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The Contribution of Transactivation Subdomains 1 and 2 to p53-Induced Gene Expression Is Heterogeneous But Not Subdomain-Specific[1,2]

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Abstract

Two adjacent regions within the transactivation domain of p53 are sufficient to support sequence-specific transactivation when fused to a heterologous DNA binding domain. It has been hypothesized that these two subdomains of p53 may contribute to the expression of distinct p53-responsive genes. Here we have used oligonucleotide microarrays to identify transcripts induced by variants of p53 with point mutations within subdomains 1, 2, or 1 and 2 (QS1, QS2, and QS1/QS2, respectively). The expression of 254 transcripts was increased in response to wild-type p53 expression but most of these transcripts were poorly induced by these variants of p53. Strikingly, a number of known p53-regulated transcripts including *TNFRSF10B*, *BAX*, *BTG2*, and *POLH* were increased to wild-type levels by $p53^{QS1}$ and $p53^{QS2}$ but not $p53^{QS1/QS2}$, indicating that either subdomain 1 or 2 is sufficient for p53-dependent expression of a small subset of p53-responsive genes. Unexpectedly, there was no evidence for $p53^{QS1}$- or $p53^{QS2}$-specific gene expression. Taken together, we found heterogeneity in the requirement for transactivation subdomains 1 and 2 of p53 without any subdomain-specific contribution to p53-induced gene expression.

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Keywords: p53, gene expression, microarray, transcription factor, apoptosis.

Introduction

The p53 tumor suppressor plays a pivotal role in preventing oncogenic transformation [1]. More than half of all human cancers is associated with alterations in p53 [1]. Decreased p53 activity is associated with hereditary cancers [2] and p53 nullizygous mice are cancer-prone [3]. The p53 protein is a sequence-specific transcription factor that can regulate the expression of a plethora of genes [1]. This protein is activated and accumulates in cells in response to a variety of cellular stresses and thus is an important regulator of stress gene regulation [1].

The p53 protein is a modular protein with several well-characterized functional domains. The C-terminus of p53 is required for oligomerization and contains sequence-independent DNA, DNA damage, and RNA binding activities [4]. This region is dispensable for p53 to function as a transcriptional activator [5,6]. The central third of p53 contains the sequence-specific DNA binding domain required for p53 to function as a transcriptional activator [7]. The majority of tumor-associated p53 mutations fall within the DNA binding domain [8]. The N-terminus of p53 contains an activation domain (AD) that is also required for sequence-specific transcriptional activation [9,10].

The N-terminal 73 amino acids of p53 expressed as a fusion protein with the DNA binding domain of the yeast GAL4 protein functions as an activator of GAL4-dependent gene expression [10]. The minimal transactivation domain was subsequently localized to the N-terminal 42 amino acids of p53 [9] and critical hydrophobic amino acids (Leu-22 and Trp-23) within this acidic region were found to be important for transactivation [5,11,12]. The mutation of these residues (L22Q/W23S) decreased the ability of the N-terminal 42 amino acids of p53 to function as an AD [5,11,12]. The $p53^{L22Q/W23S}$ variant and the murine equivalent ($p53^{L25Q/W26S}$) are commonly used as transactivation-deficient versions of p53 [13–17]. Intriguingly, the L22Q/W23S variant (hereafter referred to as the QS1 variant) reportedly retains some p53 activity despite a profound transactivation defect [13,18–20]. Specifically, the QS1 variant retains the ability to induce apoptosis in some cellular contexts but is unable to induce G_1 arrest [13,14,19,20]. Intriguingly, the homozygous QS1 knock-in mice undergo embryonic lethality although p53 is not required for embryonic development [14,21]. The QS1 variant is not equivalent to the complete loss of p53.

Abbreviations: MALDI-TOF, matrix-assisted laser desorption/ionization time of flight; AD, activation domain; MEF, mouse embryonic fibroblast

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A second functional transactivation subdomain in the N-terminus of p53 has also been identified through a similar strategy. Amino acids 43 to 73 of p53 fused to the DNA binding domain of GAL4 were able to drive Gal4-dependent reporter gene expression and two critical hydrophobic amino acids (Trp-53 and Phe-54) were again critical for this activity [5,11,20]. Like the QS1 variant of p53, the W53Q/F54S variant (hereafter referred to as the QS2 variant) is defective in sequence-specific transactivation, when the expression of a small number of well-characterized p53 target genes was assessed [16,18–20]. Intriguingly, the QS2 variant of p53 was reported to retain the ability to induce p53-dependent G_1 arrest but not p53-dependent apoptosis [20]. Therefore, despite the fact that the QS1 and QS2 variants of p53 have defects in sequence-specific transactivation, they exhibit some distinct biologic activities. This has led several laboratories to hypothesize that these domains function independently in regulating distinct subsets of p53 target genes [14,16,19–21].

Before this study, the relative contribution of these two AD subdomains to p53-mediated gene expression had not been assessed. Here we used recombinant adenoviruses expressing wild-type p53, $p53^{QS1}$, $p53^{QS2}$, and $p53^{QS1/QS2}$ to drive p53-dependent gene expression in colorectal carcinoma cell lines in which endogenous p53 expression had been abolished by gene targeting. Gene expression was assessed using Affymetrix Oligonucleotide microarrays containing over 50,000 features. The expression of 254 transcripts was increased in response to $Adp53^{wt}$ infection and approximately 10% of these transcripts was also induced by the QS1 and QS2 variants but not the compound mutant. A small number of these genes were induced to wild-type levels by the QS variants; however, the fold increase in expression of the transcripts induced by the QS1 and QS2 variants was strongly correlated. These results indicate that the two subdomains cooperate to activate transcription of most p53 target genes. Our work also identified another subgroup of p53 target genes that appear to use either subdomain interchangeably.

Materials and Methods

Cell Culture and UV Treatment

The HCT116 $p53^{-/-}$ cell line was kindly provided by Dr. Bert Vogelstein (John's Hopkins University). Cells were maintained in McCoy's 5A media supplemented with 10% fetal bovine serum (Wisent, St. Bruno, Quebec, Canada). Adenovirus constructs expressing $p53^{wt}$, $p53^{QS1}$, $p53^{QS2}$, and $p53^{QS1/QS2}$ were kindly provided by Dr. Ruth Slack (University of Ottawa, Ontario, Canada). The adenovirus Ad-$BHG^{\Delta E1\Delta E3}$ (Ad-empty) control was generously provided by Dr. Frank Graham (McMaster University, Canada). Viruses were propagated using human embryonic kidney (HEK293) cells and cesium chloride gradient purification [22]. Virus titers were determined in HEK293 cells by standard methods [22] and titers are expressed as plaque-forming units per milliliter (pfu/ml). Cell lines were routinely tested for mycoplasma contamination.

RNA Isolation and Quantitative Reverse Transcription–Polymerase Chain Reaction (RT-PCR)

HCT116 $p53^{-/-}$ cells at 70% to 80% confluence were infected at a multiplicity of infection of 25 with indicated adenovirus in serum-free media for 1 hour. Growth medium containing 10% fetal bovine serum was replaced and cells were returned to the incubator for the indicated time. Infected cells were collected and total RNA was isolated using the RNeasy RNA isolation kit (Qiagen, Valencia, CA) according to manufacturer's specifications. Five micrograms of total RNA was reverse-transcribed using a first-strand cDNA synthesis kit (MBI Fermentas, Burlington, ON, Canada). Quantitative RT-PCR was performed using the SYBR Green Fluorescent DNA stain (Invitrogen, Burlington, ON, Canada), a LightCycler 2 quantitative PCR machine (Roche Diagnostics, Mannheim, Germany), and LightCycler software version 3 (Roche Diagnostics). The primers used were ACTB (GGGCATGGGTCAGAAGGAT and GTGGCCATCTCTTGCTCGA), APAF1 (CAACGGGAGATGACAATG and CTGGAGAAAAGCAAAGGTC), BAK1 (GCCATCAGCAGGAACAGGAG and ACACCCAGAACCACCAGCAC), BTG2 (CACAGAGCACTACAAACACC and ACAAGACGCAGATGGAGC), CASP6 (GCTTTGTGTGTGTCTTCC and CTCAGTTATGTTGGTGTCC), CDKN1A (CCTCAAATCGTCCAGCGACCTT and CATTGTGGGAGGAGCTGTGAAA), TNFRSF6 (CTCATCTTAATGGCCTAATGCA and GCTTCAGTTTATAACTATCTTCAC), TNFRSF10B (GGCATCATCATAGGAGTCAC and GTCAAAGGGCACCAAGTC), TP53I3 (TCTCTATGGTCTGATGGG and TTGCCTATGTTCTTGTTG), and MafB (TGCTGAGAGAGAGAACCGAGAG and CACCACCAAGAACTCTTCCTAC).

Microarrays

Total RNA was collected from HCT116$p53^{-/-}$ cells infected for 16 hours with 25 pfu/cell of Ad-$BHG^{\Delta E1\Delta E3}$, $Adp53^{wt}$, $Adp53^{QS1}$, $Adp53^{QS2}$, or $Adp53^{QS1/QS2}$ using the RNeasy Mini kit (Qiagen). Human Genome U133plus2.0 oligonucleotide arrays (Affymetrix, Santa Clara, CA) were used for expression analysis. Experimental procedures were performed according to the manufacturer specifications at the Ottawa Genomics Innovation Centre Affymetrix GeneChip Facility (Ottawa, Ontario, Canada). Affymetrix Microarray Suite 6.0 (MAS6.0) software was used to analyze the microarray data. MAS6.0 software uses a nonparametric Wilcoxon signed rank test to determine whether statistically meaningful differences in probe cell intensities were detected between samples (change calls were determined using γ_{1H} and γ_{1L} values of 0.0025). Genes were considered to be induced if and only if they were statistically ($P \leq .0025$) increased in all experiments compared to Ad-$BHG^{\Delta E1\Delta E3}$ infected controls by an average of two-fold.

Western Blot Analysis

Total protein was extracted from cells using 1% sodium dodecyl sulfate and brief sonication. Protein samples were run on 4% to 12% Bis–Tris acrylamide gels, transferred to nitrocellulose membrane (Hybond-C; Amersham, Piscataway, NJ) and blocked with 5% skim milk–phosphate-buffered saline

with 1% Tween 20 (TBS–T). Monoclonal antibodies raised against p53 were DO-1 (Ab6; Calbiochem, San Diego, CA), Pab1801 (Ab2; Calbiochem), and Pab421 (Ab1; Calbiochem). Additional antibodies were raised against $p21^{WAF1}$ (Ab1; Calbiochem), PUMA (Ab1; Calbiochem), MDM2 (SMP14; Santa Cruz Biotechnology, Santa Cruz, CA) and MafB (P-20; Santa Cruz Biotechnology). Anti–mouse immunoglobulin (IgG) conjugated with horseradish peroxidase was used as a secondary antibody (Calbiochem), and protein bands were detected using the SuperSignal WestPico Chemiluminescent Substrate kit (Pierce, Rockford, IL) after being exposed to a film (X-OMAT; Kodak, Rochester, NY).

Immunoprecipitation and Mass Spectrometry

HCT116 $p53^{-/-}$ cells were infected with a multiplicity of infectionof 25 of $Adp53^{wt}$. Twenty-four hours postinfection cells were washed twice with PBS and scraped into PBS on ice. Cells were then treated as per manufacturer's instructions for the use of Protein A–agarose beads (Roche Diagnostics). Protein lysates were immunoprecipitated with p53 antibody Pab421. Precipitated protein extracts were run on a 4% to 12% Bis–Tris polyacrylamide gel and subsequently treated with GelCode Blue Stain Reagent according to manufacturer's specifications (Pierce). Bands of interest were excised and subjected to trypsin digestion. Matrix-assisted laser desorption/ionization time of flight tandem mass spectrometry (MALDI-TOF MS/MS) was performed at the Ontario Genomics Innovation Centre Proteomics Facility at the Ottawa Health Research Institute (Ottawa, Ontario, Canada). Peptides were identified using Mascot [23].

Results

The QS1 and QS2 Variants of p53 Are Impaired in p53-Dependent Gene Expression

HCT116 cells in which p53 had been inactivated by gene targeting ($HCT116p53^{-/-}$) [24] were infected with recombinant adenoviruses expressing wild-type, QS1, QS2, or QS1/QS2 variants of p53. Cell lysates were collected for immunoblot analysis at various times following infection using a panel of anti–p53 antibodies. The use of this panel of antibodies allowed us to distinguish between the variant forms of p53 in all experiments (Figure 1*A*). Immunoblot analysis revealed the presence of two immunoreactive bands that migrated at approximately 47 and 53 kDa (Figure 1*B*). Wild-type p53 was immunoprecipitated with Pab421 and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The bands were gel excised and analyzed by MALDI-TOF mass spectrometry. The seven peptides identified were located within the DNA binding domain of p53 (Figure 1*A* and Table W1). These peptides coupled with our panel of anti–p53 antibodies indicated that both bands represent full-length p53 (Figure 1*A*). The two forms likely correspond to

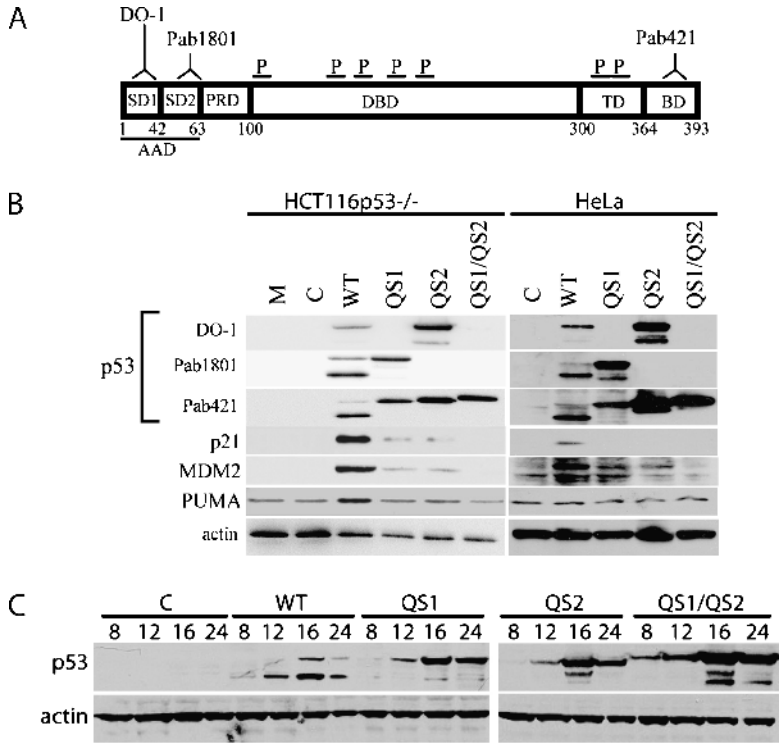

Figure 1. *Expression of transactivation subdomain variants of p53. (A) Schematic representation of epitopes recognized by the indicated monoclonal antibodies (DO-1, Pab1801, and Pab421), peptides (P) identified by mass spectroscopy (see Table W1), and p53 functional domains. SD1 and SD2 denote subdomains 1 and 2 within the acidic AD.* PRD, *proline-rich domain;* DBD, *DNA binding domain;* TD, *tetramerization domain;* BD, *basic domain. Numbers below indicate the amino acid position. (B) Immunoblot analysis of p53 expression 16 hours following infection of either $HCT116p53^{-/-}$ or HeLa cells with the indicated recombinant adenovirus, using three different anti–p53 monoclonal antibodies. M and C represent mock- and control virus–infected samples whereas WT, QS1, QS2, and QS1/QS2 denote the wild-type and variant forms of p53. Similar blots were obtained with cell lysates derived from HCT116 and MDAH041 cells (data not shown). (C) Samples were collected at 8, 16, or 24 hours and subsequently analyzed by immunoblot analysis with the Pab421 monoclonal antibody.*

differentially modified forms. However, the N- and C-termini of p53, containing the known modification sites, were not represented among the identified peptides and thus the specific modifications were not ascertained. The increased expression of these variants relative to wild-type p53 was expected because the QS variants do not induce mdm2 expression (Figure 1*B*), the ubiquitin ligase responsible for the rapid turnover of wild-type p53 [25].

To identify transcripts induced in response to Adp53^{wt}, Adp53^{QS1}, Adp53^{QS2} and Adp53$^{QS1/QS2}$ infection, microarray analysis was performed using total RNA collected from HCT116p53$^{-/-}$ cells 16 hours post infection because maximal p53 levels were achieved within this time frame (Figure 1*C*). The expression of 254 transcripts increased significantly following infection with the Adp53^{wt} virus compared to control virus infection (Table W2). Of these, only 28, 23, and 1 were induced by Adp53^{QS1}, Adp53^{QS2}, and Adp53$^{QS1/QS2}$, respectively (Figure 2*A* and Table W3). The mean induction of the Adp53^{wt}-induced transcripts was significantly higher than the fold increase in expression due to the expression of any of the QS variants (Figure 2*B*). In fact, very few of the Adp53^{wt}-induced genes appeared to be induced to wild-type levels by either the QS1 or QS2 variants (Figure 2*C*). Infection with the Adp53^{wt} virus resulted in a greater increase in gene expression even when examining genes determined to be induced in response to either Adp53^{QS1} or Adp53^{QS2} infection (Figure 2, *D* and *E*). Therefore, the majority of WT-, QS1-, and QS2-regulated genes were poorly induced by the QS variants.

Correlation between Genes Induced By the QS1 and QS2 Variants

Having determined that most Adp53^{wt}-induced genes were poorly induced by the variants, we sought to determine whether distinct subgroups of p53 target genes were preferentially responsive to the QS variants of p53. The fold change in p53 target gene expression in response to Adp53^{QS1} infection was plotted with respect to Adp53^{QS2} infection (Figure 3*B*). We observed a very striking linear correlation between the fold change in expression induced by the QS1 and QS2 variants of p53 regardless of whether the expression of Adp53^{wt}-, Adp53^{QS1}-, or Adp53^{QS2}-induced genes were considered (Figure 3*B*; R^2 values were 0.73, 0.64, and 0.61, respectively). Therefore, the Adp53^{QS1}- and Adp53^{QS2}-induced genes were induced to a similar extent by both variants (Figure 3*B* and Table W3). These results indicate that the disruption of either subdomain of p53 similarly affected the overall pattern of p53 transcriptional activation. We interpret these results to indicate that the contribution of transactivation subdomains 1 and 2 to p53-mediated gene expression was heterogeneous but not subdomain-specific.

Based on our definition of induced genes (see Materials and Methods section), the expression of 18 genes increased in response to both QS variants but 10 and 5 wild-type p53-induced transcripts appeared to be increased in response to either QS1 or QS2, respectively (Figure 3*A*). To determine whether these apparently p53^{QS1}- and p53^{QS2}-specific genes were in fact specifically and preferentially upregulated by one of the variants, the pattern of allele-specific gene expression was confirmed by quantitative RT-PCR for 11 different transcripts at several different times following viral infection. The majority of p53 target genes were poorly induced by the QS1 and QS2 variants (Figure 4*A*). Several p53 target genes were significantly induced by the QS variants but were induced more strongly by wild-type p53 (Figure 4*B*). Lastly, a few target genes were induced to near wild-type levels by p53^{QS1} and p53^{QS2} (Figure 4*C*). The quantitative RT-PCR data correlated well with the microarray analysis and none of the p53-upregulated transcripts examined displayed a subdomain-specific pattern of gene expression (Figure 4 and Tables W3 and W4). The expression of several p53-regulated proteins was assessed by immunoblot in independent cell lines and no subdomain-specific differences in protein expression were detected (Figure 1*B*). Collectively, we interpret our results to indicate that the apparently p53^{QS1}- and p53^{QS2}-specific targets were not specifically induced by a single variant. Therefore, the response of p53-induced transcripts to the QS variants was heterogeneous but not subdomain-specific.

The 254 Adp53^{wt}-induced genes were subjected to gene ontology (GO) analysis (http://www.geneontology.org/). Several genes were associated with the GO terms apoptosis (GO:0006915), cell cycle (GO:0007049), and DNA repair (GO:0006281) (Table 1), consistent with known p53 biology [26]. Of these terms, only apoptosis was statistically overrepresented ($P < .01$) based on analysis using the web-based GOstat software (http://gostat.wehi.edu.au/). Consistent with the preponderance of proapoptotic genes, Adp53^{wt} infection resulted in a significant increase in the proportion of apoptotic cells (Figure 5, *A* and *B*). Both QS variants were reduced in their capacity to induce cell death and there was no significant difference in their ability to induce apoptosis in these cells (Figure 5, *A* and *B*). Most of the apoptosis annotated genes were poorly induced by the QS1 and QS2 variants of p53 compared to wild-type p53 (Table 1). Therefore, decreased p53-dependent gene expression correlated with decreased p53-dependent apoptosis in these cells. Similarly, the cell cycle–annotated genes were poorly induced by all variants (Table 1). Interestingly, two of the three genes associated with DNA repair (*BTG2* and *POLH*) were induced to near wild-type levels by the QS1 and QS2 variants but not the QS1/QS2 variant (Table 1 and Figure 4*C*). Due to the limited number of repair-related genes, the significance of this specific observation remains unclear. Overall, our results suggest that there is substantial heterogeneity in the contribution of subdomains 1 and 2 to p53-mediated gene expression but there are no subdomain-specific effects.

Discussion

The p53 protein can act as both a positive and negative regulator of gene expression but p53 is best understood as a transcriptional activator. The p53 protein is a positive regulator of several hundred genes and this is mediated by the sequence-specific binding of p53 to consensus elements found in promoters, enhancer regions, introns, or the 5′

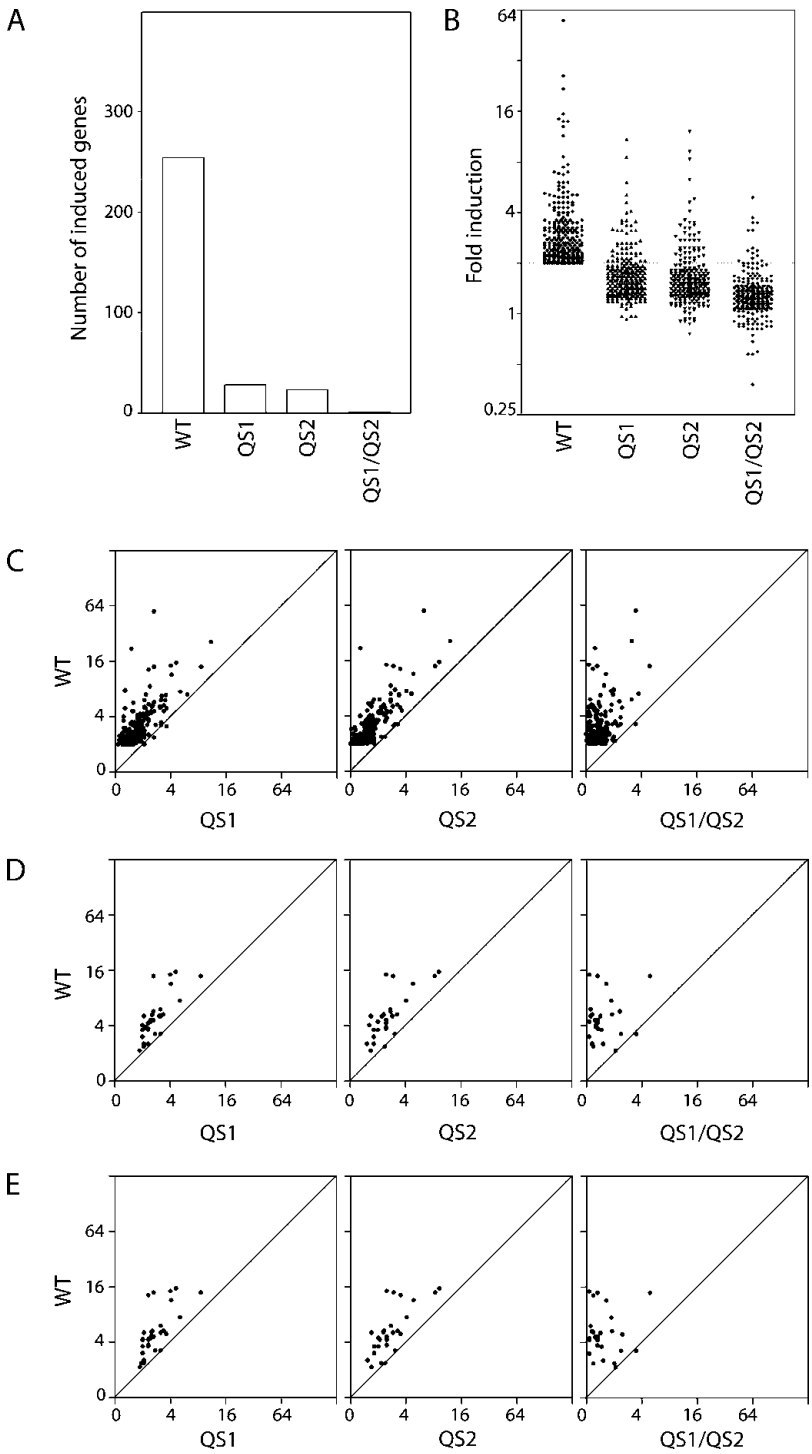

Figure 2. *Most p53 target genes are poorly induced by the QS variants. (A) Two hundred and fifty-four genes were induced by Adp53^{wt}. Of these, only 28, 23, and 1 were induced by QS1, QS2, and QS1/QS2, respectively. (B) The fold increase in expression of these 254 genes was determined following infection of cells with adenoviruses expressing wild-type, QS1, QS2, or QS1/QS2 variant of p53. The fold increase in the expression following infection with Adp53^{QS1}, Adp53^{QS2}, or Adp53$^{QS1/QS2}$ was less than the fold increase in response to Adp53^{wt} infection (one-way analysis of variance followed by a Tukey's Multiple Comparisons test, $P \leq .001$). (C–E) The fold increase in expression due to Adp53^{wt} expression was compared to the fold increase in expression due to indicated transactivation subdomain variant of p53 for Adp53^{wt}-, Adp53^{QS1}-, and Adp53^{QS2}-induced genes (C, D, and E, respectively). The 254, 28, and 23 genes induced by Adp53^{wt}, Adp53^{QS1}, and Adp53^{QS2} are listed in Tables W2 and W3.*

untranslated regions of these genes [1]. Transcriptional activation further requires the p53-dependent recruitment of the histone acetyl transferases CBP/p300, general transcription factors, and RNA polymerase II to the promoter of target genes [27–30]. The N-terminal AD is required for the recruitment of these proteins and subsequent p53-dependent gene activation [11,30]. Amino acids 1 to 42 were found to function as a minimal transcriptional AD [9,10]. However, it was subsequently shown that this minimal region was part of a larger AD with each of the two subdomains capable of supporting

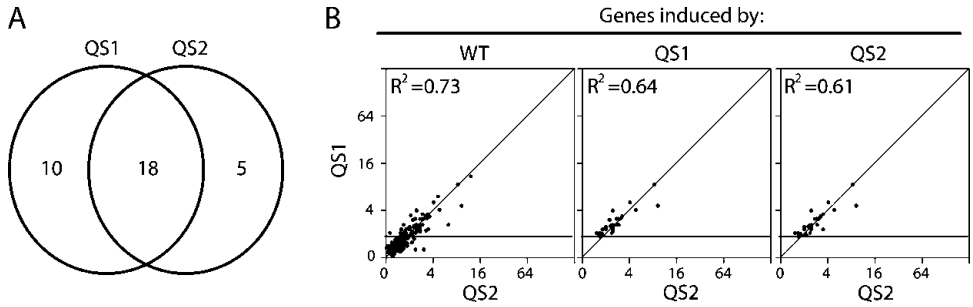

Figure 3. *Correlation between Adp53^{QS1}- and Adp53^{QS2}-induced genes. (A) A Venn diagram is used to represent the overlap between Adp53^{QS1}- and Adp53^{QS2}-induced genes, as defined in the Materials and Methods section. (B) The effect of Adp53^{QS1} and Adp53^{QS2} infection on the expression of the 254 Adp53^{wt}-, 28 Adp53^{QS1}-, and 23 Adp53^{QS2}-induced genes was determined. The genes induced by Adp53^{wt}, Adp53^{QS1}, and Adp53^{QS2} are listed in Tables W2 and W3. A very tight correlation (R^2 values are inset) between Adp53^{QS1}- and Adp53^{QS2}-induced gene expression was observed within the subset of target genes.*

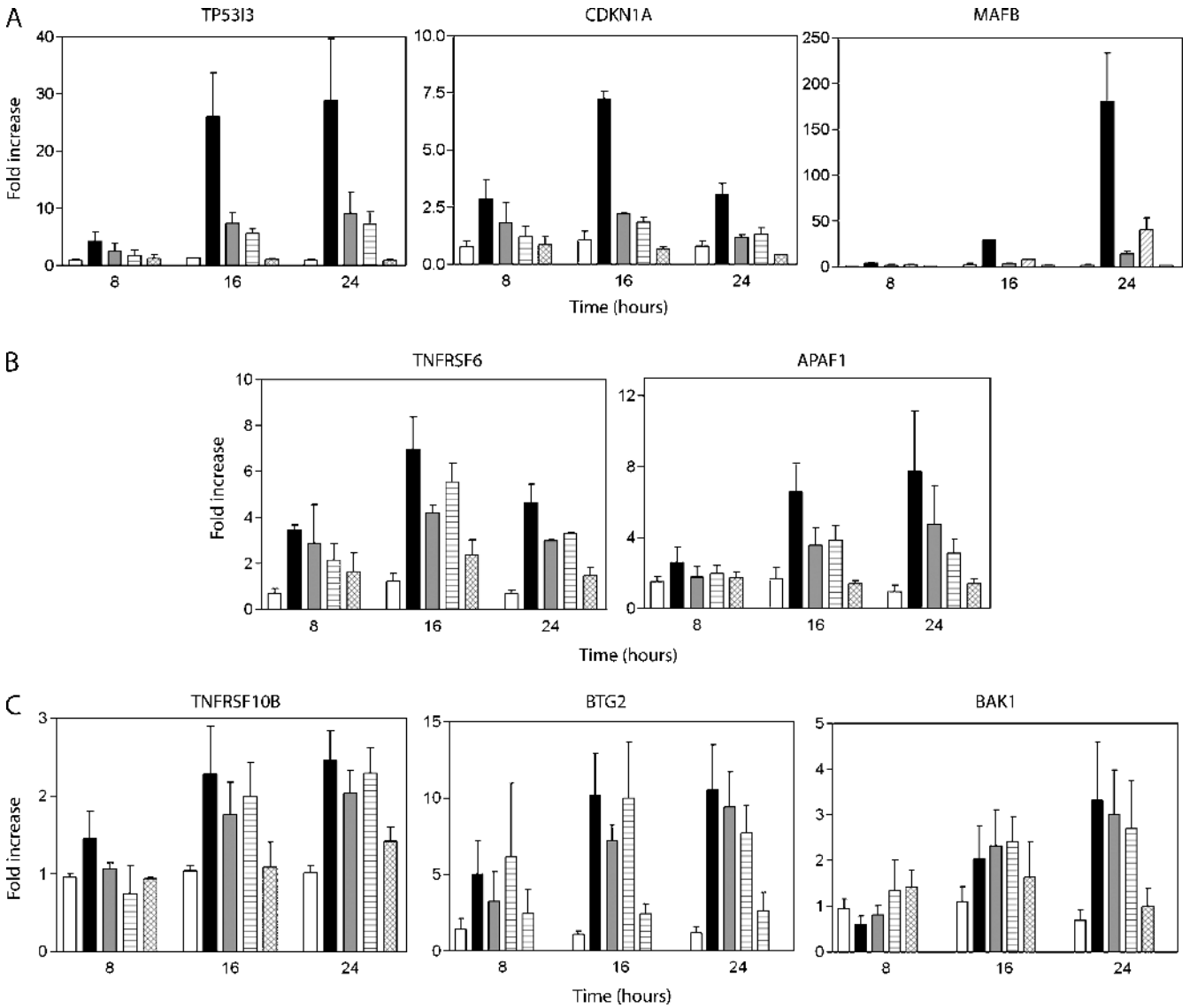

Figure 4. *Representative transcripts induced by wild-type p53. (A–C) Expression of the indicated transcript was determined by real-time RT-PCR using samples collected at the indicated time following virus infection (8, 16, or 24 hours). Expression of β-actin was used to normalize all RT-PCR results. Open, black, grey, hatched, and crosshatched bars represent control, Adp53^{wt}-, Adp53^{QS1}-, Adp53^{QS2}-, and Adp53$^{QS1/QS2}$-infected samples. Each value represents the mean fold increase in expression (± SEM) determined from a minimum of three independent experiments.*

Table 1. Adp53^{wt}-Induced Genes Involved in Apoptosis, Cell Cycle, and/or DNA Repair.

GO Term*	Locus	p53 Variant			
		WT	QS1	QS2	QS1/QS2
Apoptosis (GO:0006915)	*TP53I3*	3.9†	2.0	1.3	0.1
	TP53INP1	3.8	1.4	1.5	0.4
	APAF1	2.8	1.8	1.8	1.0
	TNFRSF6	2.4	1.8	1.7	0.9
	CASP6	1.7	0.8	0.7	0.4
	TNFRSF10B	1.5	0.9	0.8	0.5
	MDM2	1.4	0.3	0.1	0.0
	BAX	1.4	1.2	0.9	0.2
	AKTIP	1.2	0.3	0.4	0.4
	AMID	1.2	0.4	0.2	0.2
	BID	1.1	0.4	0.3	0.3
	CARD10	1.1	0.5	0.4	0.4
	BAK1	1.0	0.5	0.4	0.3
	TRAF4	1.0	0.4	−0.1	−0.2
Cell Cycle (GO:0007049)	*CDKN1A*	2.4	1.1	0.8	−0.8
	SESN1	2.0	0.9	0.8	0.1
	GAS2L1	1.4	0.8	0.6	0.4
	MDM2	1.4	0.3	0.1	0.0
	RB1	1.3	0.8	0.6	0.8
	RHOB	1.2	0.4	0.3	0.5
	PARD6G	1.2	0.7	0.6	0.4
	SFN	1.2	0.4	0.3	−0.3
	SESN2	1.1	0.3	0.2	0.0
	LATS2	1.1	0.5	0.4	0.4
	HRAS	1.0	0.5	0.4	0.2
DNA Repair (GO:0006281)	*BTG2*	2.9	2.4	2.0	0.9
	POLH	2.2	1.8	1.9	0.9
	DDB2	1.1	0.4	0.3	−0.3

*Genes involved in apoptosis, cell cycle and DNA repair were identified using the Gene Ontology database (http://geneontology.org/). Of these GO terms, only apoptosis (GO:0006915) and related GO terms were significantly over represented among the Adp53^{wt}-induced genes based on GOstat analysis (http://gostat.wehi.edu.au/).

†The mean fold increase in expression (log_2) determined from microarray experiments, as described in the Materials and Methods section.

sequence-specific transactivation when expressed as a fusion protein with a heterologous DNA binding domain [5,11].

The relative contribution of subdomains 1 and 2 to p53 activity has been examined at the cell biologic level by two laboratories. Zhu et al. [20] reported that p53^{QS1} is unable to induce G_1 arrest but retains the ability to induce apoptosis in tumor cell lines [18]. In contrast, p53^{QS2} was reportedly able to induce cell cycle arrest but was impaired in its ability to induce apoptosis in these same cell lines [20]. Cregan et al. [16] reported that overexpression of either p53^{QS1} or p53^{QS2} in neuronal cells led to similar levels of apoptosis but that forced expression of the p53^{QS2} variant in p53 nullizygous neuronal cells led to significantly more apoptosis than the p53^{QS1} variant when these cells were subsequently treated with camptothecin. Based on these studies, it was hypothesized that the p53 transactivation subdomains contribute to the regulation of distinct subsets of p53 target genes that affect the biologic activity of these variants. However, the relative contribution of the two distinct subdomains in the AD to p53-dependent transcriptional activity had remained untested.

Here we found that infection of these cells with recombinant adenoviruses expressing p53^{QS1}, p53^{QS2}, and p53$^{QS1/QS2}$ resulted in the induction of far fewer p53 target genes than Adp53^{wt} infection. Approximately 10% of the Adp53-induced genes were also increased on Adp53^{QS1} and Adp53^{QS2} infection. The identity and fold increase in expression of the p53^{QS1}- and p53^{QS2}-upregulated genes were strongly correlated, indicating that these subdomains do not contribute to the expression of distinct subsets of genes. The majority of p53-regulated genes were induced poorly by p53^{QS1}, p53^{QS2}, and p53$^{QS1/QS2}$, indicating that both subactivation domains are required to increase the expression of most p53 target genes. Conversely, a relatively small number of p53-target genes including *TNFRSF10B*, *BAX*, *BTG2*, and *POLH* were induced to near wild-type levels by p53^{QS1} and p53^{QS2}, but were poorly induced by p53$^{QS1/QS2}$. Therefore, the p53-dependent induction of this subgroup of p53 target genes requires a functional transactivation domain but subdomain 1 or 2 appears to be sufficient and used interchangeably for p53 target gene expression within this group of genes. We did not detect any subdomain-specific p53 target genes.

Like many other transactivation domains, the N-terminus of p53 is not highly conserved overall at the level of amino acid sequence; however, there is a high level of sequence conservation among rodents and primates within subdomain 1 (34% identity; see Figure W1). The region of highest homology includes amino acids 13 through 26 (93% identity), a region of p53 termed box 1 [31]. The N-terminus of p53 is rich in acidic residues characteristic of acidic ADs and is mostly unstructured under physiological conditions [32]. Box 1 contains a number of hydrophobic residues and nuclear magnetic resonance studies indicate that amino acids 18 to 26 within box 1 form a helix within the context of the larger disordered transactivation domain [32]. Mutation of Leu-22 and Trp-23 within the helical region in the QS1 variant is predicted to disrupt this region of limited secondary structure [32]. The high

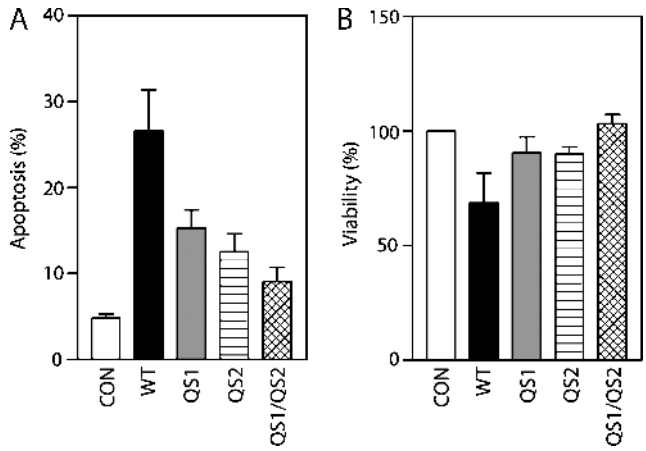

Figure 5. *Effect of QS variants of p53 on cell viability and apoptosis. Apoptosis and cell viability were assessed 48 hours following infection with either control adenovirus or adenoviruses expressing the indicated variants of p53. Apoptosis was assessed by subdiploid DNA content (A) and viability was assessed by Trypan blue exclusion (B). Each point represents the mean (±SEM) determined from three independent experiments. Adp53wt induced more apoptosis than the variants (one-way analysis of variance followed by Tukey's Multiple Comparisons test, $P \leq .01$ for apoptosis and $P \leq .05$ for viability). No significant difference in viability or apoptosis was observed when comparing QS1 and QS2 variants.*

level of conservation between mouse and human p53 has made it possible to generate knock-in mice expressing the QS1 variant from the endogenous p53 locus [14], as discussed later. It is likely that this secondary structure is important for the activity of subdomain 1.

Subdomain 2, within the transactivation domain of p53, is well conserved among primate species but is poorly conserved when the sequence comparison is extended to rodent versions of p53 (Figure W1). The limited homology between mouse and human p53 makes the QS2 variant more difficult to model in mice. Much like subdomain 1, hydrophobic residues within subdomain 2 of human p53 give rise to localized secondary structure within the mostly unstructured acidic AD (nascent turns between Met-40 and Met-44 and between Asp-48 and Trp-53) [32–34]. Regions of limited secondary structure within mostly unstructured ADs are common among transcriptional activators [12,30,34,35]. The second subdomain of p53 reportedly binds to many proteins known to interact with subdomain 1, such as mdm2, RPA, TFIID, TFIIH, and p300 [11,27,30,34,36–44]. The QS2 point mutations are thought to disrupt the localized secondary structure and would be expected to disrupt protein–protein interactions important for transcriptional activation [12,30,32–34].

As indicated above, knock-in mice expressing the QS1 variant from the endogenous p53 locus have been generated [14]. Homozygous $p53^{QS1}$ expression results in embryonic lethality; however, homozygous $p53^{QS1}$-targeted mouse embryonic fibroblasts (MEFs) were obtained [14]. Using these MEFs, the induction of five of the six p53 target genes tested was reduced in the QS1-expressing MEFs compared to control cell lines following doxorubicin treatment [14]. The single gene induced by the QS1 variant of murine p53 was *BAX* [14] and we similarly found that the human QS1 variant was able to upregulate *BAX* expression. Unexpectedly, we found that the QS1 and QS2 variants similarly increased the expression of *BAX* along with three other known p53 target genes (*TNFRSF10B*, *BTG2*, and *POLH*). Therefore, only one of the two subdomains appears to be necessary and sufficient for p53-dependent gene expression of this subset of p53 target genes. We interpret the heterogenous requirement for subdomains 1 and 2 to indicate that the requirement for specific protein–p53 AD interactions must vary in a p53 target gene–specific manner.

In summary, our results suggest that compound mutations of critical hydrophobic amino acids in either subdomain 1 or 2 decrease the affinity of the AD for cofactors or other components of basal transcription apparatus that are rate-limiting for p53-dependent gene expression, at most p53-induced promoters. Surprisingly, the induction of a small subset of p53-responsive genes, including *BAX*, *TNFRSF10B*, *BTG2*, and *POLH*, is not limited by mutations in either of the subdomains alone. Therefore, this latter group of genes appears to have a less stringent requirement for as yet unidentified protein–protein interactions. Importantly, we did not find any genes that were preferentially induced by any single AD variant. Our results support a model in which the transcription activation subdomains of p53 contribute equally to p53-dependent target gene expression.

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Table W1. Peptides Identified By MALDI-TOF.

Peptide	Band (kDa)		Position
	47	53	
1	TYQGSYGFR	TYQGSYGFR	102–110
2	KPLDGEYFTLQIR	KPLDGEYFTLQIR	321–333
3	KKPLDGEYFTLQIR	KKPLDGEYFTLQIR	320–333
4	TCPVQLWVDSTPPPGTR	TCPVQLWVDSTPPPGTR	140–156
5		ERFEMFR	336–342
6		QSQHMTEVVR	165–174
7		VEYLDDRNTFR	203–213
8		CSDSDGLAPPQHLIR	182–196

Table W2. Genes Induced following Adp53^{wt} Infection.

Probe Set(s)*	Gene Symbol†	Induced
218559_s_at	*MAFB*	5.8‡
205249_at	*EGR2*	4.7
224646_x_at, 224997_x_at	*H19*	4.5
1554340_a_at	*C1orf187*	4.0§
210609_s_at	*TP53I3*	3.9
211421_s_at	*RET*	3.8
225912_at	*TP53INP1*	3.8
205569_at	*LAMP3*	3.7
232165_at, 232164_s_at, 208156_x_at	*EPPK1*	3.5
212942_s_at	*KIAA1199*	3.1
210090_at	*ARC*	3.0
201235_s_at, 201236_s_at	*BTG2*	2.9
219583_s_at	*PATA7*	2.8
226913_s_at	*SOX8*	2.8
204859_s_at, 211554_s_at	*APAF1*	2.8
215785_s_at, 220999_s_at	*CYFIP2*	2.8
231115_at	*GTPBP2*	2.6
212070_at	*GPR56*	2.6
214890_s_at	*DKFZP564J102*	2.6
213268_at, 1555370_a_at	*CAMTA1*	2.5
242517_at	*GPR54*	2.5
232289_at	*FLJ14167*	2.5
204780_s_at, 215719_x_at, 216252_x_at, 204781_s_at	*TNFRSF6*	2.4
202181_at	*KIAA0247*	2.4
212907_at, 228181_at	*SLC30A1*	2.4
222546_s_at, 218180_s_at	*EPS8L2*	2.4
227306_at	*FLJ21245*	2.4
202284_s_at	*CDKN1A*	2.4
205286_at, 205287_s_at	*TFAP2C*	2.3
206153_at	*CYP4F11*	2.3
205493_s_at, 205492_s_at	*DPYSL4*	2.3
206832_s_at	*SEMA3F*	2.3
1557701_s_at	*POLH*	2.2
201578_at	*PODXL*	2.2
213469_at	*PGAP1*	2.2
206277_at	*P2RY2*	2.2
231928_at	*HES2*	2.2
204855_at	*SERPINB5*	2.2
203865_s_at	*ADARB1*	2.2
203045_at	*NINJ1*	2.1
235205_at	*LOC346887*	2.1
224724_at	*SULF2*	2.1
221577_x_at	*GDF15*	2.1
210367_s_at	*PTGES*	2.1
238542_at, 221291_at	*ULBP2*	2.0
228315_at	N/A	2.0
218346_s_at	*SESN1*	2.0
204379_s_at, 204380_s_at	*FGFR3*	2.0
230356_at	Transcribed locus	2.0
203722_at	*ALDH4A1*	2.0
202307_s_at	*TAP1*	1.9
219099_at	*C12orf5*	1.9
224793_s_at	*TGFBR1*	1.9
219936_s_at	*GPR87*	1.9
212496_s_at, 212492_s_at	*JMJD2B*	1.9
203946_s_at	*ARG2*	1.9
39248_at	*AQP3*	1.9
205278_at	*GAD1*	1.8
207813_s_at	*FDXR*	1.8
203570_at	*LOXL1*	1.8
235230_at	*PLCXD2*	1.8
209050_s_at, 209051_s_at	*RALGDS*	1.8
219597_s_at	*DUOX1*	1.8
213568_at	*OSR2*	1.8
218032_at	*SNN*	1.8
227964_at	*FKSG44*	1.8
209712_at, 209711_at	*SLC35D1*	1.7
225160_x_at, 229711_s_at	*MGC5370*	1.7
238335_at	*DNAJA5*	1.7
235467_s_at	*KCNC4*	1.7
215411_s_at	*TRAF3IP2*	1.7
203310_at	*STXBP3*	1.7
56256_at	*SIDT2*	1.7
228115_at	N/A	1.7
201032_at	*BLCAP*	1.7
208978_at	*CRIP2*	1.7
209693_at	*ASTN2*	1.7
227247_at	*PLEKHA8*	1.7
227295_at	*IKIP*	1.7
209790_s_at	*CASP6*	1.7
213716_s_at	*SECTM1*	1.7
210138_at	*RGS20*	1.7
205483_s_at	*G1P2*	1.7
31846_at, 209885_at	*RHOD*	1.6
201412_at	*LRP10*	1.6
227522_at	*LOC134147*	1.6
219322_s_at, 236381_s_at	*WDR8*	1.6
201963_at	*ACSL1*	1.6
235434_at	N/A	1.6
223754_at	*MGC13057*	1.6
1556194_a_at	N/A	1.6
241348_at, 219239_s_at	*ZNF654*	1.6
212510_at	*GPD1L*	1.6
336_at	*TBXA2R*	1.6
202627_s_at, 202628_s_at	*SERPINE1*	1.5
55081_at, 221779_at	*MICAL-L1*	1.5
212992_at	*C14orf78*	1.5
221215_s_at, 234730_s_at	*RIPK4*	1.5
233550_s_at, 223748_at	*SLC4A11*	1.5
219358_s_at	*CENTA2*	1.5
1555609_a_at	*WIG1*	1.5
227728_at, 231370_at, 203966_s_at	*PPM1A*	1.5
209295_at, 210405_x_at, 209294_x_at	*TNFRSF10B*	1.5
204061_at	*PRKX*	1.5
232946_s_at	*NADSYN1*	1.5
212800_at, 1552618_at, 212799_at	*STX6*	1.5
227420_at	*MGC17791*	1.5
1560228_at	*SNAI3*	1.5
202023_at	*EFNA1*	1.4
201834_at, 201835_s_at	*PRKAB1*	1.4
229616_s_at	*LOC196996*	1.4
203068_at	*KLHL21*	1.4
212812_at	N/A	1.4
227221_at	N/A	1.4
225864_at	*NSE2*	1.4
202587_s_at	*AK1*	1.4
226022_at	*SASH1*	1.4
212558_at	*SPRY1*	1.4
201302_at, 201301_s_at	*ANXA4*	1.4
208258_s_at, 209729_at, 31874_at	*GAS2L1*	1.4
218007_s_at, 238935_at, 222487_s_at	*RPS27L*	1.4
211833_s_at	*BAX*	1.4
231269_at	*ASCC3*	1.4
220161_s_at	*EPB41L4B*	1.4
222451_s_at	*ZDHHC9*	1.4
226604_at, 226600_at	*SMILE*	1.4
242705_x_at	*LRPAP1*	1.4
201117_s_at	*CPE*	1.4

Table W2. (*continued*)

Probe Set(s)*	Gene Symbol†	Induced
242463_x_at	*ZNF600*	1.4
203695_s_at	*DFNA5*	1.4
205386_s_at	*MDM2*	1.4
203132_at	*RB1*	1.3
204060_s_at	*PRKX, PRKY*	1.3
210962_s_at	*AKAP9*	1.3
219938_s_at	*PSTPIP2*	1.3
223342_at	*RRM2B*	1.3
227134_at	*SYTL1*	1.3
205109_s_at	*ARHGEF4*	1.3
224690_at	*C20orf108*	1.3
213271_s_at	*KIAA1117*	1.3
221840_at	*PTPRE*	1.3
226782_at	*SLC25A30*	1.3
225049_at	*BLOC1S2*	1.3
224862_at	*GNAQ*	1.3
202071_at	*SDC4*	1.3
224901_at	*SCD4*	1.3
225734_at	*FBXO22*	1.3
226805_at	*C20orf142*	1.3
211675_s_at	*MDFIC*	1.3
220520_s_at	*FLJ20130*	1.3
204547_at	*RAB40B*	1.3
202409_at	*LOC492304*	1.3
203224_at, 203225_s_at	*RFK*	1.2
218168_s_at	*CABC1*	1.2
238480_at	*C18orf17*	1.2
227204_at	*PARD6G*	1.2
211272_s_at	*DGKA*	1.2
226302_at	*ATP8B1*	1.2
204160_s_at	*ENPP4*	1.2
224733_at	*CKLFSF3*	1.2
212099_at	*RHOB*	1.2
201565_s_at	*ID2*	1.2
228937_at	*FLJ38725*	1.2
221012_s_at, 223132_s_at	*TRIM8*	1.2
215646_s_at, 221731_x_at, 211571_s_at	*CSPG2*	1.2
218527_at	*APTX*	1.2
226483_at	*FLJ32370*	1.2
203115_at	*FECH*	1.2
202351_at	*ITGAV*	1.2
209075_s_at	*NIFUN*	1.2
222874_s_at	*CLN8*	1.2
222820_at	*TNRC6C*	1.2
225848_at	*FLJ31413*	1.2
209513_s_at	*HSDL2*	1.2
221640_s_at	*LRDD*	1.2
213038_at, 36564_at	*IBRDC3*	1.2
224618_at	N/A	1.2
244467_at	*LOC440829*	1.2
226580_at	*BRMS1L*	1.2
224461_s_at	*AMID*	1.2
212968_at	*RFNG*	1.2
202546_at	N/A	1.2
202392_s_at	*PISD*	1.2
209260_at	*SFN*	1.2
218373_at	*FTS*	1.2
218251_at	*MID1IP1*	1.2
202672_s_at	*ATF3*	1.2
217297_s_at	*MYO9B*	1.2
217889_s_at	*CYBRD1*	1.2
225223_at, 225219_at	*SMAD5*	1.1
212637_s_at, 212638_s_at	*WWP1*	1.1
218415_at	*VPS33B*	1.1
227776_at	N/A	1.1
221732_at	*CANT1*	1.1
226214_at	*MIR16*	1.1
225473_at	*FLJ44670*	1.1

Table W2. (*continued*)

Probe Set(s)*	Gene Symbol†	Induced
209286_at	*CDC42EP3*	1.1
210026_s_at	*CARD10*	1.1
209584_x_at	*APOBEC3C*	1.1
218627_at	*FLJ11259*	1.1
204493_at	*BID*	1.1
216080_s_at	*FADS3*	1.1
228347_at	*SIX1*	1.1
44040_at	*FBXO41*	1.1
235119_at	*TAF3*	1.1
209558_s_at	*HIP1R*	1.1
223195_s_at, 223196_s_at	*SESN2*	1.1
212124_at	*RAI17*	1.1
203216_s_at, 203215_s_at, 210480_s_at	*MYO6*	1.1
210260_s_at, 208296_x_at	*TNFAIP8*	1.1
220007_at	*FLJ13984*	1.1
230563_at	*RASGEF1A*	1.1
202794_at	*INPP1*	1.1
200704_at	*LITAF*	1.1
225334_at	*C10orf32*	1.1
34206_at	*CENTD2*	1.1
223474_at	*C14orf4*	1.1
228098_s_at	*MYLIP*	1.1
227013_at	*LATS2*	1.1
221843_s_at	*KIAA1609*	1.1
214434_at	*HSPA12A*	1.1
228220_at	*FCHO2*	1.1
36711_at	*MAFF*	1.1
213587_s_at	*C7orf32*	1.1
225604_s_at	*C9orf19*	1.1
203409_at	*DDB2, LHX3*	1.1
218066_at	*SLC12A7*	1.1
218764_at	*PRKCH*	1.1
203499_at	*EPHA2*	1.1
235252_at	*KSR*	1.1
201473_at	*JUNB*	1.1
214435_x_at, 224880_at	*RALA*	1.0
205442_at	N/A	1.0
222687_s_at	*PHCA*	1.0
224617_at	N/A	1.0
203537_at	*PRPSAP2*	1.0
203728_at	*BAK1*	1.0
212983_at	*HRAS*	1.0
225319_s_at	*FLJ14775*	1.0
203367_at	*DUSP14*	1.0
229746_x_at, 231819_at	*CEBPZ*	1.0
225347_at	*ARL10B*	1.0
218288_s_at	*MDS025*	1.0
223385_at	*CYP2S1*	1.0
202755_s_at	*GPC1*	1.0
212966_at	*HIC2*	1.0
216041_x_at	*GRN*	1.0
235688_s_at	*TRAF4*	1.0
35160_at	*LDB1*	1.0
201494_at	*PRCP*	1.0
227357_at	*TAB3*	1.0
207076_s_at	*ASS*	1.0
202286_s_at	*TACSTD2*	1.0
209184_s_at	*IRS2*	1.0

*Probe sets used to identify features on the Affymetrix microarrays. Multiple probe sets are listed if individual genes were represented more than once on the array.

†Official gene symbols were determined using the Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez).

‡Log_2 (fold increase in expression) resulting from infection with adenoviruses expressing the indicated variant of p53.

§The values highlighted in yellow were increased in response to one or more variants of p53 and details are provided in Table W3.

Table W3. Genes Induced in Response to Infection with Ad-p53^{QS1} and/or Ad-p53^{QS2}.

Gene Symbol	Variant of p53			
	WT	QS1	QS2	QS1/QS2
QS1 and QS2				
*C1orf187**	15.5[†]	4.6	9.2	0.6[‡]
TP53I3[§]	14.4	4.0	2.5	1.1
RET	13.9	8.6	8.3	4.9
TP53INP1	13.9	2.6	2.9	1.3
EPPK1	11.4	4.1	4.8	1.6
BTG2	7.5	5.1	4.1	1.9
GPR56	6.1	3.1	2.7	1.1
FAS	5.3	3.4	3.2	1.9
KIAA0247	5.3	2.5	2.3	1.1
EPS8L2	5.1	3.1	2.9	1.4
PODXL	4.6	2.6	2.5	1.2
PGAP1	4.6	2.5	2.5	1.3
SERPINB5	4.4	2.5	2.0	1.1
NINJ1	4.3	2.3	2.5	1.3
TAP1	3.7	2.3	2.5	1.3
C12orf5	3.6	2.3	2.0	1.5
DNAJA5	3.2	3.1	3.0	2.4
PTPRE	2.4	2.1	2.4	1.2
QS1 not QS2				
CAMTA1	5.8	2.6	2.7	2.3
FLJ21245	5.1	3.0	2.2	1.1
CDKN1A	5.1	2.1	1.7	0.6
ULBP2	4.1	2.0	1.6	1.3
Transcribed locus	3.9	2.1	2.5	1.3
TGFBR1	3.6	2.0	1.8	1.5
KCNC4	3.2	2.7	0.9	3.5
LRP10	3.0	2.0	1.8	1.1
BAX	2.5	2.3	1.8	1.1
ASCC3	2.5	2.1	1.5	1.5
QS2 not QS1				
LAMP3	13.0	2.3	3.5	1.2
SLC30A1	5.2	2.5	2.3	1.2
CYP4F11	4.9	3.6	3.5	2.5
LOC346887	4.3	2.0	2.1	1.1
SLC25A30	2.4	1.9	2.1	2.0

Genes induced by one or both variants of p53 are grouped together.
*Official gene symbols were determined using the Entrez Gene database (http://www.ncbi.nlm.nih.gov/sites/entrez).
[†]Fold increase in expression following infection with adenoviruses expressing the indicated variant of p53. values are ordered by decreasing fold change in expression following Adp53^{wt} infection.
[‡]The values highlighted in yellow were not considered to be induced because they did not meet statistical criteria described in the Materials and Methods.
[§]The fold induction of the gene highlighted in green in response to Adp53^{QS1} and Adp53^{QS2} infection was reduced more than two-fold compared to the level induced in response to Adp53^{wt} infection.

Table W4. Correlation between Microarray and RT-PCR Results.

Transcript	Variant of p53							
	WT		QS1		QS2		QS1/QS2	
	M*	RT	M	RT	M	RT	M	RT
TP53IP3	+++[†]	+++	+	+	+	+	–	–
MAFB	+++	+++	–	–	–	–	–	–
APAF1	++	++	–	+	–	+	–	–
CDKN1A	++	++	+	+/–	–	+/–	–	–
SERPINB5	++	++	+	+	+	+	–	–
MDM2	+	+	–	–	–	–	–	–
DDB2	+	+	–	–	–	–	–	–
TNFRSF6	++	++	++	++	++	++	–	–
BTG2	+	+	+	+	+	+	–	–
TNFRSF10B	+	+	+/–	+	+/–	+	–	–
BAK1	+	+	–	+	–	+	–	–
CASP6	+	+	–	+/–	–	+/–	–	–

*M and RT denote microarray and RT-PCR expression data, respectively.
[†]The number of + symbols indicates the relative increase in transcript level, – indicates that the transcript was not increased by the variant, and +/– indicates that the transcript is marginally increased.

'*' denotes sequence identity.
':' denotes conservative substitutions.
'.' denotes semiconservative substitutions.
═ marks residues mutated in the human QS variants of p53.

Figure W1. *Amino acid sequence alignments of the N-terminal transactivation domains of p53 of rodent and primate origin. Sequence of the first 72 amino acids of p53 from representative primate and rodent species was performed using CLUSTAL W software (Thompson JD, Higgins DG, and Gibson TJ (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice.* Nucleic Acids Res **22**, *4673–4680.) hosted on http://scbr.bii.a-star.edu.sg and accessed through http://p53.bii.a-star.edu.sg. (A) Sequence comparisons were performed for (A) human (*Homo sapiens*), African green monkey (*Chlorocebus sabaeus*) and rhesus monkey (*Macaca mulatta*) p53, (B) mouse (*Mus musculus*), rat (*Rattus norvegicus*), and Chinese hamster (*Cricetulus griseus*) p53, and (C) all six species listed in A and B. Legends for A, B, and C are inset in the lower left corner of panel C.*