed 3-fold or more by long (50 hr) treatment with vitamin D3 when mutp53 was absent, but this effect was strongly attenuated in the presence of mutp53 (Figure 5C, compare lanes 5 and 10). These results are consistent with the data obtained for the *CYP27B* gene (Figures 3F and 3G). Hence, in addition to augmenting the transactivation of many vitamin D3-inducible genes, mutp53 also relieves vitamin D3-mediated repression of a relatively large subset of genes.

Cluster 3 (green) contains genes whose expression was downregulated more than 4-fold by the combination of mutp53 and vitamin D3. The basal expression level of most genes in the cluster was repressed by mutp53 even without vitamin D. Vitamin D3, however, caused a further repression in cells expressing mutp53 but not in mutp53 knocked down cells. Hence, these genes are repressed by vitamin D3 in a mutp53-dependent manner.

Apoptosis-Regulating Genes Are Differentially Affected by Vitamin D and Mutp53

The microarray analysis in Figure 5 demonstrated that mutp53 can modulate the activity of VDR in a gene-specific manner. The impact of mutp53 on the biological response to vitamin D suggested that mutp53 might modify the transcriptional program of VDR in a manner conducive to increased resistance to apoptosis. qRT-PCR analysis of individual transcripts further supports this conjecture (Figure S4A). Thus, mutp53 augmented the ability of vitamin D to upregulate genes reported to promote survival and neoplastic transformation [e.g., SEMA3C (Moreno-Flores et al., 2003), Wnt5A (Ripka et al., 2007), and CSF3R] or to be overexpressed in cancer [e.g., Klk6 (Klucky et al., 2007)]. Remarkably, mutp53 prevented the vitamin D-dependent repression of MAP2K5 and FGFR2, survival-promoting genes overexpressed in some cancers (Acevedo et al., 2007). Conversely, the combination of mutp53 and vitamin D repressed the proapoptotic XAF1 (Lee et al., 2006), CYFIP2 (Jackson

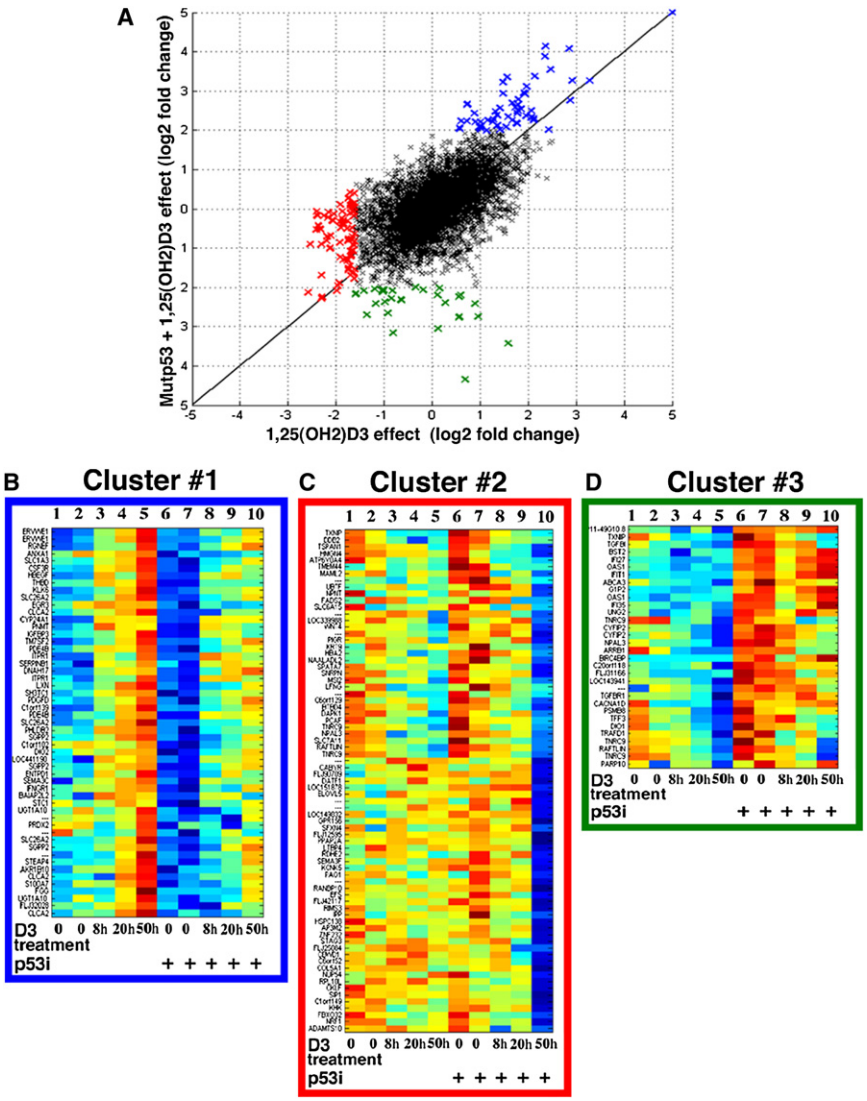

Figure 5. Expression Array Analysis of the Effects of Vitamin D3 and Mutp53

(A) Graphic representation of the relative effects of 1α25(OH)$_2$D3 (vitamin D) and mutp53 on gene expression. The x axis shows a log2 scale of 1α25(OH)$_2$D3 fold activation/repression, calculated by dividing the expression level reading of each individual gene in the p53i-D3 50 hr sample by the average of the two corresponding p53i basal level (no vitamin D) samples; this represents the effect of 1α25(OH)$_2$D3 in the absence of mutp53. The y axis shows a log2 scale of the combined effect of 1α25(OH)$_2$D3 and mutp53, calculated by dividing the expression level reading of each gene in the LacZi-D3 48 hr sample by the average of the two corresponding p53i basal level (no vitamin D) samples. Different colors identify genes belonging to the three different clusters denoted by the same colors in (B)–(D).

(B) Cluster 1 (blue), obtained by imposing a 4-fold upregulation cutoff threshold on the y axis, contains genes highly induced by the combination of mutp53 and 1α25(OH)$_2$D3 (50 hr). Values of gene expression range from −0.7 (blue) to +0.6 (red).

(C) Cluster 2 (red) contains genes that were repressed more than 3-fold by 1α25(OH)$_2$D3 in the absence of mutp53; most of those genes were repressed less well by 1α25(OH)$_2$D3 when mutp53 was present.

(D) Cluster 3 (green) includes genes whose expression was significantly repressed by the combination of mutp53 and 1,25(OH)2D3. See also Figures S4A–S4F.

et al., 2007), DAPK1 (Raval et al., 2007), and TXNIP (Billiet et al., 2008) genes. Interestingly, the first three are transcriptional targets of wtp53 (Jackson et al., 2007; Lee et al., 2006; Martoriati et al., 2005). The strong repression of those genes may underpin in part the cytoprotective program installed by vitamin D in mutp53-harboring cells.

To demonstrate that the transcriptional effects of mutp53 siRNA were not due to off-target effects, we performed a rescue experiment using an siRNA-resistant p53R175H expression plasmid. As shown in Figures S2B and S2C, restoration of mutp53 expression reversed the effect of mutp53 knockdown on specific gene expression. Furthermore, it also reversed the proapoptotic effects of the knockdown (Figure S2D). The expression pattern of these genes in additional experimental systems showed a similar trend to that of SKBR3 (Figures S4B–S4D), attesting to the generality of the transcriptional cross-talk between mutp53 and VDR.

The differential behaviors of the different clusters and the identities of some of the genes offer an attractive explanation for the mutp53-dependent antiapoptotic effects of vitamin D3.

DISCUSSION

The present study reveals a functional and physical interaction between mutp53 and the vitamin D transcriptional regulatory pathway. Specifically, mutp53 is tethered to chromosomal regions containing VDRE elements, presumably through association with VDR and to augment transcription from promoters containing such elements. Moreover, in cells harboring p53 mutations, mutp53 cooperates with vitamin D3 to elicit an antiapoptotic state. This surprising effect of mutp53 is likely due to its ability to modulate, qualitatively and quantitatively, the transcriptional program orchestrated by VDR and appears to involve augmented expression of survival genes along with reduced expression of proapoptotic genes.

Vitamin D participates in diverse biological processes such as calcium homeostasis, cell proliferation, and cell differentiation (for review see Deeb et al., 2007). VDR is believed to be constantly transported into the nucleus; however, after activation by vitamin D3, this transport is greatly enhanced (Yasmin et al., 2005). Ligand binding induces conformational changes in VDR, exposing surfaces for transcriptional coactivator binding and dimerization. The dimerization partner is usually RXR, which is required for full transactivation by VDR. Dimerization enables high affinity interaction with the VDRE. Transcriptional coactivators, physically recruited by VDR, then initiate transcription.

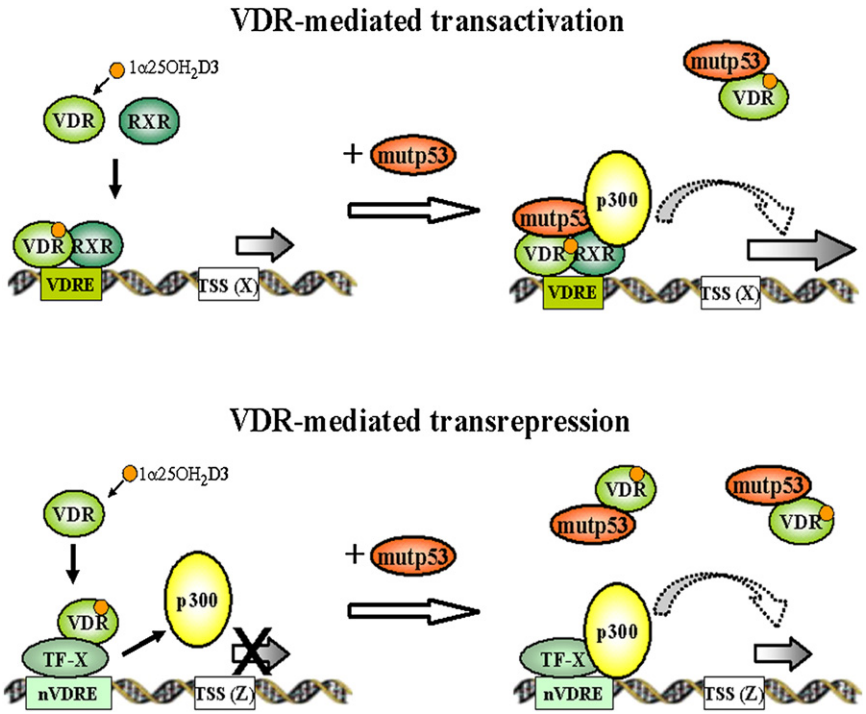

Figure 6. Cartoon of the Proposed Model for the Crosstalk between Mutp53 and the VDR Pathway

The model displays the impact of mutp53 on VDR-mediated transactivation (top) and transrepression (bottom). TSS, transcription start site; TF-X, positive transcription site (e.g., VDIR) that is displaced/inactivated by VDR. See Discussion for further details.

We show that VDR and mutp53 (and also wtp53) engage in a physical interaction, which is significantly enhanced by vitamin D3 treatment. The high endogenous levels of mutp53 in tumor cells probably enable this interaction to exert significant biological effects. Moreover, mutp53 increases the nuclear accumulation of VDR. Notably, in lung tumors, increased nuclear localization of VDR correlates with higher histological grade (Menezes et al., 2008). Remarkably, some enhancement of VDR nuclear accumulation by mutp53 can be seen even in the absence of added vitamin D. Thus, the binding of mutp53 to VDR might alter the latter's conformation in a way that mimics the effect of vitamin D3.

Increased nuclear localization of VDR is probably not the sole explanation for the effect of mutp53. Based on our findings, we propose the following additional mechanisms (Figure 6). In the case of transactivation, VDR recruits mutp53 to VDREs in target genes, and mutp53 increases VDR-dependent transcription by augmenting the recruitment of additional transcriptional coactivators, such as p300 (Figure 6, top). Indeed, p300 binds to the p53 TAD, which is intact in all cancer-associated hotspot p53 mutants.

The mechanism behind vitamin D3-mediated transrepression is less well characterized. A distinct nVDRE was identified in the promoters of several vitamin D3-repressed genes. nVDRE binds the transcriptional activator VDIR, which in turn recruits p300 and activates transcription. VDR, activated by vitamin D3, does not directly bind the nVDRE, but instead associates with VDIR. This leads to dissociation of p300 and recruitment of HDACs to repress transcription of the target gene. In contrast to the positive effect of mutp53 on VDR-mediated transactivation, mutp53 actually reverses VDR-mediated transrepression. In fact, the majority of genes repressed by vitamin D3 in SKBR3-p53i cells were derepressed in cells expressing endogenous p53R175H (Figure 5, red cluster). As an example, VDR binds the nVDRE-containing region of the *CYP27B* promoter only in the absence of mutp53 and presence of vitamin D3, and this leads to dissociation of p300 (Figure 3G) and transcriptional repression (Figure 3F). We therefore propose that while VDR can repress transcription by interfering with the binding and activity of positive transcription factors (Figure 6, bottom, TF-X), such as VDIR, engagement of VDR by mutp53 relieves this interference, allows recruitment of transcriptional coactivators, and restores transcription.

Vitamin D3 can induce apoptosis either alone or in conjunction with other drugs (Colston et al., 1992; Nakagawa et al., 2005). Moreover, vitamin D3 has been reported to possess anti-tumor activities (Kerner et al., 1989). VDR knockout mice exposed to the chemical carcinogen DMBA develop more skin tumors and lymphomas than wild-type mice, and nearly all VDR knockout mice display in situ hyperplasia of the mammary gland (Zinser et al., 2002a, 2002b). The notion that vitamin D3 is capable of exerting anti-cancer effects has spurred attempts to develop vitamin D3 analogs as cancer chemotherapy agents.

However, in addition to the well documented proapoptotic effects, there are circumstances where vitamin D3 exerts antiapoptotic effects, including increased cell survival following UV trauma and protection of some cancer cell lines from killing by cytotoxic drugs. An inhibitory effect of vitamin D3 on TNF-α-induced apoptosis and on TRAIL and Fas ligand-induced apoptosis, accompanied by a decrease in Bax and upregulation of Bcl2 and p21, has also been described (Wang et al., 1999; Duque et al., 2004). However, in SKBR3 and MDA-MB-231 cells, p21 and Bcl2 protein levels did not change significantly following either mutp53 knockdown or vitamin D3 treatment (Figure S3J). Interestingly, VDR is upregulated in several types of cancer, including breast and ovarian carcinomas (Friedrich et al., 1998, 2002). Moreover, elevated VDR was reported to correlate with tumor stage (Menezes et al., 2008; Sahin et al., 2005). Thus, the VDR pathway can lead to either death or survival, depending on the cellular context.

To date, no satisfactory mechanism was proposed to account for the seemingly conflicting effects of VDR/vitamin D3. Our findings now provide a possible explanation. Perhaps the most significant finding is that mutp53 can convert vitamin D3 from a proapoptotic agent into an antiapoptotic one. In support of this notion, vitamin D3 suppresses death receptor-mediated

apoptosis in OVCAR3 ovarian carcinoma cells (Zhang et al., 2005b), which harbor endogenous mutp53. Conceivably, some cancers might have evolved a mechanism that allows them to capitalize more efficiently on the survival route of VDR while evading its proapoptotic effects. Our data suggests that acquisition of GOF p53 mutations may constitute one such mechanism. Obviously, p53 mutations alone are not sufficient to drive the conversion of the VDR pathway into an antiapoptotic one, as not all mutp53-expressing tumor-derived cell lines are equally protected by vitamin D3. Thus, additional alterations most likely cooperate with mutp53 to orchestrate the antiapoptotic response to vitamin D3. Identification of such cooperating factors remains an important challenge.

Vitamin D and its derivatives are being extensively explored as cancer-preventive and even cancer-therapeutic agents. Our findings call for extra caution in exploring this approach. Conversely, individualized cancer therapy might be implemented also in the context of vitamin D3 treatment, where the p53 mutation status of the tumor may serve as an important aid for outcome prediction.

EXPERIMENTAL PROCEDURES

Cell Lines

H1299 human non-small cell lung cancer cells and SKBR3 breast cancer cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics. Where indicated, charcoal-stripped serum was included instead of regular serum. MCF7 human breast cancer cells stably expressing shRNA targeting p53, as well as vector control cells (gift of R. Agami, Netherlands Cancer Institute) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 2 mM L-glutamine, and antibiotics. The MDA-MB-231 breast cancer cell line and the SW480 colorectal cancer cell line were maintained in DMEM supplemented with 10% FCS and antibiotics. EP156 epithelial cells were grown as described previously (Kogan et al., 2006). SKBR3-D8 cells were grown under the same conditions as parental SKBR3, plus puromycin (1 μg/ml) and blasticidin (10 μg/ml). All cell lines were grown at 37°C in a humidified atmosphere of 5% CO_2 in air.

Plasmids

Expression plasmids for wild-type human p53 and mutant p53R175H were gifts from C.C. Harris (National Cancer Institute, Bethesda, MD). Reporter plasmids were constructed by inserting a 64 bp DNA fragment containing either three tandem VDRE sequences (AGGTCAnnnAGGTCA) separated by 5 bp spacers or a derivative that contained two point mutations in each half VDRE site (mVDRE: ATATCAnnnATATCA) upstream of the luciferase reporter gene within the PGL3-promoter plasmid (Promega).

Luciferase Assays

Cells were seeded in 24 well culture dishes. Each well was transfected with VDRE-luciferase or mVDRE-luciferase, together with increasing amounts of various p53 expression plasmids and β-galactosidase plasmid. Luciferase activity was assayed 48 hr after transfection. Each plasmid combination was transfected into three identical wells. Luciferase assays were performed using (D)-luciferin (Roche). Luminescence was determined with the aid of a Rosys-Anthos Lucy 3 luminometer. Luciferase values were normalized to β-galactosidase activity.

Immunofluorescence Microscopy

Immunofluorescence microscopy was performed as described previously (Kalo et al., 2007). Incubations with antibodies for VDR and/or p53 were carried out for 45 min at 22°C, with three extensive washes after each step. Cells were mounted with Prolong Antifade (Invitrogen). Fluorescence digital images were recorded on a Zeiss Axio Imager microscope (Carl Zeiss).

Coimmunoprecipitation

Coimmunoprecipitation was performed as described previously (Kalo et al., 2007). Cells were treated with 100 nM $1\alpha25(OH)_2D3$ for 3 hr. Cells were then scraped into ice-cold PBS and lysed with lysis buffer. Monoclonal anti-p53 antibody PAb240 or control anti-SV40 Large T antigen Pab419 antibody were incubated with 30 μl protein A for 1 hr at room temperature and added to the lysate. Immune complexes were precipitated overnight at 4°C. The immunoprecipitated material was washed and pellets were resuspended in SDS sample buffer and subjected to western blot analysis.

Chromatin Immunoprecipitation

ChIP was performed as described previously (Stambolsky et al., 2006) using protein A beads crosslinked by DMP to anti-p53 polyclonal antibodies, anti-HA antibodies, or anti-VDR antibodies (C20; Santa Cruz Biotechnology).

DNA samples were extracted using PCR clean-up mini-columns (Promega). Real-time PCR was performed using Sybr Green as described above.

Promoter Array Analysis

Samples of immunoprecipitated DNA and 0.02% of the input DNA (calibrated to be the equivalent of background binding) were amplified by linker-mediated PCR and subjected to ChIP-on-chip analysis as described (Odom et al., 2004), using 1 μg of polyclonal anti-p53 antibody (H47; homemade) and the Hu13K human promoter array. A whole-chip error model (Simon et al., 2001) was used to calculate confidence values for each spot.

Analysis of Transcription Factor Motifs

Search for enriched transcription factor binding motifs in microarray data used the MathInspector library of 414 PSSMs maintained by Genomatix (Release 4.1) (Quandt et al., 1995) and a customary promoter to PSSM assignment score (Elkon et al., 2003). A threshold on this score was defined, above which a PSSM was considered assigned to a promoter. For this purpose, the promoters of coregulated genes were used for a range of potential values of the calculated threshold score, using the hypergeometric statistics, and the group's specificity score (Hughes et al., 2000) of the motif relative to the genes in the cluster. A threshold score that minimizes the hypergeometric probability function was then identified and adopted.

Cell Death Assays

To monitor apoptosis, cultures were subjected to a caspase activity assay (Promega). To that end, cells were seeded in 96 well plates. The next day, either $1\alpha25(OH)_2D3$ or DMSO was added. After an additional 24 hr cells were subjected to different treatments (e.g., anti-cancer drugs) for various times and harvested by adding lysis buffer to the cells, and fluorescence was monitored several hours later. Values were normalized either for cell number or for readings of a WST1 kit assay (Roche), as indicated in the corresponding figure legends. For cell viability assessments, the trypan blue dye exclusion method was used, counting a minimum of 150 cells/assay and expressing data as a percentage of dead (dye including) cells.

ACCESSION NUMBERS

The accession numbers assigned in the ArrayExpress public database to the SKBR3 ChIP-on- chip experiment is E-MEXP-2538 and to the U2OS ChIP-on-chip experiment is E-MEXP-2537. The accession number in the GEO public database for the SKBR3 expression array experiment is GSE19670.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and two tables and can be found with this article online at doi:10.1016/j.ccr.2009.11.025.

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