Identification of Cell Cycle Regulatory Genes as Principal Targets of p53-mediated Transcriptional Repression*§

Received for publication, December 30, 2005, and in revised form, June 19, 2006 Published, JBC Papers in Press, June 23, 2006 DOI 10.1074/jbc.M513901200

Kevin B. Spurgers§¹, David L. Gold*, Kevin R. Coombes#, Nicole L. Bohnenstiehl§¹, Brian Mullins⁴, Raymond E. Meyn⁷‡, Christopher J. Logothetis**, and Timothy J. McDonnell†§²

From the Departments of *Molecular Pathology, **Biostatistics, ‡Experimental Radiation Oncology and §Genitourinary Medical Oncology, the University of Texas M. D. Anderson Cancer Center and the #University of Texas Graduate School of Biomedical Sciences, Houston, Texas 77030

Historically, most studies attribute p53 function to the transactivation of target genes. That p53 can selectively repress genes to affect a cellular response is less widely appreciated. Available evidence suggests that repression is important for p53-induced apoptosis and cell cycle arrest. To better establish the scope of p53-repressed target genes and the cellular processes they may affect, a global expression profiling strategy was used to identify p53-responsive genes following adenoviral p53 gene transfer (Ad-p53) in PC3 prostate cancer cells. A total of 111 genes, 0.77% of the 14,500 genes represented on the Affymetrix U133A microarray, were repressed more than 2-fold (p ≤ 0.05). Validation of the array data, using reverse transcription-PCR of 20 randomly selected genes, yielded a confirmation rate of >95.5% for the complete data set. Functional over-representation analysis was used to identify the cell cycle regulatory genes exhibited a highly significant enrichment (p ≤ 5 × 10⁻⁵) within the transpressed targets. 41% of the repressed targets are cell cycle regulators. A subset of these genes exhibited repression following DNA damage, preceding cell cycle arrest, in LNCaP cells. The use of a p53 small interfering RNA strategy in LNCaP cells and the use of p53-null cell lines demonstrated that this repression is p53-dependent. These findings identify a set of genes not known previously to be down-regulated by p53 and indicate that p53-induced cell cycle arrest is a function of not only the transactivation of cell cycle inhibitors (e.g. p21) but also the repression of targets that regulate proliferation at several distinct phases of the cell cycle.

Instances of DNA damage, resulting in gene mutation or deletion, are critical events that contribute to tumor formation. Accordingly, cells possess sophisticated mechanisms to detect and respond to DNA damage. One such mechanism is the activation of the p53 tumor suppressor protein. High levels of activated and stabilized p53 protein accumulate in the nucleus in response to various forms of cell stress, including DNA damage (1, 2). Activated p53 can induce cell cycle arrest, DNA repair processes, and apoptosis. These cellular outcomes are thought to minimize the accumulation of deleterious mutations that could eventually contribute to a malignant phenotype (3). The importance of this pathway is highlighted by the observation that over 50% of cancers have acquired p53 gene mutations, ostensibly allowing the tumors to bypass this p53 checkpoint (4, 5).

Although transcription-independent functions exist, p53 mediates its effects largely by regulating the expression of downstream target genes (6, 7). Most studies investigating p53 function have focused attention on the genes transactivated by p53. However, it is recognized that repression of target genes may be important for p53-induced cell death and cell cycle arrest. For example, trichostatin A, a histone deacetylase inhibitor, inhibits the repression of p53 targets and the ability of p53 to induce apoptosis (8). Additionally, cell cycle regulators such as cdc2 and cyclin B can be repressed by p53 (9, 10). Beyond apoptosis and cell cycle arrest, p53 can affect other cellular processes including DNA repair, senescence, and differentiation (1). Despite documented examples (11, 12), relatively little is known regarding the genes transcriptionally repressed by p53 and what roles they play in mediating p53 function. These repressed genes are of further interest in that they may potentially be overexpressed as a consequence of somatic p53 mutation during cancer progression.

Using high density oligonucleotide microarrays we identified a total of 111 genes that were significantly repressed following adenoviral p53 gene transfer (Ad-p53) in PC3 prostate cancer cells. Functional over-representation analysis was used to objectively identify which cellular processes were influenced by p53-mediated repression. Notably, nearly half of the repressed genes (45 of 111 genes) are involved in cell cycle regulation. This represents a highly significant enrichment of this functional category within the repressed targets compared with the microarray as a whole. Importantly, several cell cycle genes were repressed after genotoxic stress only in cells harboring wild type p53 alleles and before cell cycle arrest is evident. The p53-dependent nature of this repression was further demonstrated using a p53 knockdown strategy. These findings identify cell cycle regulatory genes previously unrecognized as p53-
responsive and indicate that p53-induced cell cycle arrest is a function of not only the transactivation of cell cycle inhibitors (e.g., p21), but also the repression of targets that regulate proliferation at several distinct phases of the cell cycle.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The PC3 and LNCaP prostate carcinoma cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cell culture conditions, along with the generation and characterization of stable vector control (PC3-BN) and BCL2-overexpressing (PC3-BCL2) PC3 cells have been described (13, 14). Where noted, the cells were treated with etoposide (Sigma) dissolved in Me2SO added directly to BN) and BCL2-overexpressing (PC3-BCL2) PC3 cells have been described (16) and were carried out by the M. D. Anderson Cancer Center (Houston, TX). Construction of the recombinant, replication-deficient, type 5 adenovirus expressing human p53 (Ad-p53) and the experimental parameters and procedures used in this study have been described (13, 15). Briefly, PC3 cells were infected with control virus expressing β-galactosidase (Ad-LacZ) or with Ad-p53 at 1,000 virus particles/cell. RNA was harvested 19 h after infection. Target preparation and Affymetrix (Santa Clara, CA) U133A GeneChip hybridizations have been described (16) and were carried out by the M. D. Anderson Cancer Center Microarray Core Facility (Houston, TX).

**Array Data Analysis**—The arrays were preprocessed with dCHIP version 1.3 software (www.dchip.org). Gene expression was obtained after normalization with the Perfect Match only model (17). dCHIP array level quality metrics were examined to identify outliers. The arrays were also assessed for brightness, alignment calibration, and spatial variation following the methods of Gold et al. (16).

Initially, a detection filter was applied to retain only those probe sets called present by dCHIP in all six replicates of at least one biological factor combination. Of the 22,283 probe sets on the U133A microarray, 12,308 met this detection criterion. The 12,038 detectable probe sets had a significant F-statistic versus the alternative that at least one term was not zero. 5,080 of the 12,038 detectable probe sets had a significant F-statistic at the Bonferroni adjusted level. Gene discovery proceeded with a pass for significant p53 effects exceeding a fold change of 2, corresponding to |αg| ≥ 1 on the log2 scale. Individual t-statistics for the hypotheses $H_0$: 1 < αg < 1 $H_1$: αg ≤ −1 or αg ≥ 1 were tested at a 0.05 Bonferroni adjusted significance level. While accounting for p53-BCL2 interactions, we found 391 probe sets with significant change coincidental with p53 expression beyond a fold change of 2. These 391 probe sets correspond to 335 unique genes (224 up-regulated and 111 repressed following p53 expression).

**Hierarchical Cluster Analysis**—We compared relative sources of variation between arrays by using hierarchical cluster analysis. We required probe sets to have expression levels above the median signal on at least six arrays to be included in the analysis ($n = 10,625$ probe sets). Hierarchical cluster analysis was applied with complete linkage and Euclidean distance on log2 expression. Furthermore, the respective profiles for probe set targets of p53 were examined on the log2 scale.

**Estimation of Array Data False Positive Rate**—A random number generator following a discrete uniform distribution was used to select 20 differentially expressed genes for verification. To estimate the fraction ϕ of “true positives” on our list of differentially expressed genes, we modeled the number of successful confirmations as a binomial random variable $X = \text{Binom}(N, ϕ)$. A Bayesian statistical method was used to estimate the probability distribution of ϕ. We looked at various beta prior distributions, $ϕ = \text{Beta}(2p, 2(1 − p))$, corresponding to different choices of the parameter $p$, the prior probability of confirmation. The posterior distribution of ϕ, conditional on confirming $k$ of $N$ genes, is another beta distribution, $\text{Beta}(2p + k, 2(1 − p) + N − k)$. All 20 of the genes tested were successfully confirmed, giving posterior distributions of the form $\text{Beta}(2p + 20, 2(1 − p) + 20)$. We computed the expected value and 95% confidence interval for the confirmation rate ϕ for a wide range of prior distributions.

**RT-PCR**—For RT-PCRs, 3 μg of total RNA was combined with 500 ng of oligo(dT) primer (Invitrogen) in a final volume of 12 μl, heated at 70°C for 10 min, and then placed on ice. Reverse transcription reactions (20 μl) were then assembled with final concentrations of 1X reaction buffer, 10 mM dithiothreitol, 0.5 mM dNTPs, and 100 units of Superscript II reverse transcriptase (Invitrogen). Two percent of the RT reaction was

\[ Y \sim \text{Normal}(\mu, \sigma^2) \]

for $i = j = 1–2$, $k = 1–6$, and $g = 1–12,308$, in α for the presence of a p53 effect, β for a BCL2 effect, and $\alpha\beta$ for p53–BCL2 interaction. Here μ accounts for expected log2 expression. Unexplained random variation $\epsilon$ was assumed normally distributed with expectation 0 and variance $\sigma^2$. We explored possible hybridization day and RNA extraction contributions to variation in gene expression across arrays. These were not considered, agreeing with hierarchical cluster analysis and thus were omitted from Equations 1 and 2. Genes with significantly altered expression were identified using an F-statistic pass at the 0.05 Bonferroni-corrected significance level ($p < 0.05/12,038$) to test the hypothesis that the control factors in Equation 1 were simultaneously zero,

\[ H_0: \alpha_g = \beta_g = \alpha\beta_g = 0 \quad \text{(Eq. 2)} \]

$H_0$: at least one factor $\neq 0$.

---

3 The abbreviations used are: CHX, cycloheximide; RT, reverse transcription; siRNA, small interfering RNA; BrdUrd, bromodeoxyuridine; GO, gene ontology.
used as input for each PCR. PCRs (50 μl) were assembled with final concentrations of 1X buffer, 1.5 mM MgCl2, 0.2 mM dNTPs, 0.2 μM of each primer, and 2.5 units of Taq polymerase (Invitrogen). Primers were obtained from Sigma-Genosys (The Woodlands, TX). The sequence of each primer is available upon request. PCR cycle parameters were as follows: initial step of 95 °C for 5 min and then 20-35 cycles of 95 °C for 30 s, annealing temperature of 59–61 °C for 30 s, 72 °C for 45 s, and a final step of 72 °C for 5 min. The cycle number that allows for end point analysis during linear amplification was determined empirically.

Functional Over-representation Analysis—The Expression Analysis Systematic Explorer software package was used to identify biological themes that are significantly enriched in our list of p53-repressed genes (18). Gene categories with an Expression Analysis Systematic Explorer score of ≤0.05 were considered significantly enriched. Bonferroni multiplicity correction was not employed.

siRNA Transfection—LNCaP cells were transfected with p53-specific or p21\textsuperscript{waf1}-specific siRNA SmartPool duplexes or nontargeting siRNA number 1 (Dharmacon, Lafayette, CO) at a final concentration of 10 nM. Transfections were performed using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol. A total of 30 and 60 μl of Lipofectamine 2000 were used for each 60-mm and 10-cm plate, respectively. Transfection medium was replaced with fresh complete medium after 7 h.

Western Blot—Western blots were generated and probed as described (13). The primary antibodies used were anti-p53 at 1:1,500 (Bp53-12; Santa Cruz Biotechnology, Santa Cruz, CA), anti-p21 at 1:500 (Ab-1; Calbiochem, San Diego, CA), and anti-actin at 1:1,000 (AC-40; Sigma).

BrdUrd Incorporation—BrdUrd incorporation was measured in LNCaP cells using the BrdUrd Flow Kit (BD Pharmingen, San Diego, CA) following the manufacturer’s protocol with some modifications. BrdUrd was added directly to culture medium at a final concentration of 10 μM. Forty minutes after the addition of BrdUrd, the cells were harvested with trypsin, washed once in 1X phosphate-buffered saline, and fixed in 100 μl of Cytofix/Cytoperm for 30 min at room temperature. Incubation with Cytoperm Plus and a second fixation with Cytofix/Cytoperm were omitted. The cells were read on a FACScalibur flow cytometer (BD Biosciences, San Jose, CA).

RESULTS

Identification of p53-responsive Genes—The microarray expression profiling experiments performed here were originally designed to assess p53-responsive gene expression and the extent to which this process could be modulated by BCL2 (13). BCL2 expression is associated with androgen-independent prostate cancer, tumor progression, and resistance to therapy (19, 20). Additionally, the BCL2 proto-oncogene can inhibit p53-dependent apoptosis and may act, in part, by modulating the transcriptional response of select p53 target genes (21, 22). We used Affymetrix U133A microarrays and an adenovirus p53 (Ad-p53) expression system to identify p53-responsive genes in stable vector control (PC3-BN) and BCL2 overexpressing (PC3-BCL2) PC3 prostate cancer cells (13). For each cell line, the probes for the arrays were generated from RNA harvested 19 h after infection with control adenovirus (Ad-LacZ) or Ad-p53.

Microarray experiments and subsequent statistical analysis were designed to minimize the effect of inherent experimental variation. Six replicate hybridizations (with probes from two independent virus infections and RNA extractions) were performed for each cell line/treatment combination. Hierarchical cluster analysis was used to assess sources of variation across the arrays. The most dominant feature affecting the clustering was p53 expression (not shown). RNA extraction or hybridization day were insignificant sources of variation across arrays.

To identify p53-responsive genes, control virus treatments were compared with Ad-p53 treatments. A total of 111 genes were repressed (fold change ≥ 2, p ≤ 0.05) in both PC3-BN and PC3-BCL2 cell lines following p53 expression. The repression of these 111 genes was not affected by BCL2 overexpression. No genes were detected that were repressed in one cell line and not the other. The complete list of p53-repressed genes is available as supplementary data (supplemental Table S1).

Objective Assessment of Array Data Reliability—Given the nature of microarray experiments and the statistical methods employed, the presence of false positives is inherent in any evaluation of differentially expressed genes. With this in mind, we objectively assessed the false positive rate within our final data set. In addition to p53-repressed genes, we also identified genes transcriptionally activated in response to p53. Twenty genes were chosen at random (10 activated and 10 repressed) and tested for a response to p53 by RT-PCR. The expression patterns of all 20 genes were validated (Fig. 1 and not shown). Given this data, we used a Bayesian statistical method to estimate the false positive rate for the complete data set. Using a neutral, uniform, prior probability for confirmation, the estimate of the confirmation rate is 95.5% with a 95% confidence interval of 86.7–100%. Thus, ~4.5% of the genes identified as differentially expressed are expected to be false positives.

Even with a very low expected frequency of false positives, our data set does contain at least some false negative results. For example, survivin is strongly down-regulated in PC3 cells following Ad-p53 treatment (13). This gene is represented on the U133A microarray but was not identified as a p53-repressed target in our analysis. Despite the presence of some false negatives, our data set shows considerable overlap with published expression profiling studies looking at p53-responsive gene expression and also identifies novel p53 target genes (23, 24).

Functional Over-representation Analysis—Functional over-representation analysis was performed to objectively identify biological processes potentially affected by p53-mediated transcriptional repression. Specifically, the percentage of p53-repressed genes with a given gene ontology (GO) annotation was compared with the percentage of genes on the Affymetrix U133A GeneChip with the same annotation. A significant p value (p ≤ 0.05) indicates that the observed percentage of p53-repressed genes with a given annotation could not likely occur by chance given the frequency of genes on the microarray with
LNCaP cells are still synthesizing DNA 8 h after treatment (Fig. 1). In concordance with these data, LNCaP cells were significantly enriched (p < 0.05) in our list of p53-repressed genes (Fig. 2). The GO annotation “cell cycle” shows the most significant enrichment. Specifically, 45 of the 111 p53-repressed genes identified in our study are involved in cell cycle regulation (Table 1). Most of these genes also have more specific GO annotations suggesting that several phases of the cell cycle are affected by p53-mediated transcriptional repression. X indicates that the gene product has a GO annotation for that specific cell cycle phase (Table 1). Genes that harbor consensus p53 DNA-binding sites, based on the data of Hoh et al. (25), are also indicated. Other GO categories of interest significantly enriched within the repressed targets include DNA repair, nucleotide metabolism, and DNA packaging (supplemental Table S2).

**p53-dependent Repression of Cell Cycle Genes Following DNA Damage**—To assess the potential relevance of our findings, we determined whether any of these genes respond to endogenous p53 after DNA damage. Several genes were tested for a response to DNA damage in LNCaP cells (p53 wild type) and the p53-null PC3 cell line (26, 27). Etoposide, a chemotherapeutic agent that causes DNA double strand breaks, induced p53 protein accumulation in LNCaP cells (Fig. 3A). Induction of the cell cycle inhibitor p21\(^{wap}\), a well characterized direct p53 target, was also observed. In concordance with these data, LNCaP cells undergo cell cycle arrest in response to etoposide treatment. As indicated by BrdUrd incorporation, a significant fraction of LNCaP cells are still synthesizing DNA 8 h after treatment (Fig. 3B). However, the cells are fully arrested 24 h after treatment with few, if any, exhibiting BrdUrd incorporation. By scoring cells with a sub-G1 DNA content as apoptotic, we confirmed that LNCaP cells are not undergoing apoptosis over this time course (Fig. 3C).

For RT-PCR analysis, LNCaP and PC3 cells were mock treated, not treated, or exposed to etoposide for 4, 12, and 24 h. Several genes displayed a reduction in RNA level following etoposide treatment in LNCaP cells but not in PC3 cells, consistent with this repression being p53-dependent (Fig. 4A and supplemental Fig. S1). Importantly, several genes clearly display reduced RNA levels 4 h after treatment, prior to cell cycle arrest or apoptosis. Together, these data strongly suggest that the observed repression in LNCaP cells is p53-dependent and not a secondary effect of cell cycle status or a consequence of apoptosis.

p53 dependence was assessed in a more direct manner using siRNA to prevent accumulation of p53 following DNA damage. LNCaP cells were transfected with negative control or p53-specific siRNA or not transfected. 48 h after siRNA transfection, the cells were treated with etoposide for 4 h and then analyzed by Western blot. Pretreatment with p53 siRNA prevented the accumulation of p53 protein following etoposide treatment (Fig. 4B). Next, the genes showing a DNA damage response in LNCaP cells were examined by RT-PCR following p53 siRNA transfection and etoposide (24 h) treatment. Each gene showed a reduction in RNA level in nontargeting siRNA transfected, etoposide-treated cells, compared with control cells (Fig. 4C). This reduction did not occur or was dramatically attenuated in etoposide-treated cells that had been transfected with p53 siRNA. These findings are consistent with the interpretation that down-regulation of these genes in LNCaP cells is specifically dependent on p53 protein accumulation.

**Repression of Cell Cycle Genes Requires New Protein Synthesis but Not p21 Transactivation**—In some cases the transactivation or repression of p53 target genes is known to require new protein synthesis following p53 expression (28). In these cases, changes in gene expression are thought to occur because of effects of primary p53 targets. In particular,
some groups have suggested that p53-mediated repression of cell cycle genes requires the up-regulation of p21\textsuperscript{WD}\textsuperscript{1} protein (24). We determined whether select cell cycle regulatory genes could be repressed in the presence of the protein synthesis inhibitor CHX. LNCaP cells were treated with etoposide in the presence or absence of CHX. Without CHX, as expected, p53 and p21 protein levels increased 4 h after etoposide treatment (Fig. 5A). The addition of CHX prevented the accumulation of p21 protein but had little effect on p53. CHX did not prevent the increase in p21 mRNA following etoposide treatment, demonstrating a specific inhibition of protein synthesis and not p53-regulated transcription (Fig. 5B). Significantly, CHX prevented the transcriptional repression of each p53-repressed, cell cycle regulatory gene examined (Fig. 5B). These findings suggest that new protein synthesis is necessary for repression and that repression likely occurs through the action of other p53-regulated genes.

We next specifically investigated the requirement of p21 protein up-regulation in the repression of cell cycle genes following DNA damage. LNCaP cells were transfected with negative control siRNA or siRNA specific for p21. The cells were then treated with Me\textsubscript{2}SO or etoposide after 48 h. Western blot and RT-PCR analyses were carried out 8 h after the addition of etoposide. In the presence of negative control siRNA, p21 was efficiently up-regulated following DNA damage (Fig. 6A). This accumulation of p21 protein was inhibited by p21 siRNA transfection. However, p21 siRNA had little or no effect on the transcriptional repression of select cell cycle genes after etoposide treatment (Fig. 6B). These data suggest that the p53-dependent repression of cell cycle genes following DNA damage does not require the accumulation of high levels of p21 protein.

**DISCUSSION**

Historically, investigations of p53-regulated transcription have focused on genes transactivated by p53 (28, 29). However, recent evidence has implicated the repression of target genes in p53-induced apoptosis and cell cycle regulation (30). Furthermore, in some expression profiling studies, many more p53-repressed genes are detected than genes transactivated by p53 (23). We took a global genomic approach to better define the scope of p53-mediated repression. Our microarray and statistical procedures yielded a data set of high quality with an estimated false positive rate of less than 5%. Objective assessment of array data quality is critical for the interpretation of any expression profiling experiment but is, unfortunately, lacking in most studies.

**TABLE 1**

| Probe Symbol | Gene name G 1 | G 1/S | S | G 2/M | M | p53 site |
|--------------|---------------|------|---|-------|---|---------|
| 203968_s_at  | CDC6 Cell division cycle 6 homolog | × | × | × | × | Yes |
| 204510_at    | CDC7 Cell division cycle 7 | × | × | × | × | Yes |
| 203213_at    | CDC2 Cell division cycle 2, CDK1 | × | × | × | × | Yes |
| 203418_at    | CCNA2 Cyclin A2 | × | × | × | × | Yes |
| 204695_at    | CDC25A Cell division cycle 25A | × | × | × | × | Yes |
| 202240_s_at  | CDK4 Cyclin-dependent kinase 4 | × | × | × | × | Yes |
| 205393_s_at  | CHEK1 CHK1 checkpoint homolog (Schizosaccharomyces pombe) | × | × | × | × | Yes |
| 211814_s_at  | CCNE2 Cyclin E2 | × | × | × | × | Yes |
| 204244_at    | ASK Activator of S phase kinase B | × | × | × | × | Yes |
| 220207_at    | MCM4 Minichromosome maintenance deficient 4 | × | × | × | × | Yes |
| 202107_s_at  | MCM2 Minichromosome maintenance deficient 2 | × | × | × | × | Yes |
| 201555_at    | MCM3 Minichromosome maintenance deficient 3 | × | × | × | × | Yes |
| 201590_at    | MCM6 Minichromosome maintenance deficient 6 | × | × | × | × | Yes |
| 210983_s_at  | MCM7 Minichromosome maintenance deficient 7 | × | × | × | × | Yes |
| 201890_at    | BLM2 Ribonucleotide reductase M2 polypeptide | × | × | × | × | Yes |
| 208956_s_at  | DUT dUTP pyrophosphatase | × | × | × | × | Yes |
| 204767_s_at  | FEN1 Flap structure-specific endonuclease 1 | × | × | × | × | Yes |
| 211450_s_at  | MSH6 MutS homolog 6 (Esherichia coli) | × | × | × | × | Yes |
| 209421_at    | MSH2 MutS homolog 2 | × | × | × | × | Yes |
| 205099_at    | POLE2 Polymerase (DNA directed), epsilon 2 (p59 subunit) | × | × | × | × | Yes |
| 205053_at    | PRM1 Primase, polypeptide 1, 49 kDa | × | × | × | × | Yes |
| 204127_at    | RFC3 Replication factor C (activator 1) 3 | × | × | × | × | Yes |
| 201292_at    | TOP2A Topoisomerase (DNA) II liz 170 kDa | × | × | × | × | Yes |
| 221521_s_at  | PSF2 DNA replication complex GINS protein PSF2 | × | × | × | × | Yes |
| 209832_s_at  | CDT1 DNA replication factor | × | × | × | × | Yes |
| 202870_s_at  | CDC20 Cell division cycle 20 homolog | × | × | × | × | Yes |
| 218099_s_at  | PRC1 Protein regulator of cytokinesis 1 | × | × | × | × | Yes |
| 204641_at    | NEK2 NIMA (never in mitosis gene a)-related kinase 2 | × | × | × | × | Yes |
| 204709_at    | KIF23 Kinesin family member 23 | × | × | × | × | Yes |
| 202958_at    | UBE2C Ubiquitin-conjugating enzyme E2 | × | × | × | × | Yes |
| 203630_s_at  | MAD2L1 MAD2 mitotic arrest deficient-1 (yeast) | × | × | × | × | Yes |
| 209642_at    | BUB1 Budding uninhibited by benzimidazoles 1 homolog (yeast) | × | × | × | × | Yes |
| 203755_at    | BUB1B Budding uninhibited by benzimidazoles 1 homolog B (yeast) | × | × | × | × | Yes |
| 207828_s_at  | CENPF Centromere protein F | × | × | × | × | Yes |
| 208076_s_at  | STK6 Serine/threonine kinase 6, BTAK, STK15, AUR | × | × | × | × | Yes |
| 209259_s_at  | CSPG6 Chondroitin sulfate proteoglycan 6 (bamacan) | × | × | × | × | Yes |
| 210653_s_at  | SMCL1 SMC4 structural maintenance of chromosomes 4-like 1 | × | × | × | × | Yes |
| 204162_at    | KNT2C Ketochore-associated 2 | × | × | × | × | Yes |
| 204340_s_at  | SMCL2 Structural maintenance of chromosomes 2-like 1 | × | × | × | × | Yes |
| 210052_s_at  | TPX2 Microtubule-associated protein homolog (Xenopus laevis) | × | × | × | × | Yes |
| 219494_at    | RAD54B RAD54 homolog B (Saccharomyces cerevisiae) | × | × | × | × | Yes |
| 219148_at    | TOPK T-LAK cell-originated protein k | × | × | × | × | Yes |
| 218662_s_at  | HCAP-G Chromosome condensation protein G | × | × | × | × | Yes |
| 202431_s_at  | MYC V-Myc myelocytomatosis viral oncogene homolog (c-Myc) | × | × | × | × | Yes |
| 209464_at    | AURKB Aurora kinase B, STK12 | × | × | × | × | Yes |
An objective of this study was to identify cellular processes or pathways affected by p53-mediated transcriptional repression. One approach to identify affected pathways is to determine the function of each differentially expressed gene, look for patterns, and use this information to guide future experiments. Unfortunately, this approach can be highly subjective and becomes untenable as the number of differentially expressed genes increases. In this regard, automated annotation of gene function and objective assessment of affected cellular processes is advantageous. The Expression Analysis Systematic Explorer software package uses GO annotations to identify biological themes that are significantly enriched in a list of differentially expressed genes (18). GO is a set of controlled vocabularies that describes the molecular function(s), subcellular localization, and biological processes associated with a particular gene product. This type of analysis also has limitations. First, GO annotations are not present for all genes, and annotations that are present do not describe every known function of a particular gene product. Second, the absence of a cellular process in our analysis does not mean that p53-mediated repression does not play a role in that process. For example, repression of target genes is thought to play a role in p53-induced apoptosis. However, genes with a GO annotation of apoptosis were not significantly enriched in our list of p53-repressed targets. p53 could influence apoptosis by repressing a group of genes that is insufficient in number to show up as a significant hit in this analysis.

Genes with the GO annotation of cell cycle showed the most statistically significant enrichment in our list of p53-repressed targets. It was of interest to determine whether the cell cycle genes identified in this study could be specifically repressed by activated, endogenous p53 prior to the onset of apoptosis. The LNCaP cells exhibit p53 and p21 protein accumulation, and cell cycle arrest, in response to etoposide treatment. A, LNCaP cells were not treated (NT), mock treated with Me2SO (M), or treated with etoposide for the indicated time. Western blot analysis was performed for p53, p21 and actin. B, LNCaP cells were treated with etoposide for 8 or 24 h, or not treated (NT). Cells synthesizing DNA were scored by detection of incorporated BrdUrd. The percentages of cells positive for BrdUrd incorporation are shown. C, DNA content histograms were generated from the data in B. Cells with a sub-G1 DNA content were scored as apoptotic.

**FIGURE 3.** LNCaP cells exhibit p53 and p21 protein accumulation, and cell cycle arrest, in response to etoposide treatment. A, LNCaP cells were not treated (NT), mock treated with Me2SO (M), or treated with etoposide for the indicated time. Western blot analysis was performed for p53, p21 and actin. B, LNCaP cells were treated with etoposide for 8 or 24 h, or not treated (NT). Cells synthesizing DNA were scored by detection of incorporated BrdUrd. The percentages of cells positive for BrdUrd incorporation are shown. C, DNA content histograms were generated from the data in B. Cells with a sub-G1 DNA content were scored as apoptotic.

**FIGURE 4.** p53-dependent repression of cell cycle genes in LNCaP cells following DNA damage. A, several genes repressed in response to Ad-p53 treatment were tested for a response to DNA damage in LNCaP (wt p53) and PC3 (null p53) cells. Each cell line was mock treated with Me2SO (M) or treated with etoposide for 24 h. B, Western blot analysis of siRNA transfected, etoposide-treated LNCaP cells. The cells were transfected with negative control siRNA followed by etoposide treatment. C, transfection with p53 siRNA attenuates the repression of cell cycle genes in LNCaP cells following DNA damage as determined by RT-PCR. The cells were transfected with negative control siRNA or p53-specific siRNA or not transfected. 48 h after transfection, the cells were treated with etoposide or dimethyl sulfoxide (DMSO, control), for 24 h.
cell cycle arrest. Because the transcription of a large number of genes is known to be modulated with cell cycle progression, one can observe repression as a secondary effect of cell cycle status (31). Also, the Ad-p53 vectors used in the microarray experiments produce sustained, superphysiological levels of p53 protein. One may expect that not all of the genes identified with this method will be responsive to endogenous levels of activated p53 protein. Significantly, several genes were repressed in LNCaP cells (wt p53) in response to DNA damage well before cell cycle arrest was evident. If p53 protein accumulation is reduced in LNCaP cells following etoposide treatment using p53-specific siRNA, the observed repression of these genes is abrogated. Using these methods, we identified several cell cycle regulatory genes subject to p53-dependent transcriptional repression following DNA damage. Other investigators have also identified cell cycle genes as being responsive to DNA damage or p53 expression. These genes include, among others, cyclin A2, CDC20, CDC2, CHEK1, MCM3, MCM6, MCM7, PRC1, NEK2, and TOP2α (9, 23, 32–34). However, to our knowledge, the majority of the repressed, cell cycle regulatory genes identified in our study have not been previously recognized as p53-responsive. Other processes possibly affected by p53-mediated transcriptional repression include DNA repair, nucleotide metabolism, and DNA packaging.

This analysis suggests that p53 inhibits cell cycle progression in part by repressing the transcription of genes that act at each phase of the cell cycle, from G1 through cytokinesis (Fig. 7). In support of this idea, forced expression of repressed cell cycle targets can inhibit p53-mediated cell cycle arrest. For example, c-Myc repression is observed in DP16.1 cells undergoing cell cycle arrest in response to p53 expression (35). If c-Myc expression is enforced in these cells, the extent of G1 arrest in response to p53 is reduced. Similarly, repression of CDC25C contributes to G2 arrest in response to DNA damage (36). Unlike control cells, MCF7 cells overexpressing CDC25C fail to accumulate in G2 in response to ionizing radiation. These data support the idea that active repression of cell cycle regulatory genes contributes to p53-induced cell cycle arrest.

p53 is thought to repress the expression of some target genes through direct binding to consensus DNA-binding elements (11). p53 can also bind to novel DNA sequences that do not conform to the reported consensus sequence (37, 38). Based on the data of Hoh et al. (25), most of the genes we identified do not exhibit consensus p53 DNA-binding sites, yet many of these
genes still undergo p53-dependent repression in response to DNA damage. Even without consensus DNA-binding sites, p53 may act directly, or indirectly via a DNA-binding protein complex, at the promoters of these repressed genes. For example, our analysis, consistent with previous reports, identifies c-Myc, and PRC1 as p53-repressed targets (32, 35). Using a temperature-sensitive p53 expression system, p53 can be detected at the promoters of both of these genes. At the region of p53 enrichment, p53 DNA-binding sequences are not present. The same is true for the p53-repressed genes MAD1L1 and MAP4 (8, 39).

The absence of consensus p53 DNA-binding sequences suggests that p53 binds to a nonconsensus sequence or is acting in a complex with other DNA-binding proteins. Experiments are ongoing to determine whether p53 is present at the promoters of the repressed cell cycle genes identified in our study and what DNA sequences mediate this recruitment.

Some progress has been made in determining how p53 mediates repression once it has been recruited to the promoter of an affected gene. p53 can bind directly to the corepressor sin3a, which can recruit histone deacetylase activity to targeted promoters. By immunoprecipitation, p53 was shown to interact with sin3a and HDAC1 in MCF7 cells (8). Similarly, upon p53 expression, p53, sin3a, and HDAC1 can all be detected at the MAP4 promoter. A decrease in histone acetylation, presumably aiding in the repression of transcription, is also found on the promoter at this p53-enriched region. p53 may also influence promoter methylation to influence transcription. A direct interaction between p53 and the DNA methyltransferase DNMT1 has been detected (40). Experiments with DNMT1 null cells suggest that this protein is required for p53-mediated repression of survivin. Further, in response to DNA damage, both p53 and DNMT1 can be detected at the survivin promoter (40).

Repression of some p53-responsive genes requires the transcriptional activation of p21, a cyclin-dependent kinase inhibitor (24). p21 expression can aid the formation of RB-E2F complexes that act to repress E2F target genes. RB family and E2F family proteins are required for the repression of cell cycle regulatory genes and cell cycle arrest following DNA damage or forced p53 expression in some cases (41–43). Importantly, a large number of the p53-repressed, cell cycle regulatory genes identified in this study are known E2F targets (44). Experiments with CHX suggest that new protein synthesis is required for p53-mediated repression observed in LNCaP cells following DNA damage. These data are consistent with a requirement for p21 transactivation. However, when p21 protein accumulation after DNA damage is attenuated with siRNA, we still see the repression of cell cycle genes. This is consistent with reports that p53-dependent repression of c-Myc and CDC25C can occur in the absence of p21 (35, 36). As demonstrated by Lohr et al. (24), several genes identified in our study exhibit p21-dependent repression in doxorubicin-treated HCT116 cells. These particular genes have not yet been tested for a requirement of p21 in our experimental system (etoposide-treated LNCaP cells). Further studies are needed to determine what other proteins are necessary for the repression of particular cell cycle genes in response to p53 expression.

Another possible mechanism of repression involves the NF-Y transcription factor. The promoters of many genes that control the G2/M transition contain multiple CCAAT boxes and are regulated in a cell cycle-dependent manner by NF-Y (45). Several groups have suggested that the negative regulation of G2/M promoters following DNA damage or p53 expression depends on NF-Y (46, 47). Also dependent on NF-Y, p53 can be found at the promoters of cyclin B2, CDC2, and CDC25C, before and after DNA damage, at regions that harbor CCAAT boxes but no consensus p53 DNA-binding sites (48). Several genes identified in our study contain NF-Y-binding sites.

$\text{G}_1$ and $\text{G}_2$ cell cycle arrest checkpoints in response to DNA damage have been described in detail (49). Importantly, several mechanisms for this arrest have been elucidated, some dependent on p53-mediated transcriptional changes and some p53-independent. Data presented here, and data from other groups, support the idea that a cell cycle target gene can be impacted by transcription-dependent and -independent mechanisms. CDC25A protein, the activity of which is required for S phase entry, is rapidly degraded in response to DNA damage, resulting in $\text{G}_1$ arrest (50). To our knowledge, we provide the first evidence that CDC25A is a target for p53-dependent repression at the transcript level. As a critical regulator of cell cycle progression, it is not unreasonable for CDC25A to be the target of two arms of the DNA damage response, rapid degradation via the proteasome, and transcriptional repression by p53.

Our data further suggest that the initiation of DNA synthesis is a prominent target of p53 during the course of p53-induced cell cycle arrest. We identified several MCM (minichromosome maintenance-deficient) proteins, as well as CDC6, as targets for p53-mediated repression. These proteins participate in replication origin licensing and ensure that origins fire only once during a complete cell cycle. CDC6 binds to the origin recognition complex and aids the loading of the MCM2–7 hexamer (51). Several MCM proteins have been identified as p53-responsive in other expression profiling efforts (23).

Although there are reports that p53 can repress the transcription of a small number of cell cycle regulatory genes, this function of p53 is not widely appreciated. Data presented here substantially expand the known repertoire of cell cycle regulatory genes repressed in response to p53 activation. Importantly, p53-mediated repression of target genes likely impacts cell cycle progression at several distinct points. It can be anticipated that further characterization of new p53 transcriptionally regulated target genes will improve our understanding of the DNA damage response, multistep carcinogenesis, and response to therapy.

REFERENCES

1. Vousden, K. H., and Lu, X. (2002) _Nat. Rev. Cancer_ 2, 594–604
2. Levine, A. J., Momand, J., and Finlay, C. A. (1991) _Nature_ 351, 453–456
3. Lane, D. P. (1992) _Nature_ 358, 15–16
4. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. (1991) _Science_ 253, 49–53
5. Hollstein, M., Rice, K., Greenblatt, M. S., Soussi, T., Fuchs, R., Sorlie, T., Hovig, E., Smith-Sorensen, B., Montesano, R., and Harris, C. C. (1994) _Nucleic Acids Res._ 22, 3551–3555
6. Miñara, M., Erster, S., Zaiaka, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U. M. (2003) _Mol. Cell_ 11, 577–590
7. el-Deiry, W. S. (1998) _Semin. Cancer Biol._ 8, 345–357
8. Murphy, M., Ahn, J., Walker, K. K., Hoffman, W. H., Evans, R. M., Levine,
