A Genomic Map of p53 Binding Sites Identifies Novel p53 Targets Involved in an Apoptotic Network

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Abstract

The transcriptional activity of the p53 protein is central to its role in tumor suppression. Identification of the complete repertoire of p53-regulated genes is critical for dissecting the complexity of the p53 network. Although several different approaches have been used to characterize the p53 genetic program, we still lack a comprehensive molecular understanding of how p53 prevents cancer. Using a computational approach, we generated a genome-wide map of p53 binding sites (p53BS) to identify novel p53 target genes. We show that the presence of nearby p53BS can identify new proapoptotic members of the Bcl-2 family. We show that p53 binds to p53BS identified in the BCL-G/BCL2L14 gene and that induction of this gene contributes to p53-mediated apoptosis. We found that p53 activates the COL18A1 gene encoding the precursor for the antiangiogenic factor endostatin. We also show that p53 up-regulates the MAP4K4 gene and activates the c-Jun NH2-terminal kinase (JNK) pathway to drive apoptosis. Thus, unbiased mapping of the genomic landscape of p53BS provides a systematic and complementary approach to identify novel factors and connections in the p53 genetic network. Our study illustrates how systematic genomic approaches can identify binding sites that are functionally relevant for a p53 transcriptional program. The genetic link among p53, antiangiogenic factors, and the JNK signaling pathway adds new dimensions to understanding p53 function in highly connected genetic networks.

Introduction

The p53 tumor suppressor gene is a key regulator of the cellular response to stress and plays a critical role in preventing cancer progression. The activation of p53 in response to DNA damage or cellular stress leads to cell cycle arrest, apoptosis, or senescence, depending on the cellular context (1, 2). The ability of the p53 to function as a transcription factor is central to its role in tumor suppression (3). Mutations in the p53 gene are associated with the majority of human tumors. Such mutations often disrupt its transcriptional function leading to loss of the growth arrest or apoptotic transcriptional programs that limit the proliferation of damaged cells. These observations have fired an intensive search for p53-regulated genes that underlie its tumor-suppressive function (2).

The ability of p53 to inhibit cell proliferation in the G1 phase can be largely accounted for by its transcriptional regulation of the p21^{CIP1} gene encoding an inhibitor of cyclin-dependent kinases (4). However, the transcriptional program responsible for p53-mediated apoptosis is much less clearly defined. The finding that mice lacking the p21^{CIP1} gene, unlike p53-null mice, do not develop tumors implies that the apoptotic program plays an important role in p53 tumor suppression. Several proapoptotic p53 target genes have been identified, including BAX, DR5, PUMA, NOXA, FAS, PIDD, and APAF1 (2). The products of some of these genes are involved in the mitochondrial apoptotic pathway (e.g., Bax, Puma, and Noxa), whereas others are components of the death receptor-mediated pathway (e.g., DR5, Fas, and PIDD). Studies of knockout mice have suggested that no single p53-regulated gene can alone account for p53-induced cell death (5). Combinations of target genes are therefore probably responsible for p53-induced apoptosis in different tissues and different cellular contexts.

Despite the identification of a several dozen p53 target genes using a variety of experimental approaches, the diversity of the p53-regulated transcriptional programs remain to be fully elucidated. Defining new components of the p53 network remains a priority to understand how p53 functions to prevent tumorigenesis. A number of different approaches have been applied to the identification of p53-regulated target genes. In recent years, genomic approaches, such as serial analysis of gene expression (SAGE) or microarray analysis (6–9), have identified p53-regulated genes involved in cell cycle arrest, apoptosis, and DNA repair (2). These data have been complemented by parallel approaches that exploit genomic-scale sequence information. Distinct bioinformatic approaches have been applied to explore the presence of sequences resembling to the p53 consensus binding site and chromatin immunoprecipitation (ChIP) assays have begun to identify genomic loci where p53 binds (10–12).

It is clear, however, that we are far from a complete understanding of how the p53-regulated genetic program accounts for its major role in tumor suppression. It is only by defining the complete repertoire of p53-regulated target genes that we can appreciate its role in apoptotic responses, as well as other anticancer functions such as inhibition of angiogenesis or metastasis. The task of prescribing regulatory functions to noncoding genomic sequences is in its infancy; predicting and validating the role of cis-regulatory motifs is the first step towards dissecting regulatory transcriptional networks (13). To this end, we have generated a map of the genomic landscape of p53 binding sites (p53BS) by an unbiased, highly selective identification of putative p53 cis-acting motifs using a computational approach. We show how this genomic p53BS map can be navigated to identify novel components within the p53 genetic network. We have identified new p53 target genes that shed light on how p53 regulates angiogenesis and orchestrates the apoptotic program.
This study has unraveled a link between p53 and the c-Jun NH₂-terminal kinase (JNK) signaling pathway suggesting additional complexity and synergistic interactions between key pathways regulating cell fate.

**Materials and Methods**

**Screening for genomic p53BS.** We aligned the sequences of 21 well-characterized p53BS to construct the p53 position weight matrix (PWM). These sites come from 19 p53 target genes. When aligning these sequences, we tested both potential orientations (either DNA strand) to maximize the number of matrix positions where a given nucleotide frequency is zero. Each aligned sequence was taken on the DNA strand that maximized the number of matrix positions, where a given nucleotide frequency is zero. This optimization resulted in three alternative possible matrices with the same number of zeros. We reasoned that zero frequency values indicate positions where a particular nucleotide is deleterious for p53 binding, and we assigned these with a negative weight to counter-select sequences with nonrepressed nucleotides. We incorporated the negative weight value $-177$ (corresponding to the difference between the maximum and minimum scores of sequences with only permitted sequence variations) at 26 "invariant" zero positions and an intermediate counterweight values ($-89$) at two positions that varied between the three matrix possibilities. Scores were calculated as the sum of nucleotide frequencies at each matrix position for 20 nucleotide windows throughout the matrix possibilities.

**Inducible p53 expression.** p53-Saos-2 cells were transfected with 20 pmol siRNA using the Lipofectamine 2000 reagent (Invitrogen) according to manufacturer's instructions. Cells were incubated with doxycycline 24 hours post-transfection and apoptosis was measured 48 hours later by flow cytometry (Beckman Dickinson, Le Pont de Claix, France) analysis using CellQuest software. Apoptosis measurements were also done by trypan blue exclusion and manual counting. The results are presented as mean ± SD of triplicates.

**Promoter analysis assays.** To clone the p53BS-containing sequences of p53 target genes, we amplified DNA fragments and cloned them into a Luciferase reporter plasmid (pGL2, Promega). A 1,430 bp fragment from the COL18A1 promoter was amplified with two oligonucleotides 5'-CTCGAGG-TACCAAGAGGCTTGGATCCCTG-3' and 5'--AAGCTTGAAGCTTCCACAATCTCCAACGAC-3' and digested with KpnI and SacI enzymes before cloning. A L558 bp fragment from the BCL2 gene, containing the intronic p53BS, was amplified with two oligonucleotides 5'-CTCGAGGTCAGGTGTTCCCAGAGG-3' and 5'--GATCTCTGAGCTTCCATCAATGATCTGCCG-3' and digested with Kpn1 and Sac1 enzymes before cloning. Reporter plasmids, or an empty vector control, were transfected (using Lipofectamine 2000, Invitrogen) into p53-Saos-2 cells and cells were treated with or without doxycycline for 48 hours. Luciferase expression levels were monitored by quantitative PCR and standardized using 18s quantification.
binding site is relatively long (two palindromic decamers) and significant degeneracy has been observed. To identify potential p53BS in the human genome, we constructed a PWM (see Materials and Methods for details) that models the binding specificities of p53 by aligning the sequences of experimentally validated p53BS from the cis-regulatory regions of well-characterized p53 target genes (Fig. 1A). We focused our search on well-studied p53BS that comprise the minimal 20-nucleotide p53 recognition site identified by a nonbiased alignment algorithm. We aligned these sequences to maximize the number of matrix positions where a given nucleotide frequency is zero and introduced negative values at 28 positions to counterselect sequences with nonrepresented nucleotides. This strategy reduces the number of false positives and enhances selectivity. We chose to begin with a strict criterion for p53BS selection in order to focus on sites with a strong predictive potential. A number of p53-regulated genes contain a p53BS with a gap of 1 to 14 nucleotides between the core decamer binding sites, illustrating that p53 binding tolerates an additional level of flexibility. Identification of these “gapped” sites requires a modified approach (see Discussion). Several design features make our approach particularly selective: (i) the careful choice of biochemically validated sequences to construct the PWM, (ii) the optimization of alignment to maximize the number of forbidden positions and the introduction of a negative weight to eliminate false positives, (iii) the genome-wide selection of motifs on either strand orientation above a nonarbitrary threshold (211, corresponding to the lowest scoring true p53BS), and (iv) the unbiased search for both upstream and downstream sites.

We identified 25,044 potential p53BS in the human genome (i.e., one per 110 kb). This number increases significantly if sequences that allow for a variable gap between the decamers are also selected (see Discussion). This number is greater than previous estimates of the number of p53BS (10). Our genome-wide map of p53BS provides a panoramic view of p53 binding. We anchored our physical p53BS map to the genetic landscape by assessing the relationship between p53BS and the annotated transcription start sites.
sites for the entire set of human genes (22,883 annotated genes). Whereas 79% of the matrix genes have a p53BS within 4 kb of the start site, only 8% of the whole-genome set harbor such close sites, demonstrating enrichment for nearby p53BS in p53 target genes (Fig. 1B). Approximately 9% of the potential p53BS lie within 10 kb upstream of an annotated gene (Supplementary Table S1). To explore this map of genomic p53BS, we first analyzed the distance to the nearest p53BS for genes reported to be induced or repressed by p53 in microarray experiments (7, 9). Kannan et al. examined gene expression in human lung cancer cells expressing a temperature-sensitive p53 (7). We found that 28% of induced genes have a potential p53BS within 6 kb, representing a 5-fold enrichment compared with uninduced genes (Fig. 1C). Our analysis of gene profiling data representing p53 induction in human colon carcinoma cells from Zhao et al. (9) also found enrichment for p53BS near p53-induced genes compared with uninduced genes (Fig. 1D). We did not observe any enrichment for p53BS in the subset of genes that were repressed by p53 expression (7, 9). Thus, genes that were shown experimentally to be p53-regulated are enriched for nearby p53BS compared with noninduced or repressed genes.

The BH3-only BCL-G gene is a proapoptotic p53 target gene.

A well-characterized function of p53 is the induction of apoptosis, but the exact mechanisms are not fully understood (1, 2). Among the list of proapoptotic genes regulated by p53 are several members of the Bcl-2-like family (15, 21, 22). The Bcl-2-like family can be divided into two groups: antiapoptotic proteins such as Bcl-2 itself and proapoptotic proteins such as the BH3-only members. Our genomic p53BS map allowed us to assess for the first time the relationship between the entire family of Bcl-2-like genes and the genomic landscape of p53BS. Strikingly, we found that putative p53BS were closer to genes encoding proapoptotic Bcl-2-like proteins than to antiapoptotic genes (Fig. 2A). p53 has been described as a regulator of the expression of several Bcl-2-like genes, including BAX, and BH3-only members BBC3/PUMA and PMAIP1/NOXA (2, 15, 21, 22). Our analysis predicted that the BCL2L14/BCL-G gene might also be a p53-regulated target as it has a p53BS within the first intron. Bcl-G has been previously shown to be proapoptotic in COS-7 cells, but little is known about its transcriptional regulation (23). We found that BCL2L14/BCL-G mRNA levels were up-regulated upon activation of a tetracycline-inducible p53 allele in p53-Soas-2 cells (ref. 15; Fig. 2B-C). These results suggest that the presence of the p53BS was sufficient to identify BCL-G as a p53 target gene. To investigate this further, we cloned a 1.5-kb portion of the BCL2L14/ BCL-G promoter containing the putative regulatory p53BS in a reporter Luciferase plasmid. This plasmid was transfected into p53-Soas-2 cells and the expression of Luciferase monitored after induction of p53 with doxycycline. Induction of p53 resulted in a 4-fold activation of Luciferase expression, demonstrating that the BCL-G gene is p53-regulated (Fig. 2D).

To show that the p53 protein actually binds to the predicted p53BS in the BCL-G gene, we did ChIP experiments (Fig. 3A-B). The promoters of the CDKN1A and GAPDH genes were used as positive and negative controls in ChIP analysis (see below). We found that the intrinsic BCL-G site bound to p53 in ChIP experiments, whereas a more distant upstream site showed no binding (Fig. 3A). Our algorithm also identified two p53BS in the BBC3/PUMA gene (Fig. 3B). One of these, the most 5' , was missed in previous characterization of this promoter (15, 22). We found that both sites bind p53 in ChIP experiments (Fig. 3B). Thus, the up-regulation of these proapoptotic Bcl-2 family genes upon p53 induction is associated with binding to the p53BS that we have identified within the BCL-G and PUMA genes. To investigate whether induction of BCL2L14/BCL-G is critical for p53-induced cell death, we did RNA interference (RNAi) experiments to knockdown the gene (Fig. 3C-D). Transfection with siRNA oligonucleotides for the BCL-G transcript
A p53BS cluster identifies the endostatin-containing collagen XVIII gene as a p53 target. It is well established that clustering of TFBS contributes to gene regulation (24). The genomic p53BS map allowed us to search for genes associated with higher concentrations of p53BS. We examined clustering of p53BS in our genomic map by assessing the number of p53BS in a window around the promoter of all human genes. Using a 6-kb window, only two genes had a cluster of three sites upstream of the window around the promoter of all human genes. Using a 6-kb window, only two genes had a cluster of three sites upstream of the window around the promoter of all human genes. We cloned a 1.4-kb fragment of the COL18A1 promoter into a Luciferase reporter plasmid and found that it was p53-responsive when transfected into p53-Saos-2 cells (Fig. 4C). Furthermore, ChIP experiments showed that p53 bound very efficiently to the p53BS cluster in the COL18A1 promoter (Fig. 4D). In control experiments, we observed a 4-fold enrichment of p53 bound to the CDKN1A promoter that encodes the p21Cip1 protein, whereas there was no p53 binding to the GAPDH promoter, which is not p53-regulated (Fig. 4D). Furthermore, immunostaining and Western blotting showed that p53 induction leads to an increase in endostatin polypeptide levels (Fig. 4E). Thus, we have identified a functionally relevant cluster of p53BS that regulate expression of a gene with antiangiogenic function.

p53 regulation of the c-Jun NH2-terminal kinase signaling pathway contributes to apoptosis. The only gene in the human genome that contained a nearby p53BS cluster downstream of the promoter is the MAP4K4 gene (Fig. 5A), which encodes an upstream activator of the JNK signaling pathway (27). JNK signaling has been implicated in the cellular response to stress and apoptosis (28, 29). The MAP4K4 gene contains six potential p53BS in the first intron (Fig. 5A). We showed that four of these sites bind p53 in ChIP experiments (Fig. 5B). The induction of p53 in p53-Saos-2 cells led to a reproducible 2-fold up-regulation of the MAP4K4 mRNA (Fig. 5C). p53 induction was also accompanied by an increase in phospho-c-Jun protein levels in these cells (Fig. 5D). Hence, we identified the COL18A1 gene with antiangiogenic function, whereas there was no p53 binding to the GAPDH promoter, which is not p53-regulated (Fig. 4D). Furthermore, immunostaining and Western blotting showed that p53 induction leads to an increase in endostatin polypeptide levels (Fig. 4E). Thus, we have identified a functionally relevant cluster of p53BS that regulate expression of a gene with antiangiogenic function.

Figure 3. p53 binds to sites in the BCL-G gene that plays a role in p53-mediated apoptosis. We identified potential p53BSs in (A) the BCL2L14/BCL-G and (B) the BBC3/PUMA genes. Sequence of the p53 sites and matrix scores. We showed that p53 binds to a p53BS in the BCL2L14/BCL-G gene and two sites in the BBC3/ PUMA gene (B) using ChIP analysis experiments. We did ChIP experiments with extracts from p53-Saos-2 cells treated with (+) or without (−) doxycycline (dox) and determined the binding to the promoters by site-specific, quantitative PCR analysis. The promoters of the Gapdh and CDKN1A genes were used as negative and positive controls, respectively (see data in Fig. 4). C, we treated p53-Saos-2 cells with siRNA duplexes against BCL2L14/ BCL-G or a control gene (Luciferase) before induction of p53 with doxycycline. We measured mRNA levels of BCL2L14/BCL-G to show that the RNAi had effectively reduced expression. D, we measured the levels of apoptosis by flow cytometry after 48 hours in the presence or absence of doxycycline and siRNA against BCL2L14/BCL-G or a control gene.
We tested genetically whether JNK activation by genotoxic stress is p53-dependent by examining phospho-c-Jun staining in mouse fibroblasts from embryos with or without functional p53. c-Jun phosphorylation was induced by genotoxic stress in a p53-dependent manner, whereas the tumor necrosis factor-α–induced signal was p53-independent (Fig. 6B). Finally, to show that the JNK signaling pathway is important for p53-mediated apoptosis, we blocked components of the pathway using pharmacologic or genetic approaches. We tested whether pharmacologic inhibition of JNK activity could affect p53-induced apoptosis. We found that the specific JNK inhibitor Sp600125 reduced p53-induced cell death in p53-Soas-2 cells by around 30% to 40% (Fig. 6C). We also used RNAi assays to knockdown expression of the MAP4K4 gene and tested the effect on p53-induced cell death. siRNA against MAP4K4 lead to a significant reduction in p53-dependent apoptosis, whereas control siRNA reagents had no effect (Fig. 6D). These results suggest that the JNK signaling pathway contributes to p53-regulated apoptosis, and that at least one way that p53 regulates this pathway is via induction of the MAP4K4 gene.

Discussion

We have shown that a genome-wide approach to map p53BS motifs can identify novel bona fide p53-regulated genes. Several design features contribute to the predictive nature of our bioinformatics approach. PWMs offer more powerful selection than conventional searches for consensus binding sites and selectivity was enhanced by the choice of characterized sites to construct our PWM, the high score threshold, and negative weighting to eliminate “false-positive” motifs. Our search is not exhaustive, as we chose to focus our initial search on p53BS that contain a minimal 20-nucleotide recognition site without allowing for a variable gap of 1 to 14 nucleotides between the palindromic decamers, or the contribution of adjacent nucleotides. Our approach can, however, be easily extended to search for p53BS with a gap between the two palindromic motifs (20) and to map p53BS (or other TFBS) that are conserved between species. An initial survey of p53 PWM with variable gaps indicates that each matrix adds a large number of potential p53BS (Supplementary Table S1). Combining the maps for these additional PWM offers potential to identify novel p53 targets that have been missed by the gap-less PWM. It is noteworthy that analysis of clustering for a combined p53BS data set indicates that the COL18A1 gene shows enrichment for additional p53BS with gaps. For example, a 6-kb window around the start site contains eight upstream p53BS and four intronic p53BS when all gapped motifs are included. Thus, the combined p53BS map will provide a resource for future mining of p53 binding sites and their relationship with adjacent genes. Although bioinformatics approaches have been applied to p53 transcriptional regulation in the past (11, 12), this is the first time that genome-wide in silico prediction of p53 target genes has led to the identification of specific genes whose biological function has been experimentally shown to contribute to p53-induced apoptosis. Previous studies have tended to analyze restricted gene sets and rarely showed that the potential sites bound to p53 in vivo or that the genes identified were functionally relevant for p53 biological outcomes (11, 12). Furthermore, our analysis identified

Figure 4. p53 induces COL18A1 expression via a cluster of upstream p53BS. A, we identified a cluster of four p53BS upstream of the COL18A1 gene. The position, sequence, and PWM score of the sites are indicated. B, we analyzed the expression of COL18A1 mRNA in p53-Soas-2 cells treated with doxycycline for 24 hours by quantitative PCR analysis. C, we cloned the COL18A1 promoter containing the p53BS cluster and tested expression of the luciferase reporter gene upon transfection into p53-Soas-2 cells and treated with (+) or without (–) doxycycline (dox) for 48 hours. An empty vector plasmid was used as a negative control. D, we did ChIP experiments to show that p53 binds to the COL18A1 promoter. A single pair of primers was used to encompass the four clustered p53BS. p53-Soas-2 cells were treated with (+) or without (–) doxycycline for 24 hours before ChIP analysis with anti-p53 specific antibodies. Analysis of p53 binding to the GADPH or CDKN1A promoters served as negative and positive controls, respectively. E, ChIP experiments were done using quantitative PCR. E, we detected expression of the endostatin polypeptide using specific antibodies in p53-Soas-2 cells treated with doxycycline by immunoblot (left) or immunohistochemistry (right) experiments. p53 induction was detected with antibodies against p53 protein and nuclei were detected by 4’,6-diamidino-2-phenylindole (DAPI) staining.
novel p53 target genes that have been missed in previous genomic studies, emphasizing the need to develop multiple parallel approaches if we are to understand the full complexity of the p53 genetic network. Notably, the COL18A1 gene that we have identified here is located on chromosome 21. Previous mapping of TFBS along chromosome 21 by large-scale ChIP experiments (10) failed to detect the cluster of p53BS that we have shown here can bind p53 in our ChIP assays (Fig. 4). Again, this result emphasizes the sensitivities of different experimental systems and supports the need for multiple, complementary approaches for a comprehensive definition of the entire repertoire of p53-regulated genes. The generation of TFBS maps for other factors (e.g., E2F, Myc, and AP-1) could be superimposed on our p53BS map to provide insights into coregulated transcriptional programs in response to different stress conditions. Furthermore, we show how TFBS mapping data can be combined with microarray data (Fig. 1) to focus on up-regulated genes that are enriched for local binding sites and are therefore more likely to represent direct transcriptional targets. The data from our p53BS map allows one potentially to distinguish between direct and indirect targets suggested from microarray or SAGE analysis. The ability to identify easily the nearest upstream or downstream p53BS from a characterized or annotated gene accelerates the next steps in functional characterization (e.g., ChIP and promoter assays). We have shown how the analysis of p53BS map information can be effective in predicting functional targets for network analysis. This approach aids the integration of large data sets of genomic sequence and gene expression data to dissect functionally relevant transcriptional programs. The same PWM and tools can be easily applied to genomic sequences from other species, allowing an assessment of the p53 regulation of orthologous genes and aiding in the interpretation of phylogenetic footprinting approaches to identify functionally relevant noncoding genomic regions.

The fact that our p53BS map is truly genome-wide allowed us to assess the relationship between all known human transcripts and the local distribution of p53BS. Our analysis emphasizes the importance of mapping both upstream binding sites in the promoter region, as well as intronic p53BS within the body of the gene. Indeed, many more p53BS are found within genes than in the 10-kb upstream of genes (Supplementary Table S1). For example, we identified and characterized functionally relevant p53BS in the first intron of BCL-G and MAP4K4 genes. These observations underscore the importance of genome-wide searches rather than those restricted to small portions of cloned promoters.

Genomic TFBS maps also allow a broad analysis of the regulation of large gene families. Our novel observation that p53BS lie closer to a proapoptotic subset of the Bcl-2-like genes led to the identification of BCL2L14/BCL-G as a new proapoptotic p53 target. This is the first time this approach has been taken to predict target genes. Interestingly, the BCL-G gene maps to a tumor suppressor locus at chromosome 12p12 (30). Thus, p53 regulates multiple members of the BH3-only family of death-inducing proteins in its apoptotic program (5). Inhibition of BCL-G expression by RNAi significantly reduced, but did not completely abolish, p53-induced cell death (Fig. 3). Similar results were reported when the related PUMA gene was inhibited using antisense oligonucleotides (15). These observations support the idea that multiple avenues contribute to p53-induced apoptosis and no single effector gene can solely account for the p53-regulated cell death program. Study of other gene families is likely to lead to the identification of additional targets. Thus, the identification of the entire repertoire of p53-regulated genes is essential to understand the mode of action of p53 and the combination of multiple complementary approaches is vital.

Our search for higher local densities of p53BS led to the identification of functionally relevant p53BS clusters in the COL18A1

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**Figure 5.** p53 regulates JNK pathway activation via induction of the MAP4K4 gene. A, we identified a cluster of potential p53BS in the first intron of the MAP4K4 gene. The position, sequence, and PWM score of the sites. B, we did ChIP experiments to show that p53 binds to sites within the MAP4K4 gene. p53-Soas-2 cells were treated with (+) or without (−) doxycycline (dox) for 24 hours before ChIP analysis with anti-p53 specific antibodies. Precipitated DNA was examined by quantitative PCR analysis. C, we analyzed the expression of MAP4K4 mRNA in p53-Soas-2 cells treated with doxycycline for 24 hours by quantitative reverse transcription-PCR analysis. D, immunoblot analysis with an anti-phospho-Jun (P-Jun) antibody revealed activation of the JNK pathway upon p53 induction in p53-Soas-2 cells (left), or upon cisplatin treatment of HCT116 cells with or without p53 (right).
and MAP4K4 genes. We suggest that in addition to cell autonomous effects of p53 on growth arrest and apoptosis, p53 may regulate signaling pathways and extracellular components that affect neighboring cells. p53 was previously linked to the transcriptional control of angiogenic regulators, such as thrombospondin and VEGF (31, 32). However, these proangiogenic genes are downregulated by p53 via an unknown mechanism. Endostatin represents the first antiangiogenic factor that is directly regulated by p53. It will be interesting to test whether the antiangiogenic function of p53 is mediated by endostatin in vivo. Interestingly, the enzyme that cleaves collagen XVIII to generate endostatin, MMP2, is also encoded by a p53 target gene (33). The observation that p53 regulates both MMP2 and its substrate collagen XVIII, underlies the logic behind p53 genetic programs to ensure effective antitumor function. Furthermore, endostatin has recently been linked to regulation of a complex genetic network associated with apoptosis and proliferation (34).

The MAP4K4 gene represents a rare example of transcriptional regulation of an upstream kinase involved in JNK signaling. Notably, we did not observe a clear correlation between the extent of transcriptional induction and the number of p33BS or the efficiency of p53 binding. We envisage that the modest transcriptional induction of the upstream kinase is amplified by downstream signaling cascades. Indeed, we observed robust p53-dependent JNK activation (c-Jun phosphorylation) in HCT116 cells. The JNK signaling pathway has been implicated in apoptotic regulation in a wide variety of cell types in response to different stimuli (29). JNK activation has been shown to result in the phosphorylation of several proapoptotic proteins in the Bcl-2 family (35). As BH3-only encoding genes are also p53 target genes, this should lead to a synergistic regulation of cell fate. Once again, the activation of the JNK/MAP4K4 signaling pathway and the concomitant induction of proapoptotic JNK substrates provides insights into the logic underlying the circuitry of the p53 network.

The induction of MAP4K4 and endostatin links p53 to a wider genetic network affecting diverse cell signaling events. The recent reports that BCL2L14/BCL-G (30), components of the JNK pathway (36–38), and endostatin (26, 34) play different roles in tumor suppression suggest that these novel targets may be related to p53’s critical anticancer function. These p53 targets contribute to an interconnected genetic network that reinforces the apoptotic program initiated by p53 in response to stress.

Acknowledgments
Grant support: Association for International Cancer Research grant AICR #02-347, the European Community grant CEE HPRNCT 2002-00256, the Pasteur-Weizmann Foundation, the Association pour la Recherche sur le Cancer (ARC, France), and the Ligue Nationale Contre le Cancer (Ile de France).

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We thank Samia Ben Hassine for experimental assistance and members of the Yani laboratory for helpful discussion.

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