

Profile of gene expression regulated by induced p53: connection to the TGF- β family

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Abstract The transcription regulatory function of p53 was analyzed by using two inducible p53 systems in the human lung cancer cell line H1299. cDNA probes derived from RNA harvested 12 h after p53 induction were used to probe filters containing cDNA arrays. Over 20 genes were found to be significantly induced or suppressed by p53. The induced genes can be classified mainly as cell cycle inhibitors like p21waf, GADD45, apoptosis-related genes like Fas/APO1 and PIG3 or DNA repair genes like DDB2, DNA ligase and G/T mismatch DNA glycosylase. The suppressed genes include mainly cell cycle regulators like cyclin B1, cyclin H and kinases like c-abl, CLK1 and others. The most notable induced gene was MIC-1, encoding a TGF- β -related secretory protein, suggesting a potential paracrine component for p53 growth suppression.

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Key words: p53; Transactivation; Transrepression; DNA array; DNA repair; MIC-1

1. Introduction

Normal cells respond to DNA damage and various stress conditions (like oxidative stress, hypoxia or nucleotide depletion) by activation and stabilization of p53 [1,2]. The activity of p53 was found to be important for a variety of cellular processes including growth arrest, apoptosis, senescence and genome stability [3]. The most studied function of p53 in these cell responses is its activity as a transcription factor which regulates target genes whose products may be directly involved in the biochemical regulation of these processes. The DNA target site for p53 is composed of four palindromic copies of the consensus sequence PuPuPuCA/T with a variable linker sequence in between [4] that was found to be located in the promoter region or introns of such genes. Mutant p53 loses its ability to bind such target sites and therefore the capacity to drive the cellular processes which are turned on by wild-type (wt) p53. This loss-of-function of DNA target recognition by p53 mutant is an important step in cell transformation and over 50% of human cancers contain various such p53 mutants [5]. This indicates that the transactivation function of p53 is the basis of its function as a tumor suppressor [6]. It follows that some of the genes that are transactivated by p53 can also be useful as inhibitor of tumor cell growth and as candidates for new targets in cancer therapy. In

order to discover such genes, we used two systems of induced p53 in a human lung cancer cell line: the muristerone-induced human p53, described recently [7], and the temperature-sensitive mouse p53Val135. The cDNA derived from mRNA harvested after p53 induction was hybridized to DNA arrays on filters containing representatives of genes involved in cell cycle, stress, apoptosis and cytokines (Clontech, USA). The results revealed a pattern of p53-dependent gene expression which includes genes related to cell cycle inhibition and DNA damage repair (upregulated) and to positive regulators of cell cycle genes like cyclins and kinases (downregulated). This pattern included also some other genes associated with the extracellular matrix and most notably a secretory protein MIC-1 related to the TGF- β family. The results also demonstrate the importance of transrepression by p53 (e.g. cyclin B1) in the control of cell cycle checkpoint.

2. Materials and methods

2.1. Cell culture

The human lung cancer cell line H1299 was chosen for this study since it is null for p53 [8]. Two different inducible p53 systems were used in this work. One system consists of H1299 cells expressing the mouse temperature-sensitive mutant p53Val135 (a gift of Dr. M. Oren) which on temperature shift to 32°C will assume wt conformation. The other system consists of muristerone inducible p53 (C-30) and p21 (W-65) cell lines that were generated as described previously [7,9]. The cells were maintained in RPMI medium containing 10% fetal bovine serum (FBS). Both muristerone treatment (1 μ M) and temperature shift were carried out for a 12 h time period and then the cells were harvested for RNA isolation.

2.2. RNA preparation

Total RNA was isolated using TRIZOL reagent (Gibco BRL, Life Technologies, NY, USA). Cells were collected by centrifugation, washed once with PBS, homogenized in 2 ml of TRIZOL reagent per 100 mm cell culture plate and incubated for 5 min at room temperature. Chloroform (400 μ l) was then added and the mixture was vigorously mixed and incubated at room temperature for 3 min. After centrifugation at 14000 rpm, at 4°C, for 20 min, the aqueous phase was transferred into a fresh tube and equal volume of isopropanol was added followed by incubation at room temperature for 10 min. After centrifugation, the RNA pellet was washed once with 75% ethanol and dissolved in DEPC water. The integrity of RNA was checked on a 1% formaldehyde/agarose gel.

2.3. Northern blotting

For Northern blotting, 10 μ g of total RNA was denatured in formaldehyde buffer and run on a 1% agarose gel containing formaldehyde. The RNA was transferred onto Hybond N⁺ nylon membranes (Amersham International Plc, UK) by capillary force overnight. The transferred RNA was immobilized by baking at 80°C for 2 h and UV crosslinking. Hybridization was done at 65°C for 12 h and washing procedures were performed as in standard protocols. Autoradiogra-

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phy was done on Kodak X-ray films with intensifying screens at -70°C .

2.4. cDNA array hybridization

We used cDNA arrays of human cDNAs spotted on a nylon membrane (Atlas filter arrays, Clontech, Palo Alto, CA, USA). We have used a variety of Atlas arrays for human cDNAs including the human stress/toxicology (234 genes), apoptosis (205 genes), cytokine/receptor (268 genes) and human cancer 1.2 (1176 genes) arrays. The filters include also house keeping control cDNAs and negative controls spotted in duplicate dots. A complete list of the cDNAs and controls as well as their accession numbers is available on the web (<http://www.clontech.com/atlas/genelists/index.html>). Total RNAs were treated with DNaseI according to the manufacturer and used for cDNA synthesis. A 3 μl mix containing 5 μg of total RNA and 1 μl of 10 \times CDS primer mix (specific for each filter array, provided by manufacturer Clontech, USA) was incubated at 70°C for 2 min followed by incubation at 48°C for 2 min. To this mix, 8 μl of master mix (containing 2 μl 5 \times reaction buffer, 1 μl 10 \times dNTP mix, 3.5 μl [α - ^{32}P]dATP (3000 Ci/mmol, 10 mCi/ml, Amersham/Pharmacia Biotech, UK), 0.5 μl 100 mM DTT and 1 μl MMLV reverse transcriptase (50 U/ μl) were added, mixed and incubated for 25 min at 48°C . The reaction was terminated by adding 1 μl of 10 \times termination mix at room temperature. The radioactively labeled cDNA mix was fractionated on a Chroma Spin-200 column (Clontech Laboratories, USA) and fractions that comprise the first peak of radioactivity were pooled for each cDNA synthesis reaction. In each set of hybridization, equal counts were taken for control and experimental labeled cDNA probes. The labeled cDNA probe was then mixed with 1/10 volume of 10 \times denaturing solution (1 M NaOH, 10 mM EDTA) and incubated at 68°C for 20 min followed by the addition of 5 μl (1 $\mu\text{g}/\mu\text{l}$) of cot-1 DNA and an equal volume of 2 \times neutralizing solution (1 M NaH_2PO_4 , pH 7.0), and incubated at 68°C for 10 min. Denatured, labeled cDNA was then added to 5 ml of ExpressHyb solution (Clontech) with 1 mg of sheared salmon sperm DNA (Sigma, St. Louis, MO, USA) and mixed. This hybridization solution was added to the Atlas cDNA Expression Array membrane, which was prehybridized in 10 ml of ExpressHyb hybridization solution at 68°C for 1 h. Hybridization proceeded overnight at 68°C in a roller bottle. Membranes were washed once with prewarmed 2 \times SSC/1% sodium dodecyl sulfate (SDS) for 30 min and once or twice with 0.5 \times SSC/0.5% SDS for 30 min at 68°C with constant agitation. The membranes were exposed to Fuji X-ray films at -70°C with intensifying screens.

2.5. Analysis of hybridization signals

The cDNA microarray autoradiograms were scanned and the images were analyzed using AtlasImage 1.01 software (Clontech, USA). The background was calculated using default external background calculation which takes into consideration the background signals at blank space between the different panels of the arrays. Signal threshold was set as background-based signal threshold. The signal intensities were normalized globally using the sum method (AtlasImage, Clontech, USA). A report of differentially expressed genes was generated based on ratio and intensity differences.

3. Results and discussion

3.1. Selection of cell lines and p53 induction conditions

The identification of p53 target genes required careful selection of control cell lines. For example, the comparison of the H1299Val135 RNA at 32°C to that of H1299Val135 at 37°C resulted in many changes in the expression of heat shock genes and other genes related to the temperature of the growth conditions which were not necessarily related to p53 induction. Hence, we compared H1299Val135 RNA at 32°C to that of H1299 at 32°C . The comparison of RNA from muristerone-induced p53 [7] to RNA from the same cell line without muristerone also resulted in many differences which could be related to the possible effect of muristerone. Therefore, we chose to compare RNA from muristerone-induced p53 in the C-30 cells to the RNA of muristerone-induced

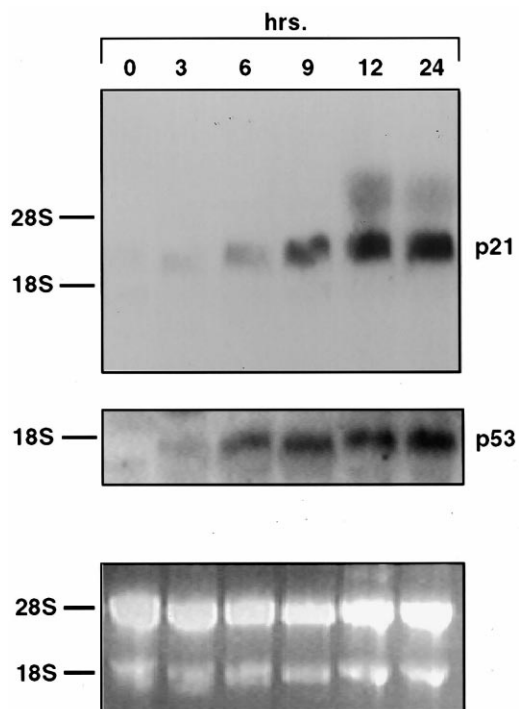


Fig. 1. Muristerone-induced mRNA of p53 and p21waf in C-30 cells. Northern blot analysis of RNA extracted from C-30 cells incubated in the presence of 1 μM muristerone for the indicated time periods.

p21waf in the W-65 cells [9]. This comparison should cancel out the secondary effect of growth arrest due to induced p21waf in both cell lines, as well as a possible effect of muristerone on gene expression.

The time for harvesting the cells after p53 induction was selected on the basis of monitoring the level of p21waf RNA resulting from p53 induction by muristerone (Fig. 1). It is shown that in C-30 cells, the p21waf, a typical p53-induced gene, is fully expressed at 12 h after muristerone induction. This time point was therefore chosen for extracting the RNA from either H1299Val135 cells following the temperature shift or from the muristerone-induced C-30 and W-65 cells.

3.2. p53-regulated genes in H1299 cell line

The results of hybridization to DNA arrays on two of the filters provided by Clontech are shown in Figs. 2 and 3 and some of the regulated genes are indicated by numbers; up-regulated genes are numbered in the upper panel and down-regulated genes in the lower panel. It is shown that the induced expression of p53 leads to both upregulation and downregulation of genes. The upregulation of genes due to transactivation by p53 was studied for many years and is dependent on the presence of the target site sequence for p53 binding in the gene of interest [6]. On the other hand, the downregulation of genes by p53, which may play an important role in its function, is not fully understood with regard to the mechanism of transcriptional repression by p53. Previous work has shown that wt p53 binds the TATA binding protein and reduces the efficiency of transcriptional initiation, thereby repressing gene expression [10]. This transcription inhibition was effective also on minimal promoters which lack p53 response element, and it was argued that additional factors may

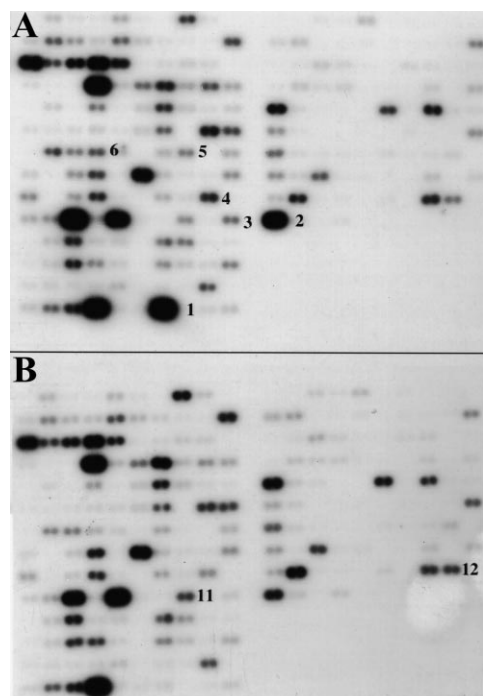


Fig. 2. Differential hybridization to stress/toxicology filters. A: RNA used for probes was derived from H1299Val135 incubated for 12 h at 32°C. B: RNA used for probes was derived from H1299 cells incubated for 12 h at 32°C. Numbers in A and B represent upregulated and downregulated genes, respectively. Upregulated: 1, p21waf; 2, PCNA; 3, DDB2; 4, LIG1; 5, MDM2; 6, Mortalin2. Downregulated: 11, topoisomerase II α ; 12, catechol *O*-methyltransferase.

confer specificity on this inhibition. Recent analysis [11] suggests that gene repression by p53 is also due to specific binding of p53 to its target site and involved indirectly histone-deacetylase (HDAC). HDAC was found to be involved in an evolutionarily conserved mechanism of gene repression by transcription factors [12]. It was shown that p53 is associated with the corepressor mSin3A and HDAC at the promoter site of the repressed gene, thus inducing chromatin condensation and transcriptional repression which is dependent on p53 [11]. Another possibility to explain p53-induced repression was based on the presence of a truncated target site, like a p53 half-site, resulting in repression rather than upregulation [13,14]. In any event, the hybridization data and the list of suppressed genes reported here make it clear that p53-induced suppression is an important element of its activity.

3.3. Pattern and classification of gene expression after p53 induction

Table 1 provides the list of genes upregulated by p53 as analyzed by screening the differences in hybridization results with the AtlasImage software. This list can be considered as a profile of the genes which may be directly related to the biological function of p53. Many of the induced genes were described before (p21waf, MDM2, PIG3, GADD45, Fas, Bax, PCNA) and they are related to the growth arrest and apoptosis function of p53 [6]. The effect of p53 on genes involved in DNA repair is markedly emphasized in our results. Genes such as damage-specific DNA binding protein 2 (DDB2), the excision repair gene ERCC5, DNA ligase 1 (DNL1 or LIG1), G/T mismatch specific thymine DNA glycosylase (TDG) and

replication protein A1 (RPA1) are important genes activated by p53 and are involved in DNA repair. An interesting new p53 upregulated gene identified in this study is MIC-1, a TGF- β like secretory protein. Fig. 4 shows the upregulation of MIC-1 on a section from the Atlas 1.2 Cancer filter (Clontech, USA) and Fig. 5 shows a Northern blot which confirms this result. This gene (also called PTGF- β) was recently isolated as a novel macrophage inhibitory cytokine [15] or as a prostate-derived differentiation factor [16] and was shown to induce cartilage formation. The promoter region of this gene contains the putative p53 target site 5'-CATCTTGCCC/AGACTTGTCT-3', 851 bp upstream to the mRNA start [17], and recent analysis demonstrated that this gene is regulated by p53 and is also activated by etoposide in a p53-dependent manner [18]. The MIC-1 (PTGF- β) product was found to be secreted and the conditioned medium from cells expressing this protein can suppress the growth of certain tumor cells provided they contain TGF- β receptors and Smad4 [18]. This indicates a connection between p53 and the growth inhibitory family of TGF- β and implies a potentially important paracrine mechanism for tumor growth suppression by p53 [19].

In addition to the upregulated genes, we could observe a high number of genes which are suppressed by induced p53 expression, suggesting that transrepression is also an important mechanism through which p53 exerts its function. Downregulated genes are shown in Table 2. This list contains mainly cyclins, NM23, topoisomerase II α and replication factor C4 (RF-C4). In addition, Table 2 contains several kinases

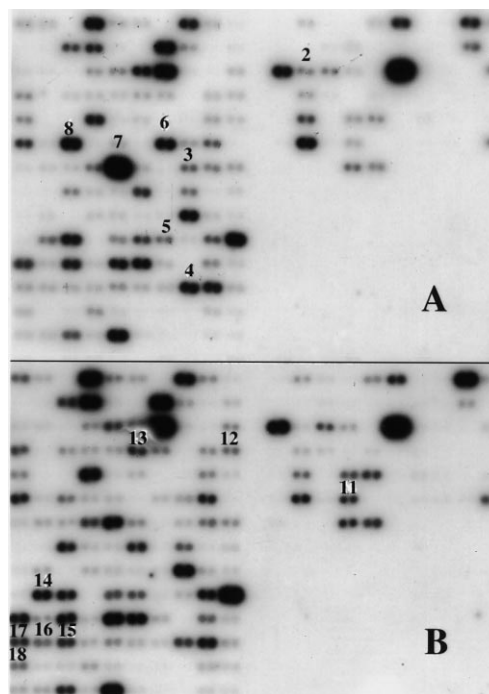


Fig. 3. Differential hybridization to apoptosis filters. A: RNA used for probes was derived from H1299Val135 cells incubated for 12 h at 32°C. B: RNA used for probes was derived from H1299 cells incubated for 12 h at 32°C. Numbers in A and B represent upregulated and downregulated genes, respectively. Upregulated: 1, retinoic acid receptor β ; 2, Fas/APO1; 3, Bax; 4, BAK; 5, PIG3; 6, MDM2; 7, PCNA; 8, p57. Downregulated: 11, PDCD2; 12, NIK; 13, E2F-5; 14, cyclin B1; 15, PLK1; 16, cyclin D1; 17, cdc25c; 18, CLK1.

Table 1
p53-induced genes in the H1299–Val135 system

Accession no.	Gene/protein name	Ratio	Category
U09578 ^a	p21waf1	38.0	Cell cycle
AF010309	PIG3, p53-induced gene 3	11.0	Apoptosis
AF019770	MIC-1, member of TGF- β family	10.0	Growth inhibitor
Z12020 ^a	MDM2	8.3	Cell cycle
M15796 ^a	PCNA		Cell Cycle
U18300 ^a	DDB2, involved in nucleotide excision repair	6.5	DNA repair
U70451 ^a	MYD88, myeloid differentiation	5.4	Receptor
X07282	Retinoic acid receptor β	4.6	Development
M60974 ^a	GADD45	3.9	Cell cycle
Z70519	Fas/APO1	3.8	Apoptosis
L35253	MAPK14	3.8	Kinase
L22474	Bax, Bcl2-associated X protein	3.7	Apoptosis
M88279	FKBP4, possible peptidyl-prolyl- <i>cis-trans</i> -isomerase	3.1	Immunophilin
L15189 ^a	Mitochondrial stress 70 precursor (Mortalin2)	3.0	Cell cycle
U22398	p57KIP2, CDK inhibitor 1C	2.4	Cell cycle
M36067	LIG1, DNA ligase 1	2.3	DNA repair
L20046 ^a	ERCC5, DNA excision repair-related	1.9	DNA repair
U51166	TDG, G/T mismatch thymine DNA glycosylase	1.8	DNA repair
D11117 ^a	HOXD3, homeobox protein	1.8	Development
U77129	MAP4K5, activator of Jun N-terminal kinase	1.6	Kinase
M63488	RPA1, replication factor A protein 1	1.6	DNA repair
U23765	BAK1, Bcl2 antagonist/killer 1	1.5	Apoptosis
S81439 ^a	TIEG, TGF- β inducible early growth response gene	1.5	Cell cycle
L05624	MAP2K1, MEK1	1.5	Kinase
U16306 ^a	CSPG2, chondroitin sulfate proteoglycan 2		ECM ^b
Z21707 ^a	ZNF197, zinc finger protein 197 (p18 protein)		Undefined

^aThese genes are induced also in the muristerone inducible p53 system.

^bECM = extracellular matrix.

including CDC like kinase 1 (CLK1) that activates protein tyrosine phosphatase PTP-1B [20]; NF- κ B inducing kinase (NIK) which is involved in the NF- κ B inducing signaling pathway [21]; Polo/cdc5 like kinase (PLK) which is highly expressed in various tumors and is required for cell division [22]; CDC7-related kinase which may be involved in regulation of G1/S transition and DNA replication [23]; rac- β kinase (AKT2 oncogene) which is an oncogene amplified in ovarian carcinoma and is activated by growth factors [24]; c-abl; NDR protein kinase which is a nuclear kinase implicated in the regulation of cell division [25]; protein tyrosine kinase 9 (PTK9); Fas-activated serine–threonine (FAST) ki-

nase which is involved in signaling for Fas-mediated apoptosis [26] and cyclin-dependent kinase 1 (cdc2 or CDK1) which is involved in G1/S and G2/M transition [27]. All kinases mentioned here are directly or indirectly involved in cell division and cell cycle control. Their suppression will lead to the suppression of cell division which is consistent with the activity of p53. Protooncogene c-abl was reported to increase the expression of p53 by neutralizing the effect of MDM2 [28]. We have observed transrepression of c-abl by overexpressed p53, suggesting that this may represent a negative feed back loop controlling the amount of p53 protein. Other notable genes that were not reported earlier to be transrepressed by p53 include K-ras2, E2F-5 and NM23a and b. NM23 genes (NDKs) were found to show reduced expression in tumors progressing to the metastatic phenotype [29] and NM23b is a transcriptional activator of c-myc [30]. By downregulating NM23b, p53 may suppress one of the c-myc activating pathway.

RF-C4 is required to assemble PCNA and DNA polymerase δ on the DNA template, and the resulting complex is essential for the elongation of the primed DNA templates during DNA replication [31]. By downregulating RF-C4, p53 may inhibit DNA synthesis. On the other hand, LIG1 (upregulated gene in this study, Table 1) interacts with FEN1 (Flap endonuclease 1) and inhibits DNA synthesis carried out by DNA polymerase δ /PCNA complex [32]. Therefore, it seems likely that p53 function is executed by both induction and suppression: inducing LIG1 that inhibits DNA synthesis and suppressing RF-C4 that promotes it, both ways leading to inhibition of DNA synthesis. The property of DNA synthesis inhibition along with induction of genes responsible for DNA repair may very well explain the role of p53 as molecular guardian of the genome.

We also observe downregulation of cyclin B1, cyclin D1

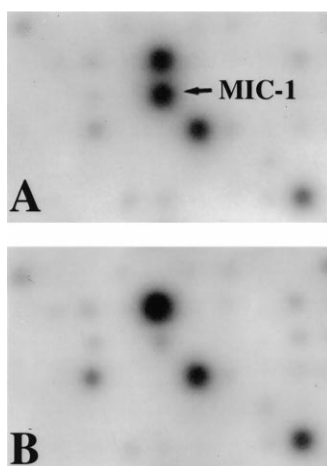


Fig. 4. Differential hybridization to MIC-1. A section of the film developed from cancer 1.2 array filter is shown. A: RNA used for probes was derived from H1299Val135 cells incubated for 12 h at 32°C. B: RNA used for probes was derived from H1299 cells incubated for 12 h at 32°C.

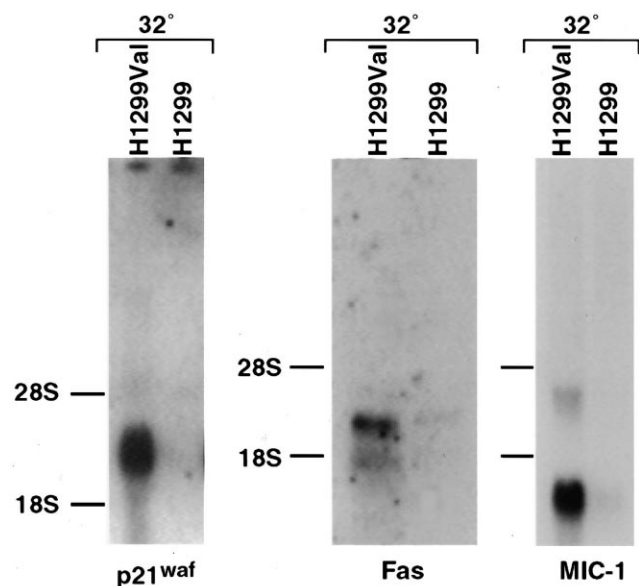


Fig. 5. Northern blot analysis of three typical induced genes, p21waf, Fas and MIC-1. RNA derived from H1299Val135 cells and H1299 cells after 12 h incubation at 32°C was separated on a 1% agarose gel and blotted onto nylon filters. The blot was hybridized to probes of p21waf, Fas and MIC-1.

and cyclin H by induced p53 (Table 2). Cyclin H is a component of CDK activating kinase [33], cyclin D1 (also known as bcl-1 oncogene) is essential for G1/S transition and is overexpressed in many tumors [34]. Cyclin B1 is essential for G2/M transition and it is well known that p53 is involved both in G1 and G2 arrest of the cell cycle [35]. It was proposed that there may be two p53-dependent pathways that can contribute to G2 arrest. One pathway was by decreasing the intracellular levels of cyclin B1 [36]. The other pathway operates by increasing the expression of 14-3-3 σ protein which prevents mi-

totic initiation by sequestering cdc25c [37], a phosphatase that activates cdc2 by dephosphorylating it at tyrosine 15 [35]. Our results show transrepression of genes involved in both these pathways. A significant downregulation of cyclin B1 was observed (Table 2). On the other hand, the downstream target of 14-3-3 σ , the cdc25c phosphatase, was downregulated, thus bringing the expected decreased activation of cdc2. Both pathways converge on cdc2 kinase activity and we have observed also downregulation of cdc2 kinase (Table 2). This indicates that p53 acts in more than one place at the G2/M checkpoint leading to G2 arrest. One should be cautious, however, in the interpretation that all the regulated genes (listed in Tables 1 and 2) are primary targets of p53. This is because of the 12 h time elapsed between p53 induction and harvesting the RNA. This time point was chosen from RNA expression data (Fig. 1) and protein expression [7] aiming at maximal expression using p21waf1 as an indicator for target gene induction. Clearly the wt p53 is functional to variable degrees even before the 12 h time point. Hence, we cannot exclude the possibility that some of the genes listed here are not primary targets of p53, but may be regulated by the products of the primary targets of p53. It is however clear that many of the genes (e.g. p21waf1, PIG3, MIC-1, MDM2, Bax, GADD45, Fas/APO1 and cyclin B1) are known to contain the consensus p53 binding site.

Several systems of p53 inducible genes were analyzed recently at the level of genomic expression, using different analytical tools [19,38]. The results are not always similar, indicating the heterogeneity of the p53 induction, depending on cell lines and gene repertoire. Also in the genes listed in Tables 1 and 2, one could see the effect of p53 on the expression of many genes but not all of them are modulated similarly in the two p53 inducible systems used in this analysis. This heterogeneity in the expression pattern could be explained by the recent findings and suggestion that the p53-dependent gene expression pattern may vary even between cell lines derived

Table 2
p53-suppressed genes in the H1299–Val135 system

Accession no.	Gene/protein name	Ratio	Category
M25753	Cyclin B1	–27.8	Cell cycle
X17620 ^a	NM23a, metastasis inhibition factor	–14.0	Kinase
L16785 ^a	NM23b, transcriptional activator of c-myc	–6.0	Kinase
L29222	CLK1, CDC like kinase 1	–4.6	Kinase
M73791	Ribosomal protein L10	–3.6	Protein synthesis
M96322 ^a	Kinase anchor protein (Gravin) 12	–3.5	Scaffold
Y10256	NIK, NF- κ B inducing kinase	–3.4	Kinase
U11791	Cyclin H	–3.3	Cell cycle
U01038	PLK, Polo/cdc5 like kinase	–3.0	Kinase
S78085 ^a	PDCD2, programmed cell death 2 gene	–3.0	Apoptosis
M54968	p21 K-ras2	–2.9	Cell cycle
U15642	E2F transcription factor 5	–2.9	Cell cycle
M34065	CDC25C, tyrosine protein phosphatase	–2.5	Cell cycle
M87339	RF-C4	–2.4	Cell cycle
AF015592	CDC7-related kinase	–2.3	Kinase
M33294	TNF receptor 1A	–2.3	Apoptosis
M77198	AKT2 oncogene, rac- β kinase	–2.3	Kinase
U77845	TRIP, TRAF interacting protein	–2.2	Apoptosis
X59798	Cyclin D1	–2.2	Cell cycle
J04088 ^a	Topoisomerase (DNA) II α	–2.1	DNA repair
M14752	c-abl protooncogene, tyrosine protein kinase	–2.0	Kinase
Z35102 ^a	NDR protein kinase	–2.0	Kinase
U02680	Protein tyrosine kinase 9	–1.9	Kinase
X86779	Fas-activated serine–threonine (FAST) kinase	–1.9	Kinase
X05360	Cyclin-dependent kinase 1	–1.8	Kinase

^aThese genes are suppressed also in the muristerone inducible p53 system.

from the same clone and depends also on the nature of p53 induction signal and on the cell type [38]. In conclusion, the p53-dependent gene expression profile showed upregulation of cell cycle inhibitor and of DNA repair genes and downregulation of cyclins and kinases that promote cell cycle and cell division. The overall pattern appears to be in line with the proposed growth suppressive function and DNA repair activity of p53. In addition, some novel genes, the expression of which is modulated by p53 (e.g. MIC-1, cyclin B1 and others), were listed which may open up new avenues for future research on growth control.

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