

Inhibitory effect of c-Myc on p53-induced apoptosis in leukemia cells. Microarray analysis reveals defective induction of p53 target genes and upregulation of chaperone genes

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We have previously demonstrated that c-Myc impairs p53-mediated apoptosis in K562 human leukemia cells, which lack ARF. To investigate the mechanisms by which c-Myc protects from p53-mediated apoptosis, we used K562 cells that conditionally express c-Myc and harbor a temperature-sensitive allele of p53. Gene expression profiles of cells expressing wild-type conformation p53 in the presence of either uninduced or induced c-Myc were analysed by cDNA microarrays. The results show that multiple p53 target genes are downregulated when c-Myc is present, including *p21^{WAF1}*, *MDM2*, *PERP*, *NOXA*, *GADD45*, *DDB2*, *PIR121* and *p53R2*. Also, a number of genes that are upregulated by c-Myc in cells expressing wild-type conformation p53 encode chaperones related to cell death protection as *HSP105*, *HSP90* and *HSP27*. Both downregulation of p53 target genes and upregulation of chaperones could explain the inhibition of apoptosis observed in K562 cells with ectopic c-Myc. Myc-mediated impairment of p53 transactivation was not restricted to K562 cells, but it was reproduced in a panel of human cancer cell lines derived from different tissues. Our data suggest that elevated levels of Myc counteract p53 activity in human tumor cells that lack ARF. This mechanism could contribute to explain the c-Myc deregulation frequently found in cancer.

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Introduction

Mutations and deletions of the *p53* tumor suppressor gene constitute one of the most common genetic

alterations in human cancer. In many neoplasias and particularly in leukemia and lymphoma, loss of normal p53 function occurs during tumor progression, and tumors without active p53 are usually more aggressive and have worse prognosis (Kirsch and Kastan, 1998; Wallace-Brodeur and Lowe, 1999). p53 promotes cell growth arrest and/or apoptosis upon genomic DNA damage. The molecular basis for the decision between induction of cell cycle arrest and induction of apoptosis by p53 appears to depend on the cell type and on the p53 protein level, with higher levels determining apoptosis and lower levels determining growth arrest or differentiation (Chen *et al.*, 1996; Ronen *et al.*, 1996; Adachi *et al.*, 1998). While p53-mediated cell cycle arrest is mostly mediated by upregulation of the cyclin-dependent kinases inhibitor *p21^{WAF1}*, the mechanisms by which p53 induces apoptosis are less clear. In recent years, a number of p53 target genes involved in apoptosis have been described, such as *IGF-BP3*, *NOXA*, *PERP* and *PUMA* (Amundson *et al.*, 1998; Fridman and Lowe, 2003; Oren, 2003).

c-Myc is a protein of the helix–loop–helix/leucine zipper (bHLHLZ) family. c-Myc heterodimerizes with the protein Max. Myc-Max dimers upregulate target genes upon binding to a specific DNA sequences (E-boxes). These include genes involved in cell cycle control, energetic metabolism, protein and ribosome synthesis and other miscellaneous functions. Also, an important number of c-Myc target genes are repressed by c-Myc (Dang, 1999; Grandori *et al.*, 2000; Li *et al.*, 2003; Orian *et al.*, 2003 <http://www.mycancergene.org>). We and others have identified the zinc-finger protein Miz1 as a partner of c-Myc. The formation of Myc-Miz1 complexes on the promoter is required for c-Myc-mediated repression of several genes, including *p21^{WAF1}* (reviewed in Wanzel *et al.*, 2003). Mitogenic stimulation of quiescent cells rapidly induces c-Myc expression, and growth arrest or terminal differentiation are usually accompanied by suppression of c-Myc expression. c-Myc activity is sufficient to drive cells into proliferation in the absence of growth factors, promoting cell cycle progression by mechanisms impinging on G1 phase control (Grandori *et al.*, 2000; Eisenman, 2001; Lutz

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et al., 2002). Consistent with these *ex vivo* effects, deregulated expression of *myc* is found in a wide array of human cancers, in most cases associated to disease progression (Nesbit *et al.*, 1999; Oster *et al.*, 2002).

Despite its role in promoting tumorigenesis, c-Myc overexpression induces apoptosis in some cell types subjected to suboptimal growth conditions, for example, deprivation of growth factors or hypoxia (for a review see Hoffman and Liebermann, 1998; Thompson, 1998). Studies in murine models have established a connection between c-Myc and p53 through upregulation of ARF. ARF, in turn, inactivates Mdm2, a protein required for efficient degradation of p53. Thus, elevated levels of c-Myc activate p53-function in cells that express ARF (reviewed in Henriksson *et al.*, 2001; Lowe and Sherr, 2003).

In many tumor cells, and in particular lymphomas, ARF is mutated or repressed by promoter methylation (Krug *et al.*, 2002), and many human lymphomas express high levels of Myc and wild-type p53 (Chang *et al.*, 1994; Preudhomme *et al.*, 1995). We have previously shown that c-Myc partially protects from p53-mediated apoptosis and that c-Myc impairs p53-mediated transactivation of *p21^{WAF1}* (*CDKN1A*) in K562 cells (Ceballos *et al.*, 2000). These cells derive from human chronic myeloid leukemia and are p53- *ARF*- and *p16^{INK4A}*-null (Delgado *et al.*, 1995, 2000; Lewis *et al.*, 2001). These former results suggest that c-Myc inhibits p53 function in an ARF-deficient situation. However, the mechanism of this phenomenon remained unexplored. In this work we used microarray analysis to investigate the mechanisms by which Myc counteracts p53 apoptosis in K562 cells. We found that c-Myc impairs the p53-mediated transactivation of multiple p53 target genes, including several well-known proapoptotic genes. Also, c-Myc reverted the p53-mediated downregulation of chaperones known to protect K562

cells from apoptosis. Moreover, we found that Myc-mediated interference with the transactivation function of p53 is not exclusive to K562 cells but also takes place in multiple human cell lines. The results indicate that c-Myc can suppress p53 apoptotic activity through an ARF-independent pathway.

Results

Microarray analysis reveals defective induction of p53 target genes in the presence of c-Myc

We previously reported that ectopic c-Myc partially inhibits p53-mediated apoptosis of K562 cells. In order to explore the possibility that c-Myc could antagonize p53-mediated apoptosis by activating or repressing genes other than *p21^{WAF1}*, we performed a microarray-

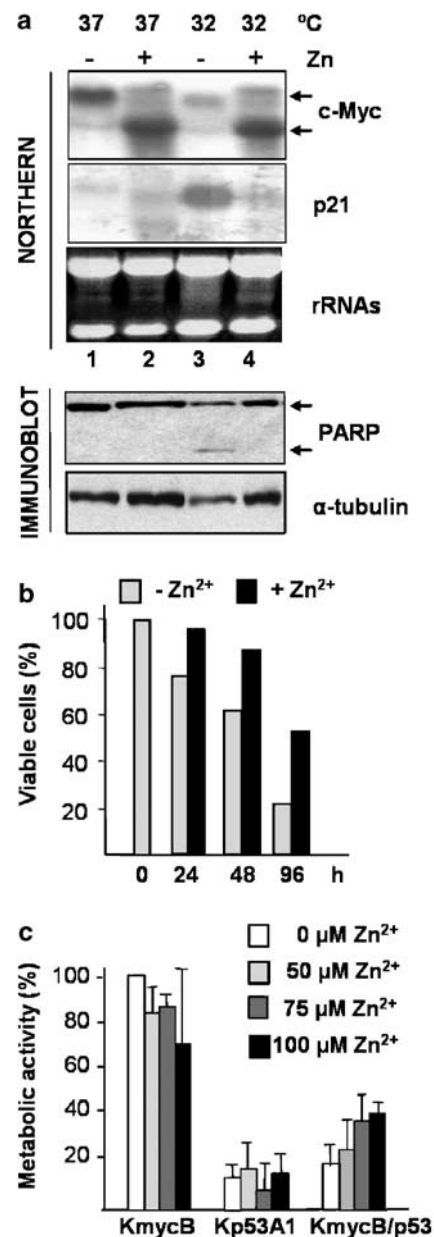


Figure 1 Induction of ectopic c-Myc and reduction of p53-mediated apoptosis in KmycB/p53 cells. (a) Upper panels: KmycB/p53 cells were incubated for 24 h at 32 or 37°C (grown in 2% serum) in the absence or presence of 75 μ M ZnSO₄ as indicated. *MYC* and *p21^{WAF1}* mRNA expression was analysed by Northern blot hybridization. The upper arrow indicates the position of the endogenous *MYC* mRNA and the lower arrow the position of the exogenous *MYC* mRNA directed by the transfected vector. A picture of the rRNAs stained with ethidium bromide is shown to assess loading and integrity of the RNAs. Lower panels: immunoblot showing apoptosis protection by c-Myc as determined by caspase-dependent proteolysis of PARP. The upper and lower arrows indicate the position of the undegraded PARP and of the smaller caspase digestion product, respectively. Aliquots of the cells of the same experiment used for Northern analysis were used to prepare lysates and subjected to immunoblot using an anti-PARP polyclonal antibody. The filter was also incubated with anti- α -tubulin antibody to assess protein loading and transfer. (b) Cell viability as assessed by Trypan blue test of KmycB/p53 cells incubated at 32°C in the absence or presence of 75 μ M ZnSO₄ for the indicated periods of time. (c) Cell viability as assessed by metabolic activity. Cells of the indicated cell lines were incubated for 48 h with the indicated concentrations of ZnSO₄ and the metabolic activity was determined by the WST-1 reduction test. Values are means \pm s.e.m. ($n = 2$ for KmycB and $n = 3$ for the other cell lines)

based analysis of gene expression. We used KmycB/p53, a K562-derived cell line, which harbors a temperature-sensitive allele of p53 and a zinc-inducible Myc gene. Activation of p53 and expression of c-Myc can be independently induced by incubating the cells at 32°C or adding zinc salts to the media, respectively (Ceballos *et al.*, 2000). The activation of p53 represses endogenous *MYC* (northern in Figure 1a, compare lanes 1 and 3), but in KmycB/p53 the addition of zinc in the presence of wild-type conformation p53 results in *MYC* expression levels similar or higher than in absence of p53 (northern in Figure 1a, compare lanes 1 and 4). As we reported earlier (Ceballos *et al.*, 2000), induction of p21^{WAF1} mRNA expression was reduced after zinc addition at 32°C (northern in Figure 1a, compare lanes 3 and 4). Also, induction of *MYC* protected K562 cells from p53-mediated apoptosis as shown by the block in PARP proteolysis (immunoblot in Figure 1a, lanes 3 and 4) and the increased fraction of viable cells in KmycB/p53 cells treated with zinc, as assessed by the Trypan blue test (Figure 1b) and by metabolic activity as measured by a tetrazolium salt reduction assay (Figure 1c).

We prepared labeled cDNA from KmycB/p53 cells incubated at 37 and 32°C for 12 h in the absence or presence of 75 μM ZnSO₄ (a concentration that achieves maximal apoptosis protection) and hybridized the microarrays containing 4608 human cDNAs and ESTs (Berwanger *et al.*, 2002) as described in Materials and methods. We prepared samples from experiments performed with 2% fetal calf serum and 0.5% fetal calf serum, as the apoptosis protection by c-Myc is more marked with low serum concentrations (not shown). We also used as controls cells incubated at 37 and 32°C (i.e. with p53 in mutant or wild-type conformation, respectively) and cells incubated in the presence or absence of 75 μM ZnSO₄ (i.e. with and without *MYC* induction).

Comparison of the microarray data with databases of known p53 target genes revealed eight p53-target genes which were significantly downregulated in the presence

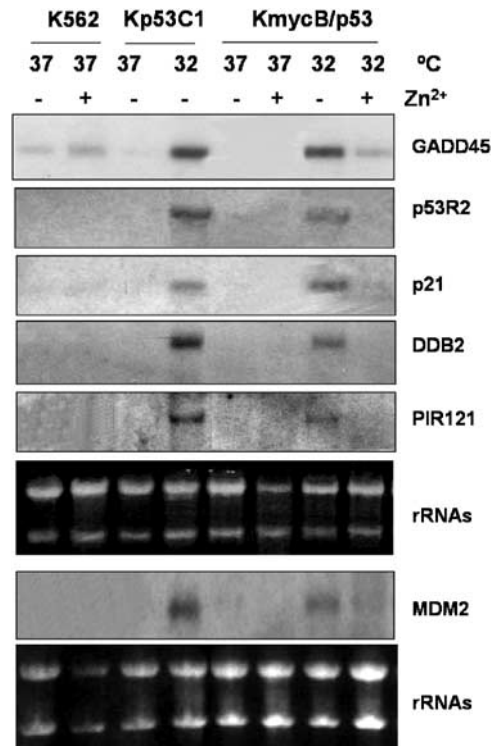


Figure 2 c-Myc antagonizes the p53-induced upregulation of p53-target genes. The cells indicated at the top of the figure were incubated for 24 h at 32 or 37°C in the absence or presence of 75 μM ZnSO₄ as indicated. Two Northern blots were prepared and were consecutively hybridized to probes of the genes indicated at the right. A picture of the rRNAs stained with ethidium bromide is shown to assess loading and integrity of the RNAs

Table 1 p53 target genes repressed by c-Myc in cell expressing active p53

	Fold repression ^a		Access. no.
<i>Cell cycle arrest</i>			
p21/Waf1, <i>CDKN1A</i> ^{b,c}	5.93	3.65	U35290
MDM2 ^{b,c} , <i>MDM2</i>	2.35	1.05	R80235
14-3-3 ^c , <i>SFN</i>	^d		
<i>Apoptosis</i>			
Heparin-binding EGF-like growth factor, <i>DTR</i>	11.15	4.66	R14663
Insulin-like growth factor binding protein 3, <i>IGBP3</i>	2.14	2.93	AA598601
Peanut-like 2, <i>PNUTL2</i>	2.55	2.13	T64878
CD95/FAS ^b , <i>TNFRSF6</i>	3.20	1.74	AA031420
p53 apoptosis effector related to PMP-22, <i>PERP</i> ^c	^d		
NOXA ^c	^d		
<i>DNA repair</i>			
Damage-specific DNA-binding protein 2, <i>DDB2</i> ^b	10.19	4.44	AA406449
Pir121 ^b , <i>CYFIP2</i>	6.14	5.17	H12043
Gadd45 ^{b,c} , <i>GADD45A</i>	^d		
p53R2 ^b , <i>RRM2B</i>	^d		

^aGene expression determined by densitometry of the signals in the microarray. Data refer to two experiments performed with 2% (left column) and 0.5% (right column) serum. Only genes whose expression decreased at least two-fold in two independent experiments are included. The corresponding gene names from the HUGO Gene Nomenclature are indicated in italics. ^bConfirmed by Northern. ^cConfirmed by promoter-luciferase assay. ^dGene not included in the array

of c-Myc (Table 1). These genes showed an upregulation of two-fold or higher by p53 (i.e. upregulated in cells incubated at 32°C relative to cells incubated at 37°C) in two independent experiments performed with 2 and 0.5% serum.

The data suggested that Myc suppresses multiple p53 target genes. To extend these results, we performed Northern blots for several other genes that were previously identified as p53 target genes and that were not present in our DNA microarrays. The analysis (Figure 2) showed that induction of *MYC* expression downregulated *p21^{WAF1}*, *DDB2*, *PIR121*, *MDM2* (as in the microarray analysis), *GADD45* and *p53R2*. Using luciferase reporter assays of p53-responsive promoters, we found that c-Myc impaired the p53-mediated activation of the promoters of *p21^{WAF1}*, *14-3-3σ*, *MDM2*, *GADD45*, *PERP*, and *NOXA* and genes, as well as the synthetic p53-responsive promoter PG13 (Figure 3).

In contrast, other known p53-responsive genes as Cyclin G1 (*CCNG1*), PIG3 (*TP53I3*) and PIG11 (*TP53I11*) did not show significant differences in the

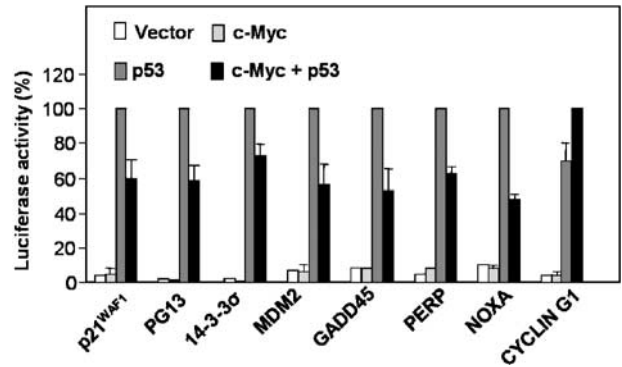


Figure 3 c-Myc impairs the p53-induced activation of p53-target promoters. K562 cells were transfected with p53 and c-Myc expression vectors and the different luciferase-reporter genes as indicated at the bottom of the figure. The luciferase activity determined in the presence of p53 was defined 100% in each case, and the other values were normalized accordingly. Reporters with PG13 and 14-3-3σ promoter constructs carrying mutations in the p53 responsive elements did not show any luciferase activity after p53 expression vector transfection. Data are mean values from three or four independent experiments, depending on the reporter. Error bars represent s.e.m.

Table 2 Genes repressed by c-Myc in K562 cells expressing active p53

	Fold repression ^a		Acces. no.
<i>Signaling</i>			
ARVCF catenin ^b , <i>ARVCF</i>	12.81	9.64	H17975
Vascular cell adhesion molecule 1, <i>VCAM1</i> ^b	2.44	3.68	H07071
Pim-1 kinase, <i>PIM1</i>	2.58	3.73	AA453663
Renin, <i>REN</i>	3.27	3.03	AA455535
Zeta-diacylglycerol kinase, <i>DGKZ</i>	2.67	2.08	AA262204
c-src tyrosine kinase, <i>CSK</i>	2.75	3.36	AA078778
Plasminogen activator/urokinase receptor, <i>PLAUR</i>	4.91	11.79	AA455222
<i>Metabolism</i>			
Valyl-tRNA synthetase 2, <i>VAR2</i>	11.59	4.11	AA464470
UDP glycosyltransferase 2 B15, <i>UGT2B15</i> ^b	4.40	3.01	T50788
Ferritin, light polypeptide, <i>FTL</i> ^{b,c}	3.68	3.41	N69252
Nitrogen fixation cluster-like, <i>NIFU</i> ^b	3.45	2.93	T55639
Solute carrier family 4, <i>SLC4A1</i>	2.73	4.00	T86708
Solute carrier family 2, <i>SLC2A5</i> ^b	2.75	4.00	H38650
Glycosyl transferase AD-017	3.00	2.27	H94897
<i>Other</i>			
α-synuclein, <i>SNCA</i>	3.75	16.56	AA455067
KIAA0042 gene product	2.84	10.41	AA477501
Myeloid leukemia factor 2, <i>MLF2</i> ^b	4.19	5.73	AA480835
Coagulation factor II (thrombin) receptor, <i>F2R</i>	5.35	2.30	AA456376
Ring finger protein, <i>RNF</i> ^b	3.99	2.19	AA054421
Transcobalamin II, <i>TCN2</i>	2.65	4.92	AA490680
α-Tropomyosin 1, <i>TPMI</i> ^c	3.22	3.11	W58092
Clk-associating RS-cyclophilin ^b , <i>CYP</i>	3.24	2.25	H14513
Keratin 8 ^b , <i>KRT8</i>	2.49	2.43	AA598517
Lymphoid-restricted membrane protein, <i>LRMP</i>	3.79	3.66	AA457051
Hypothetical protein FLJ90440	2.51	4.29	N94069
Trichohyalin, <i>THH</i>	3.01	2.51	AA258735
Ubiquitin-conjugating enzyme E2-1, <i>UBE2V1</i>	2.58	2.34	R64190
α-Thalassemia/mental retardation syndrome X-linked, <i>ATRX</i>	2.88	2.98	AA410435
Chromatin assembly factor 1, <i>CHAF1</i> ^c	3.48	2.36	AA425120
Glycoprotein M6B, <i>GPM6B</i>	2.79	2.19	AA284329
Conserved gene telomeric to α-globin cluster, <i>CGTHBA</i>	2.69	2.04	AA425757
DKFZP586P2220 protein	2.58	2.06	H81940

^aSignal changed at least two-fold in both duplicates from two independent experiments performed with 2% (left column) and 0.5% serum. The corresponding gene names as from the HUGO Gene Nomenclature are indicated in italics. ^bInduced by p53 in the microarray analysis. ^cPreviously identified as repressed by c-Myc

microarray analysis. Consistent with this observation, Myc failed to downregulate the cyclin G1 promoter in luciferase assays (Figure 3). The list of p53-target genes downregulated by c-Myc as assessed by different methods is shown in Table 1. Taken together, the results demonstrate that c-Myc reduced the p53-mediated transactivation of multiple, but not all of the p53-target genes tested. The microarray analysis also yielded other genes that were downregulated by c-Myc in K562 cells incubated at 32°C (Table 2). As indicated in the table, several of these genes (10 out of 32) were also upregulated by p53, although they have not been previously described as p53-target genes.

We considered the possibility that ectopic expression of Myc interferes with either stability or modification of p53. However, immunoblot analysis revealed that levels of p53 in Kp53A1/myc and KmycB/p53 continued unabated up to 4 days at 32°C in the presence of zinc (Figure 4a) and that both phosphorylation of p53 at serine-18 and acetylation at lysine-370 were unaffected by induction of Myc (Figure 4b). Also, nuclear localization of p53 was unmodified by c-Myc induction in KmycB/p53 cells (not shown). Alternatively, work

from our laboratories and others has demonstrated that c-Myc downregulates the p21^{WAF1} gene via binding to Miz1 (Herold *et al.*, 2002; Seoane *et al.*, 2002; Wu *et al.*, 2003). Therefore, we asked whether Miz1 was also involved in c-Myc-mediated downregulation of other p53-target promoters as p21^{WAF1}, GADD45, MDM2, NOXA and PG13. K562 cells were transfected with expression vectors encoding either wild-type c-Myc and a c-Myc mutant unable to bind Miz1 (c-MycV394D) (Herold *et al.*, 2002). Both vectors directed the expression of similar amounts of c-Myc protein and were similar in their ability to transactivate an E Box-luciferase reporter (not shown). In contrast to c-Myc, c-MycV394D did not inhibit p53-mediated transactivation of the PG13, MDM2 and NOXA promoters (Figure 5a). However, c-MycV394D was as efficient as c-Myc repressing the GADD45 and PERP promoters, suggesting that Myc inhibits some, but not all p53 target promoters through binding to Miz1 (in agreement with a recent report) (Barsyte-Lovejoy *et al.*, 2004). Three additional experiments were performed to support this

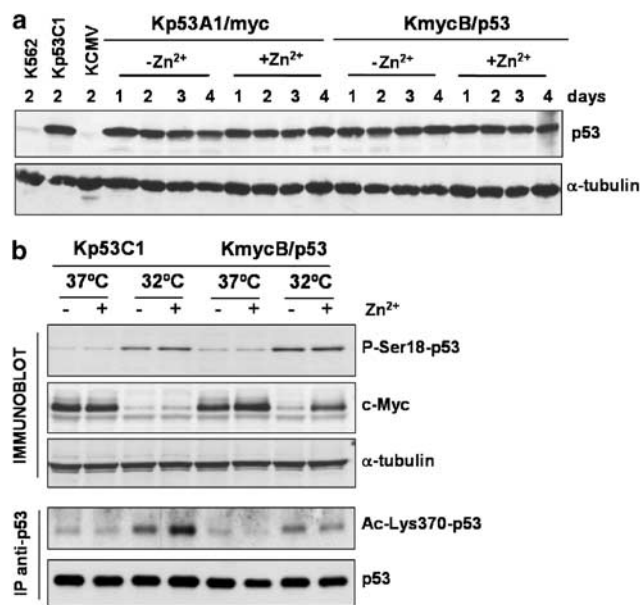


Figure 4 c-Myc does not modify p53 stability, phosphorylation and acetylation. (a) Myc induction does not enhance p53 degradation. Cells from the cell lines indicated at the top of the figure were incubated at 32°C in the absence or presence of 75 μM ZnSO₄ for up to 4 days as indicated. Lysates were subjected to immunoblot to reveal levels of p53. The filter was also incubated with anti-α-tubulin antibody to assess protein loading and transfer. (b) c-Myc does not significantly alter p53 phosphorylation and acetylation. Protein lysates were prepared from the indicated cell lines incubated for 15 h at 37° or 32° and in the presence or absence of 75 μM ZnSO₄ as indicated in the top of the panel. Upper panel: immunoblot probed with antiphospho-Ser15-p53 (corresponding to Ser18 of murine p53) and anti-Myc to show the induction of Myc by Zn²⁺ in KmycB/p53 cells and anti-α-tubulin as a loading control. Lower panel: Lysates were immunoprecipitated with anti-p53 antibody and the precipitated material was electrophoresed and immunoblotted with an antibody anti-acetyl-Lys373-p53 (corresponding to Lys370 of murine p53)

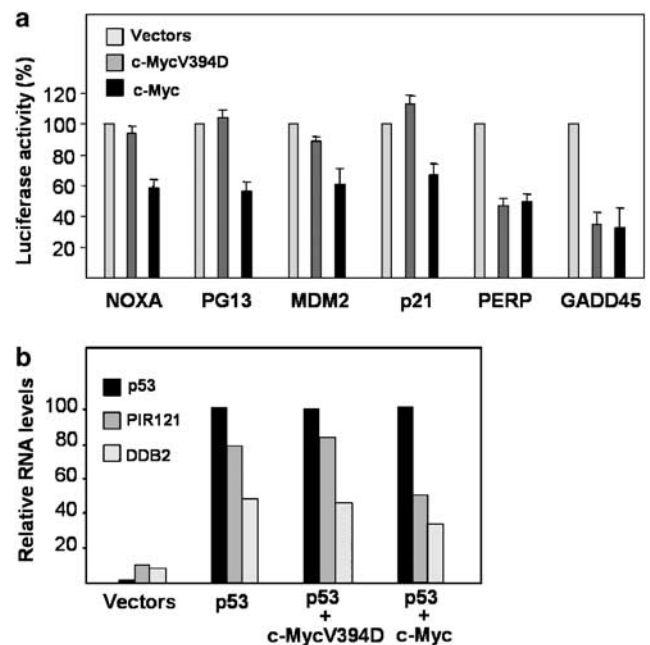


Figure 5 Binding to Miz1 is required for c-Myc inhibitory effect on p53 transactivation. (a) Effect of c-Myc and c-MycV394D on promoter activities. Kp53A1 cells were transfected with the promoter-luciferase construct indicated at the bottom and the pCEFL-Myc, pCEFL-MycV394D or the pCEFL vector as indicated. After 24 h, aliquots of the culture were transferred to 32°C (to have p53 in wild-type conformation) and luciferase activity was measured 36 h after transfection. Luciferase activities were normalized to that measured in cells transfected with the empty vector. Data are mean values from three to five independent experiments, depending on the reporter. Error bars represent s.e.m. Luciferase activity at 37°C was below 2% for all reporters. (b) Effect of c-Myc and c-MycV394D on the relative mRNA expression of p53-target genes. K562 cells were cotransfected with expression vectors for p53, wild-type c-Myc and c-MycV394D as indicated. At 24 h after transfection RNA of the transfected cells was prepared and the mRNA levels of p53, PIR121 and DDB2 were measured by real-time RT-PCR as described in Materials and methods

conclusion. First, we asked whether the above-described effect at the level of promoter activation translates into a change in the level of mRNA. To do this we cotransfected K562 cells with expression vectors for wild-type p53 and for c-Myc or c-MycV394D. At 24 h after transfection we analysed by RT-PCR the expression of DDB2 and PIR121, two p53 target genes affected by c-Myc (Table 1) and with very low expression in the absence of p53. The results (Figure 5b) demonstrate that c-Myc but not c-MycV394D reduced mRNA levels of both genes, with the effect being more pronounced for PIR121. Secondly, we transiently transfected expression vectors encoding either Miz1 or p53 into K562 cells, since we were unable to establish stable K562 cells lines expressing ectopic Miz1 (Wanzel *et al.*, 2005). By itself, Miz1 did not enhance expression of p21^{WAF1} and PIR121 (not shown). However, Miz1 synergized with p53 in the activation of both target genes, and c-Myc reduced the induction of p21^{WAF1} and PIR121 mRNA mediated by p53 and by p53 plus Miz1 (Figure 6a). Finally, we asked whether c-Myc and Miz1 bind to the promoters of p53 target genes *in vivo*. Using chromatin-immunoprecipitation, we detected both c-Myc and Miz1 at the start sites of the p21^{WAF1}, DDB2, p53R2 and NOXA promoters (Figure 6b). We concluded that at least on some p53-target promoters, the inhibition of p53 transactivation by c-Myc is mediated via binding to Miz1.

Microarray analysis reveals that c-Myc induces chaperone genes in the presence of wild-type p53

A list of the genes that were upregulated at least two-fold in K562 cells upon induction of c-Myc is shown in Table 3. Among the genes strongly upregulated by Myc were those encoding the chaperones HSP10, HSP70, HSP105 and GRP58. In order to validate the microarray results we performed Northern analysis. The results (Figure 7a) demonstrated that HSP27, HSP90 and HSP105 were strongly downregulated in Kp53C1 cells and KmycB/p53 cells incubated at 32°C (i.e. with p53 in wild-type conformation). In contrast, the three genes were upregulated in KmycB/p53 cells overexpressing c-Myc in the presence of active p53. The temperature shift or the addition of ZnSO₄ in parental K562 cells provoked no significant changes in the mRNA levels of the three chaperones (Figure 7a). As a control we also tested chaperone expression in KmycB cells, which carry a zinc-inducible MYC gene (Delgado *et al.*, 1995). The c-Myc-mediated upregulation of HSP90 and HSP27 in the presence of active p53 was also observed at the protein level by immunoblot (Figure 7b). Therefore, the upregulation of these chaperone genes in KmycB/p53 at 32°C in the presence of ZnSO₄ is a consequence of the enhanced levels of c-Myc.

An unexpected result from the microarray analysis is the absence of genes directly related to cell cycle progression or proliferation that were upregulated by c-Myc in cells expressing wild-type conformation p53. This may be explained by the fact that c-Myc could not reverse the p53-mediated growth arrest (Ceballos *et al.*,

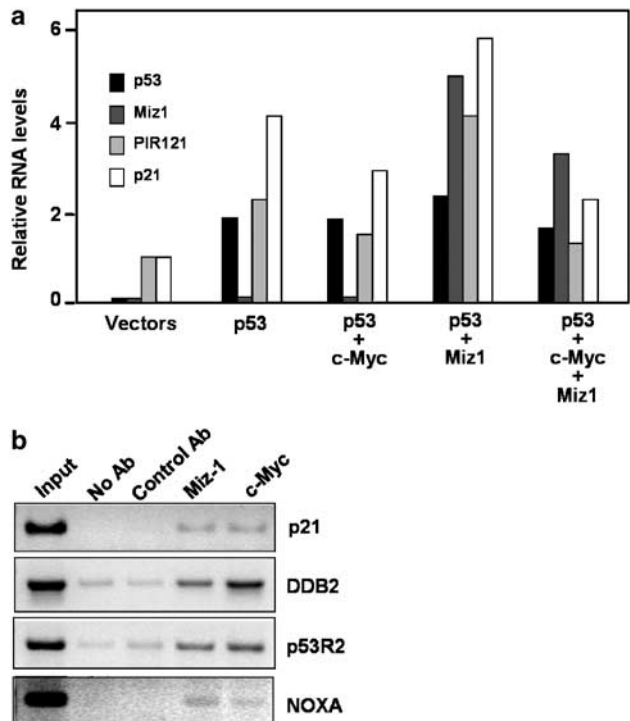


Figure 6 c-Myc and Miz1 co-activate and bind to p53-target promoters. (a) K562 cells were cotransfected by electroporation with p53, Miz1 and c-Myc expression vectors as indicated. After 24 h, the expression of p53, Miz1, PIR121 and p21^{WAF1} mRNA was analysed by real-time RT-PCR as described in the legend of Figure 5. Transfection of expression vectors for c-Myc, Miz1 and c-Myc plus Miz1 did not significantly modify the expression of PIR121 and p21^{WAF1}. Data are normalized to the expression levels determined in the transfection with the empty vectors, set at 1 for p21^{WAF1} and PIR121 and 0.1 for Miz1 and p53. (b) Chromatin immunoprecipitation demonstrating binding of c-Myc and Miz1 to the promoters indicated at the right. Cells were fixed and the chromatin was immunoprecipitated with an irrelevant antibody (as a control), anti-Myc and anti-Miz1 antibodies as indicated. A fraction of chromatin before immunoprecipitation was also analysed ('Input' lanes). For this experiment, Ls174T colon carcinoma cells were used since they express high endogenous levels of both c-Myc and Miz1

2000). However, despite its inability to promote growth, induction of Myc enhanced expression of D-type cyclins (Figure 8a) and promoted phosphorylation of the retinoblastoma protein, pRB (Figure 8b), in the presence of active p53. However, cells expressing c-Myc and wild-type p53 showed an intermediate RB phosphorylation level between that of growing cells (expressing inactive p53) and cells expressing wild-type conformation p53, which are growth-arrested and committed to apoptosis.

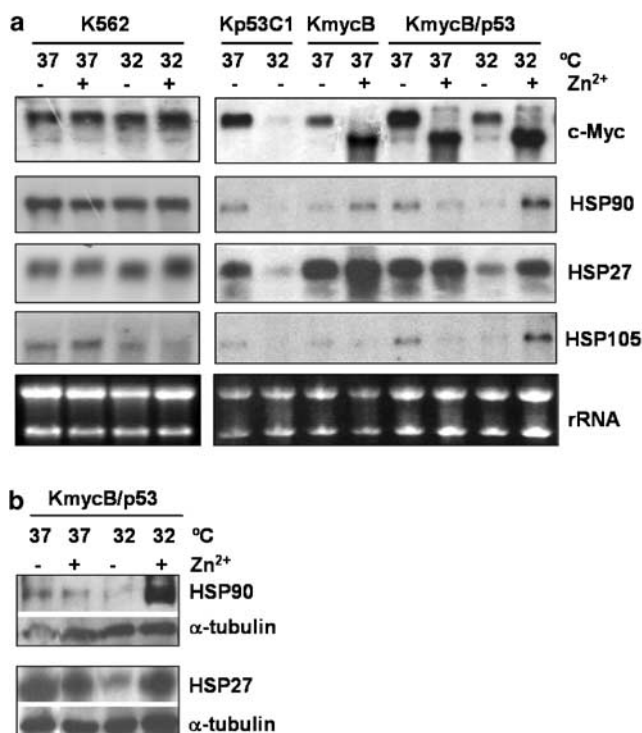
c-Myc antagonizes p53-mediated transactivation in cells derived from different tissues

All the former results have been obtained with the K562 cell line and its derivatives, which derive from human chronic myeloid leukemia in blast crisis. In order to know whether this effect of c-Myc can be extended to cells derived from other tumors, we cotransfected

Table 3 Genes induced by c-Myc in K562 cells expressing active p53

	Fold induction ^a		Acces. no.
<i>Chaperones</i>			
HSP70 protein 2, <i>HSPA2</i> ^{b,c,d}	7.87	4.75	AA455102
HSP105, <i>HSPH1</i> ^{b,c,d}	7.65	8.0	AA485036
HSP10 chaperonin, <i>HSPH1</i> ^{b,c,d}	3.39	3.34	AA48396
Glucose regulated protein 58kDa/ERP57 <i>GRP58</i>	2.00	3.43	R33030
Hsp27, <i>HSPB1</i> ^d	e		
Hsp90, <i>HSPCB</i> ^{c,d}	e		
<i>Transcription factors</i>			
FosB, <i>FOSB</i>	11.26	14.02	T62179
MYC, <i>MYC</i> ^{b,d}	3.56	1.98	AA514409
Signal transducer and activator of transcription 5B, <i>STAT5B</i>	2.49	2.56	AA282023
JUNB, <i>JUNB</i>	2.00	2.69	N94468
<i>Signaling</i>			
Insulin induced gene 1, <i>INSIG</i> ^b	5.77	4.72	H59620
Developmentally regulated GTP-binding prot. <i>DRG1</i> ^b	3.18	3.13	AA488336
NIMA-related kinase 3, <i>NEK3</i> ^b	2.96	2.97	AA490263
<i>Metabolism</i>			
Histidyl-tRNA synthetase, <i>HARS</i> ^b	3.05	3.11	H61209
Fatty-acid-Coenzyme A ligase, long-chain 1, <i>FACL1</i> ^b	2.98	2.63	T73556
Methylene tetrahydrofolate dehydrogenase, <i>MTHFD2</i> ^b	2.63	2.63	MTHFD2
Fatty-acid-coenzyme A ligase, long-chain 3, <i>FACL3</i>	2.28	2.65	W31074
UDP-N-acetylglucosamine pyrophosphorylase 1, <i>UAP1</i> ^b	2.96	2.42	N68465
<i>Other</i>			
Zinc-finger protein FLJ10697	2.46	4.47	W94105
Ig superfamily containing leucine-rich repeat, <i>ISLR</i> ^b	4.61	2.13	H62387
Decay accelerating factor for complement, <i>CD55/DAF</i>	2.41	4.16	R09561
Cystathionase (cystathionine gamma-lyase), <i>CTH</i> ^b	3.51	2.55	R07167
Delta-amino levulinic synthase 2, <i>ALAS2, MYO47</i>	2.60	2.77	T67104
Splicing factor, arginine/serine-rich 7, <i>SFRS7</i> ^b	2.05	2.77	H54020

^aSignal changed at least two-fold in both duplicates from two independent experiments performed with 2 and 0.5% serum. Metallothionein genes (induced by zinc) are not included. The corresponding gene names as from the HUGO Gene Nomenclature are indicated in italics. ^bRepressed by p53 in the microarray analysis. ^cPreviously identified as induced by c-Myc. ^dConfirmed by Northern/immunoblot. ^eGene not included in the array



several cell lines derived from different human tumor types and from other species. The tissue origin of the cell lines assayed and their p53 status are summarized in Table 4. The coexpression of wild-type p53 and c-Myc in HT29, Raji, KU812, BAF3 and HaCaT cells resulted in a decrease in p53-mediated transactivation of p21^{WAF1} promoter by 30–60% depending on the cell line (Figure 9a). As expected, p53 could not activate p21^{Waf1} promoter in HeLa, as these cells express the papillomavirus E6 protein (which mediates rapid p53 degradation). Also, we tested the TM2 cell line, a nontransformed cell line derived from mink lung epithelial cells expressing a MYC inducible gene by the tet-off system (Warner *et al.*, 1999), and MYC induction resulted in decreased p53-mediated p21^{WAF1} promoter activation (Figure 9a). We also tested the c-Myc effect in

Figure 7 c-Myc upregulated chaperone expression in the presence of wild-type conformation p53. **(a)** K562, KmycB, Kp53C1 and KmycB/p53 cells were incubated for 24 h at 32 and 37°C in the presence of 75 μM ZnSO₄. The expression of the indicated chaperone genes was analysed by Northern blot hybridization. A picture of the rRNAs stained with ethidium bromide is shown to assess loading and integrity of the RNAs. **(b)** Expression of HSP90 and HSP27 chaperones in KmycB/p53 after 24 h at 32°C with and without 75 μM ZnSO₄, as determined by immunoblot

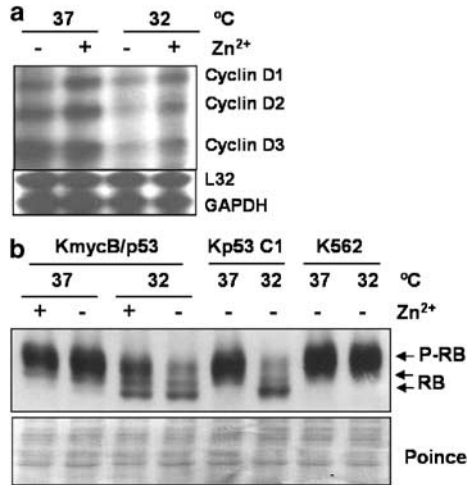


Figure 8 c-Myc induction in KmycB/p53 cells partially rescues cyclin D repression and RB hypophosphorylation mediated by wild-type conformation p53. (a) Expression of cyclins D1, D2 and D3 in KmycB/p53 cells at 32 or 37°C and in the absence or presence of 75 μM ZnSO₄ for 24 h. The expression was determined by RNase protection assay. Ribosomal protein L32 and GAPDH mRNAs are used as loading controls. (b) Expression and phosphorylation state of RB proteins in KmycB/p53, Kp53C1 and K562 cells incubated for 24 h at 32°C in the absence or presence of 75 μM ZnSO₄. 'P-RB' indicates the position of hyperphosphorylated form of RB. A picture of the immunoblot section comprising proteins between 80 and 120 kDa stained with Ponceau red is shown to assess protein transfer and lane loading

Table 4 Cell lines used in the experiments of Figure 9

Cell line	Origin ^a	p53 gene status ^b
K562	Chronic myeloid leukemia	Inactivated
Kp53A1	Chronic myeloid leukemia	Conditional, endogenous inactivated
Kp53C1	Chronic myeloid leukemia	Conditional, endogenous inactivated
KU812	Chronic myeloid leukemia	Inactivated
BV173-tsp53	Chronic myeloid leukemia	Conditional, endogenous wild type
HEL-tsp53	Acute myeloid leukemia	Conditional, endogenous inactivated
NB4-tsp53	Promyelocytic leukemia	Conditional, endogenous inactivated
Raji	Burkitt lymphoma	Inactivated
PC3-tsp53	Prostate carcinoma	Conditional, endogenous inactivated
HT29	Colon carcinoma	Inactivated
HaCaT	Keratinocytes	Inactivated
HeLa	Cervical carcinoma	Wild type ^c
BAF3	Murine lymphoid cells	Wild type
TM2	Mink lung epithelial cells ^d	Wild type

^aAll from human origin except BAF3 (mouse) and TM2 (mink). Origin and references of the lines are indicated in Materials and methods. ^b'Conditional' refers to transfectant cells expressing the p53^{Val135} mutant. 'Inactivated' refers to cells carrying mutations and/or deletions of the p53 gene. ^cFunctionally inactive due to the expression of E6 and E7 papillomavirus proteins. ^dMv1Lu transfected with inducible (tet-off) Myc gene

NB4tsp53, HELtsp53 and BV173tsp53 cells. These are leukemia-derived cell lines that express the p53^{Val135} mutant (Rizzo *et al.*, 1998). After transfection with an

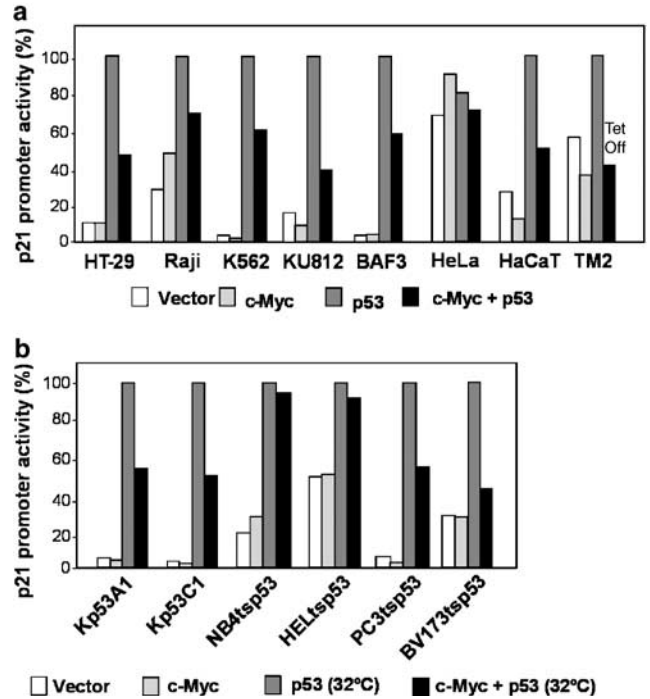


Figure 9 c-Myc impairs p53-dependent transactivation of p21^{WAF1} in cell lines derived from different tissues. (a) Cells of the indicated cell lines were cotransfected with p53 and/or c-Myc expression vectors as indicated. At 36 h after transfection, the activity of the p21^{WAF1} promoter was determined by the luciferase assay. (b) Sublines expressing the p53^{Val135} mutant were transfected with a c-Myc expression vector, incubated 16 h at 37°C and then further incubated for 24 h at 32°C (to have p53 in wild-type conformation). The activity of the p21^{WAF1} promoter was determined by luciferase assay. The luciferase activity determined in the presence of active p53 was defined 100% in each case, and the other values were normalized accordingly. Data are mean values from three independent experiments

MYC expression vector, the cells were shifted to 32°C (i.e. p53 in wild-type conformation) and the activity of p21^{WAF1} promoter was determined. The results (Figure 9b) show that the transactivation of p21^{WAF1} was reduced as compared to cells transfected with the empty vector. We also generated the line PC3-tsp53 by transduction of the PC3 prostate cancer cell line with a retrovirus encoding the p53^{Val135} mutant (see Material and methods). Transfer of PC3-tsp53 to 32°C resulted in cell growth arrest and activation of p21^{WAF1} promoter (data not shown). We found that c-Myc also reduced p53 transcriptional activity in PC3-p53ts (Figure 9b). Altogether the results indicate that impairment of p53-mediated transactivation by c-Myc occurs in different cell types.

Discussion

How does c-Myc impair the p53-mediated apoptosis in K562 cells?

We previously showed that expression of wild-type-conformation p53 in K562 cells leads to apoptotic cell

death, accompanied by downregulation of c-Myc. However, induction of ectopic Myc reduced apoptosis (Ceballos *et al.*, 2000). Although no single p53 effector molecule can account for all of p53's proapoptotic activity, there is compelling evidence that transactivation is essential for p53 to promote apoptosis (reviewed in Fridman and Lowe, 2003; Oren, 2003). Thus, one obvious explanation for the reduction of p53-mediated apoptosis by c-Myc in the K562 model is blunting the p53 transcriptional activation of target genes involved in apoptosis. We previously described that sustained c-Myc levels in cells expressing wild-type conformation p53 resulted in defective upregulation of p21^{WAF1}. p21^{WAF1} has been related in most cases to p53-mediated cell cycle arrest rather than to apoptosis, although there are also reports describing a proapoptotic role of p21^{WAF1} (Sheikh *et al.*, 1995; Chinery *et al.*, 1997; Wu *et al.*, 1998). However, in K562 p21^{WAF1} is not proapoptotic since the coexpression of antisense p21^{WAF1} did not reduce the p53-mediated apoptosis (data not shown). Thus, in our leukemia model downregulation of p21^{WAF1} by c-Myc does not switch the p53 activity from cytostatic to proapoptotic, as has been found in colon cancer cells (Seoane *et al.*, 2002). Our results are in agreement with the lack of effect of p21^{WAF1} on Myc-induced apoptosis of mammary cells *in vivo* (Bearss *et al.*, 2002). As BAX is a proapoptotic protein induced by p53, a defective upregulation of BAX in cells expressing c-Myc could explain the apoptosis reduction. However, BAX mRNA and protein levels are not downregulated by c-Myc in K562 (Ceballos *et al.*, 2000), and thus it cannot be responsible for c-Myc antiapoptotic effect in K562 expressing p53.

We therefore performed a microarray-based analysis to find other genes deregulated by c-Myc in the context of active p53 in K562 cells. This analysis, complemented with Northern and reporter luciferase assays of selected genes, yielded two results: (a) many, but not all, of p53-target genes tested were downregulated by c-Myc; and (b) chaperones and other genes downregulated by p53 were upregulated by c-Myc (Tables 1–3). Importantly, p53 target genes downregulated by Myc included not only p21^{WAF1} but also genes involved in apoptosis and in genome integrity maintenance, as well as the regulator of p53 stability *MDM2* (Table 1). Besides these known p53 target genes, c-Myc repressed other genes, whose expression was augmented in K562 expressing wild-type conformation p53 (32 genes included in Table 2). Among these, there are several genes previously associated with cell death induction, as α -synuclein (Iwata *et al.*, 2001; Zhou *et al.*, 2002) and renin (Pierzchalski *et al.*, 1997).

The mechanisms of transcriptional activation by p53 are not fully understood (for a discussion see Haupt *et al.*, 2002), and therefore how c-Myc interferes with this process is open to speculation. The fact that not all p53-target genes are affected by c-Myc (for example, cyclin *G1*, *PIG3* and *PIG11* were unchanged) suggests that c-Myc targets specific components of the p53 transcriptional activation complex. It has been

previously shown that c-Myc downregulates p21^{WAF1} expression through the formation of a complex with the zinc-finger protein Miz1 (Herold *et al.*, 2002; Seoane *et al.*, 2002; Wu *et al.*, 2003). Using a c-Myc mutant unable to bind Miz1 (c-MycV394D), we found that Miz1 is required for the impairment of p53 transcriptional activity mediated by c-Myc in promoters other than p21^{WAF1}, as *MDM2*, *NOXA* and the synthetic promoter *PG13*. Moreover, Myc and Miz1 bind to the promoter of several p53-responsive promoters *in vivo*, demonstrating that Miz1 plays a role c-Myc-mediated inhibition of multiple, but not all p53 target genes. For example, we did not detect the effect on the *PERP* and *GADD45* promoter, as recently described (Barysytte-Lovejoy *et al.*, 2004). Other potential mechanisms involve Myc-mediated degradation or modification of the acetylation or phosphorylation of p53, but we did not detect a significant Myc-induced change in protein levels, phosphorylation of Ser18 and acetylation of Lys373 of the p53 expressed in our model.

Upregulation of chaperones by c-Myc and apoptosis protection

Our microarray-based analysis revealed that 34% (21/61) of the genes modified by c-Myc in K562 cells expressing wild-type conformation p53 were upregulated. We already observed in this model that the impairment of p53-mediated gene repression of CMV immediate-early promoter (Ceballos *et al.*, 2000) was antagonized by c-Myc and it has been recently reported for stathmin and survivin (Lohr *et al.*, 2003). However, one interesting result of this work is that c-Myc dramatically upregulated the genes of chaperones *HSP27*, *HSP90*, *HSP105* and *GRP58*, some of them being repressed by p53. These results are consistent with the antiapoptotic effect of these chaperones in many models (reviewed in Jolly and Morimoto, 2000; Garrido *et al.*, 2001; Beere, 2004), including p53-mediated apoptosis (Zylicz *et al.*, 2001). Thus, chaperone upregulation by c-Myc is a mechanism that may operate in the partial rescue from p53-apoptosis mediated by c-Myc. Consistently, *HSP10*, *HSP60*, *HSP70* and *HSP90* have been described as c-Myc-target genes in microarray- and SAGE-based analysis (Coller *et al.*, 2000; Menssen and Hermeking, 2002). Apart from chaperones, other genes upregulated by c-Myc in p53-expressing cells have functions related to cell death protection in myeloid cells as *FOSB* (Yamamura *et al.*, 2000) and *STAT5* (Horita *et al.*, 2000). *JUNB* and *DRG1* have been involved in antiproliferative responses (Shaulian and Karin, 2001; Motwani *et al.*, 2002). Interestingly, the majority of the genes upregulated by c-Myc in our microarray analysis were repressed by wild-type conformation p53. It has been proposed that p53-mediated repression requires p21^{WAF1} (Lohr *et al.*, 2003), and thus it is possible that the upregulation observed for all or some of these genes is a consequence of p21^{WAF1} repression elicited by c-Myc. Thus, c-Myc provokes repression of p53 transcriptional activity and upregula-

tion of antiapoptotic chaperones, and both effects can explain the antagonistic effect of c-Myc on p53-mediated apoptosis.

The microarray data did not reveal upregulation of genes related to cell cycle progression. This is explained by the fact that c-Myc could not reverse the p53-mediated growth arrest. Although c-Myc increases RB phosphorylation in cells expressing wild-type conformation p53, it is unable to restore the levels of hyperphosphorylated RB detected in growing cells with nonfunctional p53, which can explain the inability of cells to maintain active proliferation in the presence of zinc at 32°C.

Myc and p53 antagonism: implications in tumorigenesis

The expression of a number of p53 target genes related to apoptosis is impaired by c-Myc, such as *IGBP3*, *NOXA*, *PERP*, *CD95*, *PNUTL2* and *DTR*. Of these genes, *PERP* (Ihrie *et al.*, 2003; Jeffers *et al.*, 2003) and *NOXA* (Schuler *et al.*, 2003; Shibue *et al.*, 2003) correlate more closely than *BAX* with p53-inducible apoptosis. We conclude that the impairment of p53-induced upregulation of proapoptotic genes can explain the partial protection from apoptosis mediated by c-Myc.

Interestingly, c-Myc also impaired the upregulation of p53 target genes involved in DNA repair and genome integrity maintenance functions (*DDB2*, *PIR121*, *p53R2*, *GADD45*). This function has been recently found to play a prominent role in the tumor suppressor activity of p53 *in vivo* (Liu *et al.*, 2004). Also, it is becoming increasingly clear that one consequence of deregulated *MYC* expression is genomic instability, which results in unscheduled DNA replication, gene rearrangements and chromosomal aberrations (Li and Dang, 1999; Kuschak *et al.*, 2002; Sheen and Dickson, 2002; Karlsson *et al.*, 2003). Thus, our data also support the hypothesis that deregulated *MYC* contributes to genomic instability in cells arrested by wild-type conformation p53. A previous report has described that c-Myc can overcome the p53-mediated cell cycle arrest and promotes genomic instability (Chernova *et al.*, 1998). Altogether the results support the idea that interference with the activity of p53 as 'guardian of the genome' can be one of the tumorigenic pathways used by c-Myc.

It is noteworthy that K562 derives from chronic myeloid leukemia, where it has been reported higher *MYC* expression in final blast crisis phase of the disease (Handa *et al.*, 1997; Beck *et al.*, 1998 and our unpublished results). Alterations of *TP53* are infrequent in this disease and therefore our results support the idea that deregulated *MYC* contributes to tumor progression in this leukemia through impairment of p53 function. However, we also found that the impairment of p53 transactivation by c-Myc was not restricted to K562, but it also occurs in a wide array of cell lines deriving from different tissues, including colon, epidermis, prostate and lymphoid tissue. A parallel scenery has recently been described for promyelocytic leukemia, where *TP53*

mutation is absent and the oncogenic transcription factor PML-RAR α impairs p53 transcriptional activity (Insinga *et al.*, 2004). Apoptosis protection by c-Myc is not without precedent and has been observed in other models (Wu *et al.*, 1996; Waikel *et al.*, 1999; Liu *et al.*, 2000; D'Agnano *et al.*, 2001; Ebinuma *et al.*, 2001). However, our results argue for a novel pathway for c-Myc impairment of p53-mediated apoptosis. In tumor models where c-Myc is proapoptotic, c-Myc overexpression provides a selective pressure for inactivation of the Myc-ARF-Mdm2-p53 pathway. As such, murine fibroblasts that survive c-Myc overexpression or murine lymphomas arising in E μ -myc-transgenic mice show p53 and ARF mutations or *MDM2* amplification (Zindy *et al.*, 1998; Eischen *et al.*, 1999; Inoue *et al.*, 2001; Schmitt *et al.*, 2002). Similar findings have been described for murine myeloid tumors induced by c-Myc (Haviernik *et al.*, 2003). However, K562 are ARF-null, and therefore our work demonstrates that c-Myc can impair p53 function through a mechanism not involving ARF. Of notice in this regard is the observation that in the E μ -myc lymphoma model, about 20% of the tumors did not carry detectable alterations in p53, ARF or *MDM2* (Eischen *et al.*, 1999). Similarly, *MYC* overexpression has been observed in a significant number of human tumors not showing p53 alterations, including leukemia or lymphoma (Preudhomme *et al.*, 1995; Chang *et al.*, 2000).

In conclusion, although other mechanisms may also be suggested, our results suggest an additional mechanism to explain c-Myc overexpression during tumor progression, that is, the abrogation of p53 proapoptotic functions by interfering with p53-dependent transcription. As p53 plays a pivotal role in the response of tumor cells to drug- or radiation-induced cell death, this mechanism would help to explain how c-Myc deregulation confers a selective advantage to tumor cells.

Materials and methods

Cell lines and cell viability assays

K562 cell line was obtained from the American Type Culture Collection. This cell line is derived from a human chronic myeloid leukemia in blast crisis. Kp53A1 and Kp53C1 are K562 cells expressing a mouse p53^{Val135} mutant, which adopts a 'wild-type' conformation at 32°C (Ceballos *et al.*, 2000). KmycB are K562 transfectants expressing zinc-inducible c-Myc (Delgado *et al.*, 1995). Kp53A1/myc and KmycB/p53 are K562 derivatives that express the p53^{Val135} mutant and zinc-inducible c-Myc, and have been previously described (Ceballos *et al.*, 2000). Unless otherwise stated, c-*MYC* was induced in the transfectant cell lines by addition of 75 μ M ZnSO₄ 2 h before shifting the cells to 32°C. Other cell lines used were: PC3, Raji, HeLa, KU812 (from ATCC), HT-29 (provided by Xavier Mayol, IMIM, Barcelona), HaCaT (provided by Miguel Quintanilla, IIB, Madrid), BAF3 (provided by Augusto Silva, CIB, Madrid), NB4tsp53, BV173tsp53, HELtsp53 myeloid leukemia expressing p53^{Val135} (Rizzo *et al.*, 1998) (provided by M Giulia Rizzo, Inst. Regina Elena, Roma), TM2 (derived from mink Mv1Lu cells) show inducible

expression of c-Myc (Warner *et al.*, 1999) (provided by Joan Massague and Joan Seoane, Sloan Kettering Institute, NY, USA) and Ls174T) (Wanzel *et al.*, 2005). The tissues where these cell lines were originated from are indicated in Table 4. PC3sp53 are PC3 expressing p53^{Val135}, and were obtained by retroviral transduction using the pBabe-p53Val135 vector and selected in 1 μ g/ml of puromycin. All cell lines were from human origin except BAF3 and TM2. Cells were grown in RPMI-1640 medium except TM2 and HaCaT, which were grown in DMEM medium (Life Sciences Tech). Media were supplemented with 8% fetal calf serum and gentamycin (80 μ g/ml) and cyprofloxacin (2 μ g/ml), and cultures were maintained at cell densities below 10⁶ cell per ml. Cell viability was scored in hemocytometer with Trypan blue vital staining and by measuring metabolic activity with the WST-1 tetrazolium salt reduction assay (Roche Applied Science).

Expression vectors

The following expression vectors were used: pLPC-hp53 (encoding human wild-type p53) and the corresponding empty vector pLPCX (provided by Manuel Serrano, CNIO, Madrid); pBabe-p53Val135 encoding the murine p53^{Val135} thermosensitive mutant (provided by Moshe Oren, Weizmann Institute, Rehovot), LMycSN (encoding human MYC cDNA) and the corresponding empty vector LXSX (provided by Robert Eisenman, FHCR, Seattle). The c-Myc cDNA and the mutated version c-MycV394D were subcloned in the pCEFL vector, under the control of the translation elongation factor 1 α promoter. This promoter is not affected by p53. pCEFL-Myc was constructed as described (Mauleon *et al.*, 2004) and pCEFL-MycV394D was constructed from the CMV-MycV394D vector (Staller *et al.*, 2001). pCDNA3-Miz1 (encoding human Miz1 cDNA) and the corresponding empty vector pCDNA3 (Staller *et al.*, 2001).

Transactivation assays

Cells (4–8 \times 10⁶) were transfected by electroporation at 260 v and 1 mFa in a Bio-Rad electroporator. Kp53A1 cells were cotransfected with the appropriate plasmids (3 μ g of luciferase reporter construct and 9 μ g of c-Myc or c-MycV394D vector). After 24 h of incubation at 37°C, cultures were split into aliquots and further incubated for 12 h at 32 or 37°C as indicated. Cells were lysed and the luciferase activity was measured in duplicate by a dual-luciferase reporter gene assay system (Promega Corp.). K562 and the cell lines of Table 4 cells were co-transfected with a luciferase reporter, p53 expression vector (pLPC-hp53) and c-Myc expression vector (LMycSN) or the corresponding empty vectors. Luciferase reporter plasmids were: pGL3-Waf1-Luc, containing 2.3-kb of p21^{WAF1} promoter upstream of the firefly luciferase gene; Cyclin G1-Luc, MDM2-Luc (the three reporters provided by Moshe Oren, Weizmann Institute, Rehovot); PG13-P4-luc, carrying a synthetic p53 responsive promoter and PG13mut-P4-Luc (carrying a mutated p53 binding sequence as negative control) (el-Deiry *et al.*, 1993), 14-3-3 σ and 14-3-3 σ -mutated (the four reporters provided by Bert Vogelstein, John Hopkins University, Baltimore), GADD45-luc (provided by Linda Penn, University of Toronto), PERP-luc (provided by Tyler Jacks, MIT, Boston), NOXA-luc (provided Tsukasa Shibue, University of Tokyo). In total, 1 μ g of the pRL-TK plasmid encoding for *Renilla* luciferase (Promega) was cotransfected in each case to normalize for transfection efficiency. Promoter activity was defined as the ratio between light units generated by the firefly and *Renilla* luciferases.

Northern, RNase protection and immunoblot analysis

Total RNA was isolated from cells with the Qiagen RNA isolation kit and Northern blots were prepared and hybridized to ³²P-labeled probes, according to standard procedures. Probes for human MYC, p21^{WAF1} and rat histone H4 were as described (Ceballos *et al.*, 2000). Probes for HSP27, HSP70, HSP90 α and HSP105 genes were fragments from human cDNA clones. RNase protection assays for cyclins D and controls L32 and GAPDH genes were carried out with the RiboQuant kit (BD Pharmingen) using probes labeled with [α -³²P]UTP and T7 RNA polymerase, according to the manufacturer's protocol. For immunoblots, cells were lysed and protein electrophoresed and immunoblotted as described (Ceballos *et al.*, 2000). The following antibodies were used: anti-Retinoblastoma, anti-c-Myc, anti-p21^{WAF1} and anti-poly(-ADP-ribose)polymerase (PARP) (all rabbit polyclonals from Santa Cruz Biotech), anti-p53 monoclonal Pab240 (Santa Cruz Biotech.), monoclonal anti-phospho-p53 (Ser15) (Cell Signaling), rabbit polyclonal anti-acetyl-p53 (Lys373) (Upstate Biotechnology), anti-HSP27 and anti-HSP90 (monoclonals from Stressgene) and α -tubulin (rabbit polyclonal provided by Nicholas Cowan, New York University). Immunocomplexes were detected with peroxidase-conjugated secondary antibodies (Cappel) and chemiluminescent method (ECL, Amersham). The goat polyclonal anti-p53 M19 (Santa Cruz Biotech.) was used for p53 immunoprecipitation.

Real-time PCR analysis

K562 cells (1 \times 10⁶) were co-transfected with 1 μ g of pLPC-hp53 and 3 μ g of pCEFL-c-Myc or pCEFL-c-MycV394D by nucleofection with a Cell Line Nucleofector. At 24 h after transfection, total RNA was isolated from cells using RNeasy Kit (Qiagen). First-strand cDNA was synthesized from 1 μ g of total RNA using SuperScriptTM II RNase Reverse Transcriptase (Invitrogen). PCR primers sequences for human p53, c-Myc, Miz1, PIR121, p21^{WAF1}, DDB2, p53R2 and ribosomal protein S14 (RPS14) cDNAs are available upon request. For real-time PCR, an SYBR Green PCR kit (Qiagen) was used. Reactions were performed in duplicate for RPS14 and the test genes on the same 96-well plate for each individual experiment using on an ICycler iQTM Detection System (Bio-Rad). The data were normalized to the RPS14 RNA level in each case.

Chromatin immunoprecipitation

Chromatin immunoprecipitation from Ls174T cells (Wanzel *et al.*, 2005) was performed as described previously (Strutt and Paro, 1999; Eberhardy *et al.*, 2000). Chromatin was immunoprecipitated with anti-c-Myc (antibody N-262, Santa Cruz Biotech), anti-Miz1 (C-19, Santa Cruz Biotech.) and anti-cdk2 as a control antibody. PCR of immunoprecipitated DNA was amplified with the following primers: for DDB2 5'GAA ACG CCC AGA AAC CCA GAA GAC3' and 5'CAC AGC GCC AGG AAA GGT AGG ATT3' (423 bp); for p53R2 5'CGG GCC GGC GCA GGG AGA GT3' and 5'CGG CGA GGG CGG GCG GAC AG 3' (375 bp); for p21^{WAF1} 5'CGA GCG CGG GTC GCC TCC TTG AG3' and 5'CGG CCC GGG GTC CCC TGT TGT CT3' (201 bp); for NOXA 5'CGT CCC GCG TCC GCT CCC ATA AC3' and 5'TGA GCC CGC CCC AGC CGA GAC CT 3' (460 bp). Conditions for PCR were: 94°C 30 s, 62°C (for p53R2 65°C) 45 s, 72°C 60 s, 38 cycles. For NOXA and P53R2, 5% DMSO was added to the PCR reaction. For each gene, primers surrounding the transcription start site were used for the analysis.

Preparation of labeled cDNA and hybridization of microarrays

Total RNA from KmycB/p53 cells was isolated with a Qiagen RNA isolation kit, and labeled with Cy5- and Cy3-dCTP as described (Berwanger *et al.*, 2002). Labeled cDNAs were mixed prior to microarray hybridization. The cDNAs mixed were from: (a) cells incubated for 24 h at 37 or 32°C (for genes induced/repressed by p53 in wild-type conformation); (b) cells incubated for 24 h at 37°C in the absence or presence of 75 µM ZnSO₄ (for genes induced by c-Myc); and (c) cells incubated for 24 h at 32°C in the absence or presence of 75 µM ZnSO₄ (for genes induced/repressed by c-Myc in the presence of p53). Two sets of experiments were performed with RPMI-2% fetal calf serum and RPMI-0.5% fetal calf serum, respectively.

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