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Transcriptional activation by p53, but not induction of the p21 gene, is essential for oncogene-mediated apoptosis

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The p53 tumor suppressor limits cellular proliferation by inducing either G_1 arrest or apoptosis, depending on the cellular context. To determine if these pathways are mechanistically distinct, we have examined the effects of different p53 mutants in *p53* null primary mouse embryo fibroblasts. We chose this system as it is highly physiological and ensures that the interpretation of the results will not be confounded by the presence of endogenous p53 or oncoproteins which target p53. Using single cell microinjection assays for both G_1 arrest and apoptosis, with loss-of-function and chimeric gain-of-function mutants, we have demonstrated that transcriptional activation is critical for both processes. Replacement of the p53 activation domain with that of VP16, or replacement of the p53 oligomerization domain with that of GCN4, reconstituted both G_1 arrest and apoptosis activities. However, despite the importance of transcriptional activation in both processes, the target gene requirements are different. The p21 cyclin-dependent kinase inhibitor, which has been shown to be a direct target of p53 and a component of the radiation-induced G_1 arrest response, is dispensable for oncogene-induced apoptosis, suggesting that these two p53-dependent transcriptional pathways are distinct.

Keywords: apoptosis/G_1 arrest/p53/p21/transcriptional transactivation

Introduction

The *p53* tumor suppressor gene is a critical regulator of tumorigenesis, as evidenced by the fact that p53 is lost through mutation or inactivation by viral oncoproteins in >50% of human cancers (Hollstein *et al.*, 1991; Levine, 1993). Moreover, individuals with Li–Fraumeni syndrome, who inherit a mutant *p53* allele, are highly predisposed to various cancers (Malkin, 1993). Additionally, germline mutation of the *p53* gene in mice causes pronounced cancer predisposition, with 100% of mutant animals developing tumors within 3–6 months after birth (Donehower *et al.*, 1992; Jacks *et al.*, 1994; Purdie *et al.*, 1994; Tsukada *et al.*, 1994).

p53 is known to suppress cellular proliferation through two mechanisms, each operating in distinct contexts (reviewed in Haffner and Oren, 1995). For example, in normal fibroblasts, p53 induces G_1 arrest in response to DNA-damaging agents, presumably to allow the cells to perform critical repair functions before progressing through the cell cycle. In other contexts, such as in abnormally proliferating cells or in irradiated thymocytes, induction of p53 leads to programed cell death or apoptosis. Both of these functions may contribute to tumor suppression, and their simultaneous inactivation following *p53* mutation may explain the high frequency of *p53* mutation in cancer. Moreover, p53 has been linked to apoptosis in response to cytotoxic agents, making an understanding of its mode of action important for cancer therapy (reviewed in Lowe, 1995).

p53 has been shown to activate transcription from specific DNA binding sites as well as repress transcription in a binding site-independent manner (Oliner, 1993; Prives, 1994). It is thought to induce G_1 arrest by activating expression of genes such as p21/CIP 1, a cyclin-dependent kinase (CDK) inhibitor known to inhibit cell cycle progression through binding of cyclin–CDK complexes (El-Deiry *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993; Dulic *et al.*, 1994). However, it is likely that p53 also activates some other gene(s) to effect G_1 arrest, as p21 deficiency only partially abolishes the irradiation-induced G_1 checkpoint, while p53 deficiency completely abolishes it (Kastan *et al.*, 1992; Brugarolas *et al.*, 1995; Deng *et al.*, 1995). A possible candidate is the p53-responsive *GADD 45* gene, whose activity has been shown to be sufficient to inhibit entry into S-phase (Smith *et al.*, 1994). It is also possible that other functions of p53, such as transcriptional repression or stimulation of DNA repair (Wang *et al.*, 1995), could contribute to the growth arrest response.

The mechanism by which p53 induces apoptosis has been more elusive. Two studies have demonstrated that p53-dependent cell death can occur even in the presence of transcriptional and translational inhibitors, suggesting that transcriptional transactivation by p53 is dispensable for this function (Caelles *et al.*, 1994; Wagner *et al.*, 1994). Independent studies in HeLa cells supported these findings by showing that p53 mutants that are unable to activate transcription can induce apoptosis (Haupt *et al.*, 1995). Together, these experiments suggest that either p53-mediated gene repression or some activity of p53 distinct from transcriptional transactivation is important for programed cell death. In addition, Sabbatini *et al.* have shown that a p53 double point mutant [termed p53(22–23)] which is defective in both activation and repression, is compromised for the induction of apoptosis, suggesting that either of these activities, or perhaps the structural integrity of the amino-terminus, is important for induction of cell death (Sabbatini *et al.*, 1995). Reconciling these disparate findings and interpreting these experiments conclusively has been difficult due either to the expression of endogenous wild-type p53 in the systems in which

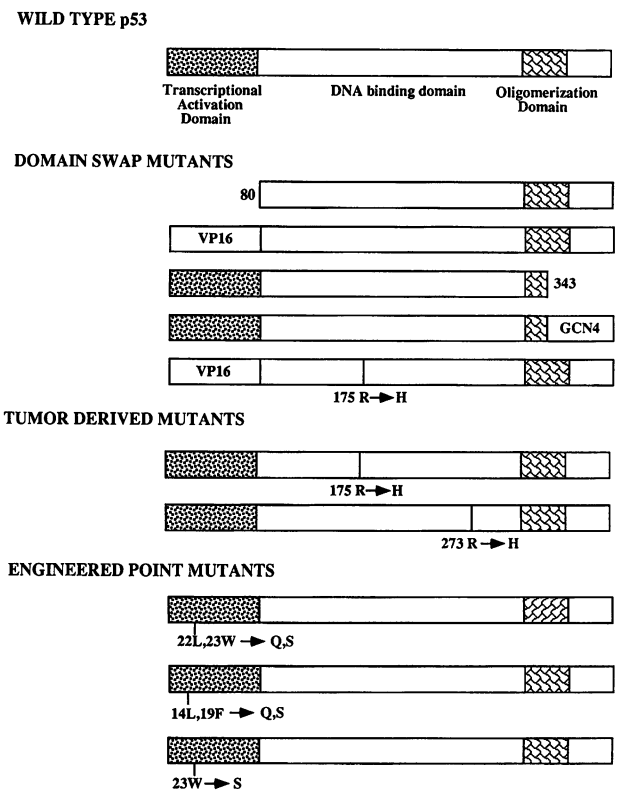


Fig. 1. p53 domain structure and mutant constructs. The p53 DNA binding, transactivation and oligomerization domains are indicated. Three classes of mutants are depicted. Domain swap mutants include deletions of the activation and oligomerization domains, as well as fusions to the VP16 activation and GCN4 dimerization domains. Tumor-derived mutants include 175 R-H, which has a completely altered structure, and 273 R-H, which is only affected in DNA binding. Engineered point mutants include three site-directed mutants, whose transactivation activities are compromised on two different synthetic reporter genes.

mutants were tested or to the presence of viral oncoproteins which target p53. In addition, each of these studies provides negative, rather than positive, evidence.

Clues to the molecular mechanism by which p53 suppresses cellular proliferation have also come from the analysis of *p53* mutations found in human tumors. The majority of these mutations occur within highly conserved clusters in the central core of the protein, which has been shown to be involved in sequence-specific DNA binding (Friend, 1994). These are generally single base point mutations which have the consequence of either altering the conformation of p53 or blocking its ability to bind specific DNA sequences. Since sequence-specific DNA binding is necessary for p53-mediated transactivation of various target genes, it is likely that disruption of the transcriptional activation function of p53 is important for tumor progression.

Here, we identify the functional domains of p53 required for both G_1 arrest and apoptosis. We have used *p53* null primary cells to eliminate any effects from endogenous p53 protein. Moreover, we have examined both loss-of-function and gain-of-function mutants. These experiments demonstrate unequivocally that transcriptional activation is necessary and sufficient for G_1 arrest and apoptosis. In addition, we have examined the requirement of the p53 target gene, p21, for oncogene-induced apoptosis.

Table I. G_1 arrest activities of p53 mutants

Construct	Percent BrdU$^+$ cells	No. of cells examined
pCEP 4	92	24
p53	32	204
p53(80–393)	78	162
p53–VP16	26	140
p53(1–343)	54	265
p53–GCN4	35	113
Uninjected cells:		
Cells re-entered into S	79	
Cells not arrested in G_1	29	

G_1 arrest data obtained from the co-injection of p53 mutants and β-galactosidase in several experiments are summarized. For each construct, the total number of β-galactosidase-positive cells examined, ~18 h after injection, is indicated. The percentage of these which are BrdU positive is also indicated. The percentages of uninjected cells re-entering the cell cycle upon serum stimulation (79%) and cells that continued to enter S-phase, even after serum starvation (29%), are also indicated.

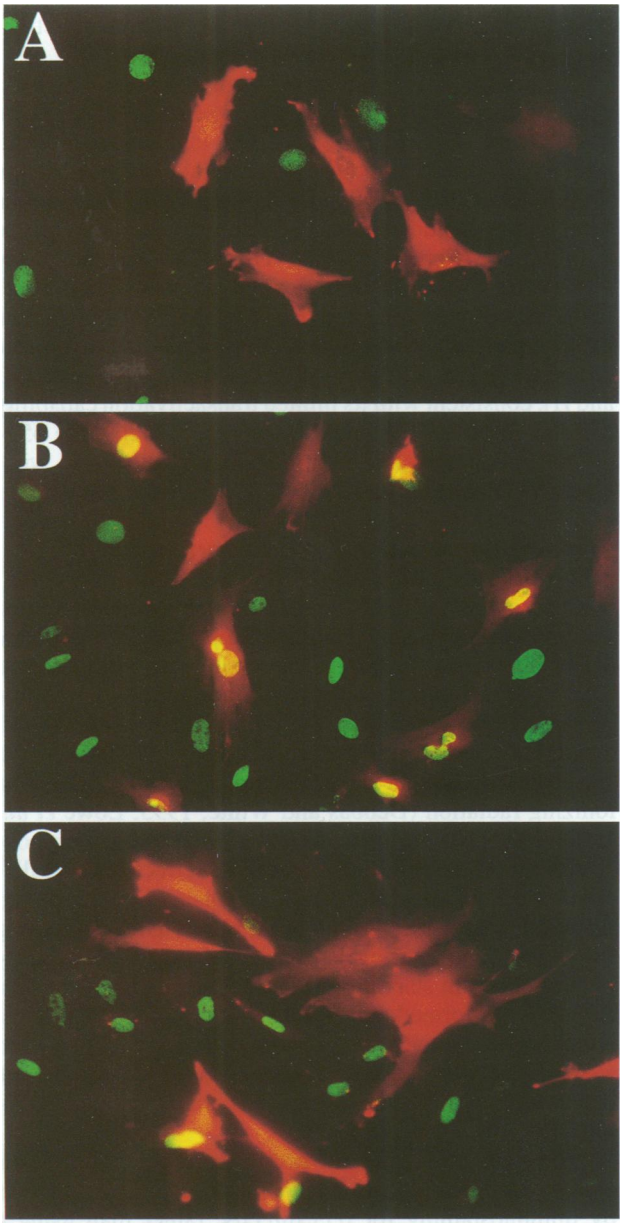
Although p21 induction is a component of p53-dependent G_1 arrest, it is dispensable for apoptosis. These results suggest that distinct programs of gene expression are important for p53-mediated G_1 arrest and apoptosis.

Results

Transcriptional activation is important for p53-mediated G_1 arrest

The domain structure of p53 is depicted in Figure 1. The DNA binding domain resides in the central 200 amino acids of the protein, with the transcriptional activation domain and the tetramerization domain at the amino- and carboxy-termini, respectively (reviewed in Prives, 1994). In an effort to establish whether separate functions of p53 are involved in G_1 arrest and apoptosis, we aimed to determine conclusively the role of p53-mediated transcriptional activation for both functions. To this end, we tested several deletion and domain swap mutants of p53 in G_1 arrest and apoptosis assays (Figure 1). These included p53(80–393), which lacks the transcriptional activation domain, as well as a fusion of this mutant with the exogenous activation domain from the herpes simplex virus VP16 protein. In addition, we tested p53(1–343), which lacks part of the oligomerization domain, and p53–GCN4, in which the GCN4 coiled-coil is substituted for the p53 oligomerization domain. Although monomeric p53 is capable of activating transcription, it does so less efficiently than wild-type p53 (Stenger *et al.*, 1994; Subler *et al.*, 1994). Both the p53–VP16 and the p53–GCN4 proteins have been shown previously to have growth suppression activity in a colony-forming assay (Pietenpol *et al.*, 1994). Whether this growth suppression reflected G_1 arrest or apoptosis, however, is unclear.

Wild-type human p53 and a collection of mutant variants were first assayed for their ability to induce G_1 arrest. Early passage *p53*-deficient mouse embryo fibroblasts (MEFs) were synchronized in G_0/G_1 by serum starvation and then microinjected with either wild-type or mutant p53 expression constructs. Since p53 has a short half-life and can be detected only for several hours after injection (data not shown), we co-injected a β-galactosidase expres-



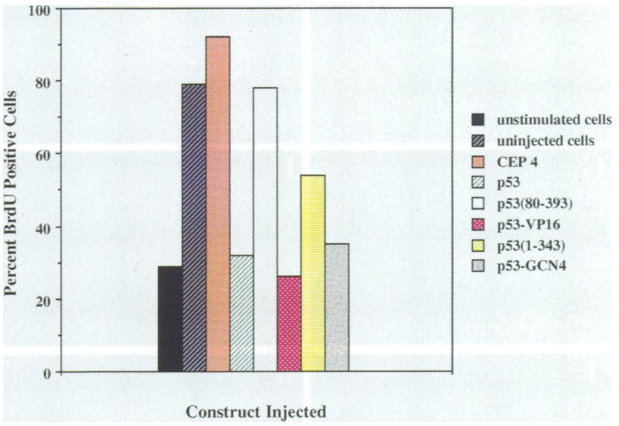


Fig. 2. Transcriptional activation is important for p53-induced G_1 arrest. Cells have been co-injected with β-galactosidase and p53 expression constructs and stained with anti-β-galactosidase antibodies (red staining). Cells that have entered S-phase and thus incorporated BrdU were detected by staining with anti-BrdU antibodies (green staining). Photographs represent superimposed images of β-galactosidase and BrdU staining (overlap is seen as yellow staining). (**A**) Wild-type p53 prevents entry of cells into S-phase. (**B**) A p53 deletion mutant lacking the amino-terminal activation domain, p53(80–393), fails to impede entry of cells into S-phase. (**C**) A p53–VP16 chimera prevents entry of cells into S-phase. (**D**) Data from various mutants are summarized in bar graph form. All data are derived from Table I.

sion plasmid to mark those cells that were injected successfully. The majority of cells expressing β-galactosidase were shown also to express p53 several hours after injection (data not shown); a representative experiment showing p53 expression levels several hours after co-injecting p53 and β-galactosidase expression plasmids is shown in Figure 3. Following injection, cells were stimulated to re-enter the cell cycle by serum addition, in the presence of bromodeoxyuridine (BrdU). Cells were labeled with BrdU for ~18 h and then processed for immunofluorescence to determine the percentage of injected cells which had successfully entered S-phase.

By counting the number of β-galactosidase-positive cells that were also BrdU positive, we determined that exogenous wild-type p53 was able to block entry of cells into S-phase (Table I, Figure 2A). Only 32% of the cells injected with wild-type p53 entered S-phase, as compared with ~80% of uninjected cells (Table I) or cells injected with an empty expression vector (Table I and data not shown). The unexpectedly high percentage of cells which were still able to enter S-phase in the wild-type p53 injections is likely to reflect both the fact that a significant proportion of cells were not growth arrested successfully by serum starvation (29%; Table I) and that some β-galactosidase-positive cells failed to co-express p53. Injection of the p53(80–393) mutant lacking the transactivation domain failed to impede S-phase entry, indicating that this domain is required for p53-mediated G_1 arrest (Figure 2B and Table I). Replacement of the p53 activation domain with that of VP16 reconstituted the G_1 arrest response to levels comparable with wild-type p53 (26%; Table I, Figure 2C). Thus, transcriptional transactivation by p53 is sufficient to induce G_1 arrest.

Microinjection of a p53 mutant with the oligomerization domain deleted produced a partial G_1 arrest response; the percentage of cells arrested upon injection of this mutant (54%) was intermediate between the percentages for wild-type p53-injected cells and uninjected cells. This partial effect may be explained by the fact that carboxy-terminal deletion mutants are several fold less active than wild-

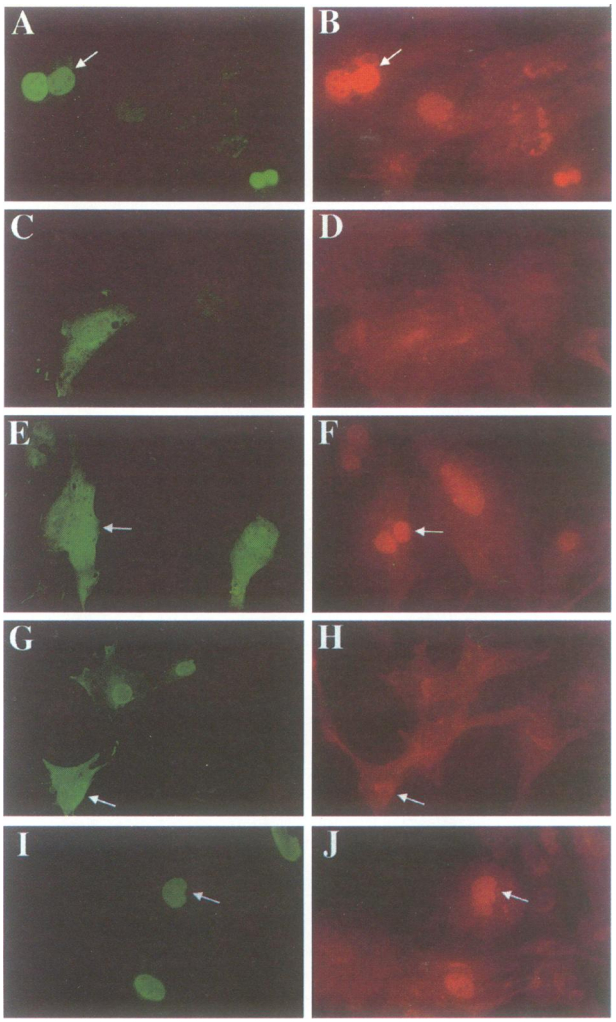


Fig. 3. p21 induction by various p53 mutants. p53 injection leads to induction of the p21 gene product. Cells were injected with both β-galactosidase and p53 expression plasmids, as in the G_1 arrest assays, and immunostained for p53 and p21 6–7 h after injection. p53 staining (green; A, C, E, G and I) and p21 staining (red; B, D, F, H and J) co-localizes to nuclei; representative cells are indicated by arrows. Cells have been injected with wild-type p53 (**A** and **B**), p53 (80–393) (**C** and **D**), p53–VP16 (**E** and **F**), p53(1–343) (**G** and **H**) and p53–GCN4 (**I** and **J**). Some injected cells stained strongly for p21, but only faintly for p53 because of its rapid turnover.

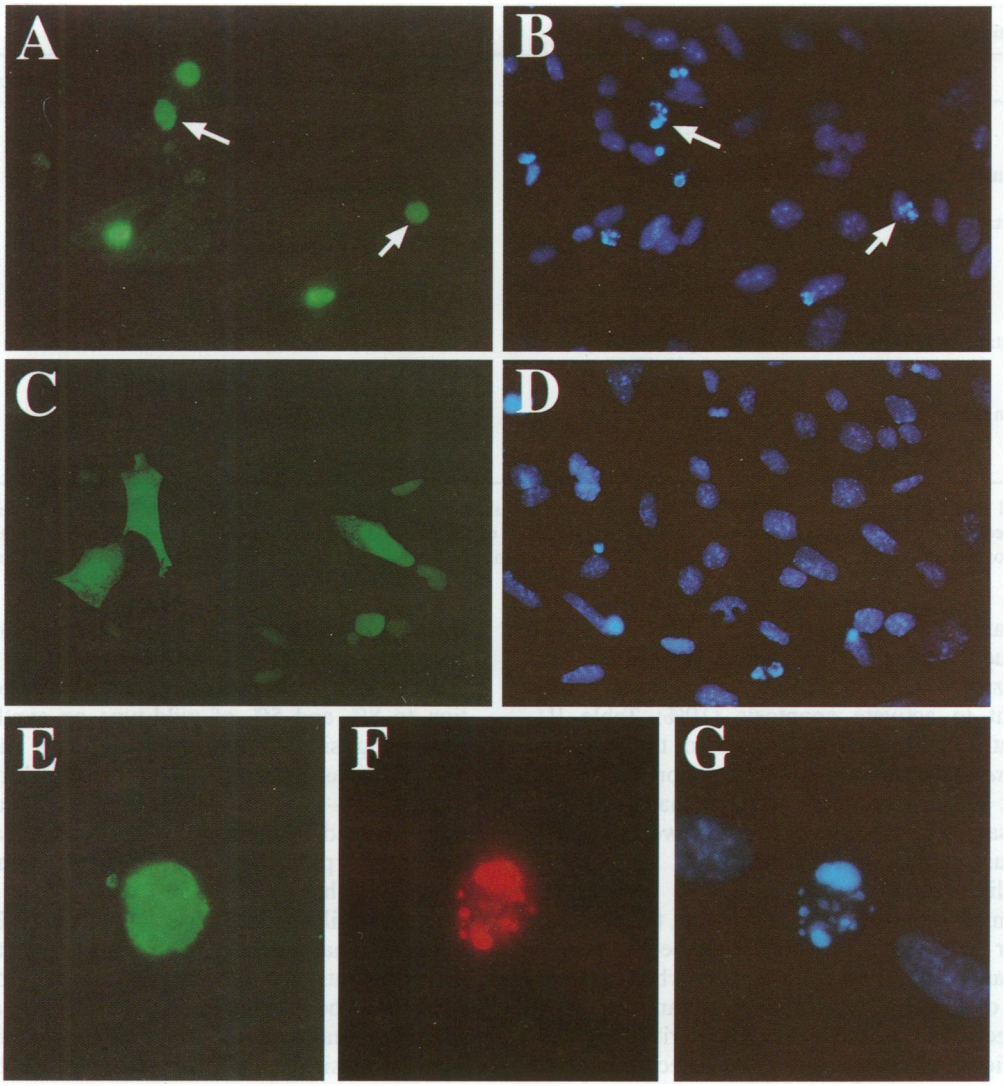
type p53 in transcriptional activation assays (Stenger *et al.*, 1994; Subler *et al.*, 1994), and thus may require particularly high expression levels to produce an effect. Importantly, replacement of the p53 oligomerization domain with the coiled-coil dimerization motif of GCN4 led to cell cycle arrest capacity nearly commensurate with wild-type p53 (35%), indicating that oligomerization is necessary for efficient G_1 arrest function. Immunofluorescence analysis demonstrated that all mutants are expressed (Figure 3) and localize to either the nucleus or both the nucleus and cytoplasm. The presence of protein in the cytoplasm may reflect especially high protein expression, which may occur more readily with certain mutants of p53 than with wild-type p53. Taken together, these data demonstrate that transcriptional transactivation is both necessary and sufficient for p53-mediated G_1 arrest function. Moreover, the fact that the p53–VP16 fusion is as active as wild-type p53 in our assay suggests that all G_1 arrest functions of p53 involve transcriptional transactivation.

The transcriptional activity of these p53 mutant proteins was confirmed by examining the expression of a known downstream target of p53, the CDK inhibitor p21. Wild-type p53 was able to induce p21 within 7 h after injection (Figure 3A and B). As expected, the p53(80–393) mutant lacking the amino-terminal activation domain failed to induce p21 (Figure 3C and D), while the fusion of this region to the VP16 activation domain restored p21 induction (Figure 3E and F). The p53(1–343) mutant lacking part of the oligomerization domain was capable of inducing low levels of p21 in some cells (Figure 3G and H), consistent with its limited transactivation capability and its partial growth arrest phenotype. Finally, the p53–GCN4 fusion activated p21 indistinguishably from wild-type p53 (Figure 3I and J).

Transcriptional activation is important for p53-mediated apoptosis

We next examined the functional domains of p53 required for apoptosis. To this end, we employed *p53*-deficient MEFs infected with an E1A-expressing retrovirus because they serve as a model system for tumorigenesis and cancer therapy. Cells expressing E1A undergo programed cell death in a p53-dependent manner, particularly after serum starvation, treatment with chemotherapeutic agents or ionizing radiation (Debbas and White, 1993; Lowe *et al.*, 1993, 1994a,b). We injected human *p53* expression constructs into these cells and assessed the effects by visualization of cell and nuclear morphology using DAPI staining. As is the case with wild-type (p53+/+) E1A-expressing cells, we found that levels of apoptosis were particularly high after serum starvation (Figure 5 and data not shown), and thus performed our assays under those conditions. Numbers of normal and apoptotic cells were totaled from a series of independent experiments to determine the percentage of p53-expressing cells that exhibited apoptotic features.

Wild-type p53 induced apoptosis efficiently in this system. In this assay, we were able to detect injected cells by immunostaining for p53 directly, rather than using a β-galactosidase marker, because p53 protein is stabilized in the presence of E1A (data not shown; Lowe and Ruley, 1993). At 6–12 h after injection, most of the p53-expressing cells maintained normal morphology (data not shown). However, by 16–25 h, the number of p53-expressing cells had greatly diminished. Moreover, many of the p53-positive cells had become rounded, with shrinking cytoplasm, blebbing nuclei and condensed chromatin, classical signs of apoptosis (Figure 4, Table II). These cells also stained positively by TUNEL analysis (Figure 4F), which measures fragmented DNA, further confirming that cell death occurred via apoptosis. Examination of all p53-positive cells observed in multiple experiments indicated that 59% of these cells were apoptotic. Co-injecting the empty pCEP 4 expression vector along with a β-galactosidase marker and immunostaining for β-galactosidase demonstrated that there was a background incidence of apoptosis of ~3%, confirming that p53 expression is necessary for high levels of death. Additionally, it must be stressed that the percentage of cells induced to die by p53 simply represents those observed at discrete



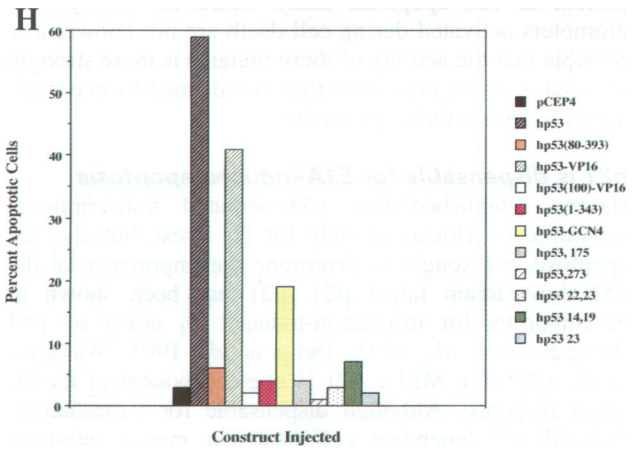


Fig. 4. Apoptosis is induced by microinjection of wild-type but not mutant p53 into E1A-expressing cells. (**A**) p53-expressing cells exhibit rounded morphology. (**B**) DAPI staining confirms that these cells are undergoing apoptosis, as nuclei are blebbing and chromatin is condensed. Representative cells (A) and their corresponding apoptotic nuclei (B) are indicated by arrows. (**C**) p53(80–393) mutant-expressing cells exhibit normal morphology. (**D**) DAPI staining confirms the normal nuclear morphology of these cells. (**E**) High magnification view of a rounded p53-expressing cell undergoing apoptosis. (**F**) Same cell as in (E), examined by TUNEL analysis. (**G**) Same cell as in (E) and (F), stained with DAPI to show the nuclear phenotype. (**H**) Data from various mutants are summarized in bar graph form. All data are derived from Table II.

windows of time. Since cell death occurs with temporal heterogeneity, it is likely that we have missed some apoptotic cells and thus underestimated the total number induced to die.

In contrast to wild-type p53, tumor-derived p53 mutants 175R-H and 273R-H failed to induce apoptosis above background levels (1 and 4% respectively, Table II). Importantly, while mutant 175R-H is known to have a grossly disrupted conformation, mutant 273R-H retains the native conformation but lacks a residue critical for interaction with DNA (Cho *et al.*, 1994). Therefore, while mutant 175R-H would be expected to be inactive regardless of the domains of the protein required to induce cell death, mutant 273R-H might retain activity if sequence-specific DNA binding were dispensable for cell death. However, our results strongly indicate that this is not the case.

Next, p53 domain-swap mutants were examined for their ability to induce apoptosis. The oligomerization-

Table II. Apoptotic activities of p53 mutants, 16–25 h post-injection

	Construct	Percent apoptotic cells	Total No. of cells examined
	pCEP4	3	88
	hp53	59	205
Tumor-derived mutants	hp53,175	4	183
	hp53,273	1	263
Domain swap mutants	hp53(80–393)	6	140
	hp53–VP16	41	102
	hp53(1–343)	4	251
	hp53–GCN4	19	202
	hp53(100)VP16	2	52
Domain swap mutants with β-gal marker	hp53(80–393)	5	229
	hp53–VP16	29	128
	hp53,175–VP16	5	128
Activation domain point mutants	hp53 22,23	3	216
	hp53 14,19	7	229
	hp53 23	2	242

The data obtained 16–25 h after injection of p53 into E1A-expressing populations are depicted. The total number of p53-positive cells examined is listed. For the injections with the β-galactosidase marker, the total number of β-galactosidase-positive cells is listed. The percentage of these p53- or β-galactosidase-positive cells which are apoptotic is also indicated. These numbers represent the sums of values from different experiments.

defective mutant p53(1–343) was greatly impaired in its ability to induce cell death (4%; Table II). Adding the GCN4 dimerization domain partially restored the ability of this mutant to activate apoptosis (19%; Table II), suggesting that it is oligomerization and not the carboxy-terminus *per se* that is important for function. The p53–GCN4 mutant is somewhat less efficient than wild-type p53 in this assay, at least in the time frame we examined, perhaps because it can only dimerize as opposed to tetramerizing like wild-type p53.

To detect amino-terminal deletion proteins, the antibody used for other p53 derivatives could not be used, as it recognizes an amino-terminal epitope. Thus while optimizing detection of these amino-terminal mutants, we performed co-injection experiments of these derivatives with the β-galactosidase marker plasmid and detected injected cells with antibodies to β-galactosidase. The p53(80–393) protein failed to induce cell death, reinforcing the importance of the amino-terminus for p53 function (5%; Table II). As with G_1 arrest, a fusion of the p53(80–393) mutant to the VP16 activation domain resulted in a protein competent for inducing apoptosis (29%; Table II). Importantly, a control p53–VP16 fusion carrying the 175 mutation, which disrupts wild-type p53 structure, was unable to induce cell death (5%; Table II). Similar results were obtained by injection of p53(80–393) or p53–VP16 alone without the β-galactosidase marker plasmid and direct detection of p53 (Table II). These findings indicate that p53-mediated transcriptional activation or repression, rather than some other function of the amino-terminus, is important for apoptosis. To examine the importance of transcriptional repression, we tested the p53(100–393) fusion to VP16, which is defective in transactivation function but retains repression function (Pietenpol *et al.*, 1994). This mutant was incapable of inducing apoptosis in our assay, indicating that repression is not sufficient for the induction of cell death (2%; Table II). These data indicate that transcriptional activation by p53 is necessary and sufficient for the induction of apoptosis as well as for G_1 arrest.

To confirm the significance of the transcriptional activation domain, several point mutants defective in transcriptional activation were assayed for their ability to induce apoptosis. p53(22–23) is a double point mutant in the conserved activation domain that reduces activation function to 8% and 5% of wild-type on synthetic reporters containing sites from the mdm-2 and muscle creatine phosphokinase promoters, respectively (Lin *et al.*, 1994). The p53(14–19) and p53(23) mutants are also somewhat compromised in their ability to transactivate these p53-responsive promoters, but less so than p53(22–23); p53(14–19) has 41% and 56% of wild-type transcriptional activity, while p53(23) has 74% and 22% of wild-type transcriptional activity. Interestingly, injection of any of these mutants into E1A-expressing cells did not induce apoptosis above background levels, indicating that full transcriptional activity is critical for inducing cell death. This is consistent with the inactivity of the p53(1–343) protein in this apoptosis assay. Since the endogenous promoters activated during cell death are not known, it is possible that the activity of these mutants is more strongly affected on those promoters than is indicated from experiments using synthetic promoters.

p21 is dispensable for E1A-induced apoptosis

Having established that p53-mediated transcriptional activation is critical not only for G_1 arrest, but also for apoptosis, we sought to determine the importance of the p53 downstream target p21. p21 has been shown to be important for irradiation-induced G_1 arrest by p53 (Brugarolas *et al.*, 1995; Deng *et al.*, 1995; Waldman *et al.*, 1995). In MEFs, p21 is one component of the G_1 arrest response. Although dispensable for γ-irradiation-induced p53-dependent cell death in mouse intestinal crypts (Brugarolas *et al.*, 1995) and thymocytes (Deng *et al.*, 1995), its role in oncogene-mediated cell death was unclear. Thus, we tested the requirement for p21 in E1A-induced cell death. To this end, wild-type, *p53–/–* and *p21–/–* MEFs were infected with an E1A-expressing retrovirus. Cells were serum starved and examined by trypan blue exclusion over the course of 72 h, to determine the incidence of cell death (Figure 5). This p53-dependent loss of viability has been shown previously to be due to apoptosis (Lowe *et al.*, 1993, 1994b). As expected, exten-

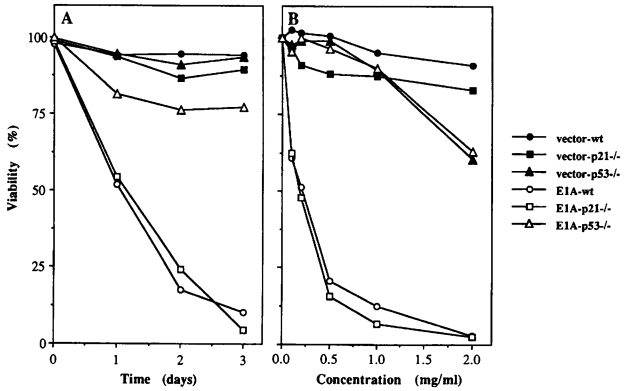


Fig. 5. Oncogene-induced cell death occurs in the absence of p21. Wild-type and p21-deficient mouse embryo fibroblasts expressing E1A undergo apoptosis upon serum starvation or treatment with adriamycin. Wild-type, *p53–/–* and *p21–/–* mouse embryo fibroblasts infected with either an empty vector-containing retrovirus or an E1A-expressing retrovirus were serum starved for varying lengths of time or treated with varying doses of adriamycin for 18 h. Viable cells, based on counting at least 200 cells by trypan blue exclusion at each point, are plotted. The graphs represent the average of data from two independent infections.

sive cell death was observed with wild-type cells but not *p53–/–* cells (Figure 5A). Importantly, the levels of apoptosis observed in the wild-type and *p21–/–* cells were indistinguishable, indicating that p21 is dispensable for oncogene-mediated cell death. Similar results were obtained upon treatment of cells with the chemotherapeutic agent adriamycin (Figure 5B). This cell death was dependent on the presence of E1A, as cells infected with a non-recombinant retrovirus did not undergo apoptosis in response to serum starvation or adriamycin treatment (Figure 5A and B). These data demonstrate that although p53 induces G_1 arrest and apoptosis by activating transcription, it does not require induction of precisely the same set of target genes, indicating that at least parts of these pathways are distinct.

Discussion

We have used a single cell microinjection assay and a battery of mutants, including both loss-of-function and gain-of-function mutants, to demonstrate conclusively that transcriptional activity of p53 is necessary and sufficient for both its G_1 arrest and apoptotic activities. Chimeric activator proteins in which either the activation domain of p53 was replaced with that of VP16 or the oligomerization domain was replaced with that of GCN4 substituted for wild-type p53 function, arguing strongly that oligomerization and activation, rather than the amino- and carboxy-termini of p53 *per se*, are important for both p53 functions. These findings are important given that p53 has been shown to interact via its amino- or carboxy-terminus with various other proteins without clear functional significance.

Although p53 generally is believed to mediate G_1 arrest by the activation of specific genes, this mechanism is not fully understood. p21 is clearly a component of the p53-mediated G_1 arrest in response to DNA-damaging agents (Brugarolas *et al.*, 1995; Deng *et al.*, 1995; Waldman *et al.*, 1995). However, at least in MEFs, the absence of p21 only partially impairs arrest; therefore, at least one other component is also important. The fact that p53–VP16 can substitute for wild-type p53 in this assay suggests that transcriptional function of p53 is sufficient for G_1 arrest. It will be interesting to determine whether the p53-responsive *GADD45* gene is another necessary component of the G_1 arrest response, or if some novel gene is involved.

In contrast, the mechanism of p53-mediated cell death has been widely disputed due to disparate findings that are difficult to reconcile. In two instances it was shown that transcription and translation inhibitors did not impair the ability of p53 to induce apoptosis, implicating another role for p53 in this process (Caelles *et al.*, 1994; Wagner *et al.*, 1994). It is unclear if these results were due to a peculiarity of the immortalized cell lines studied, or if these inhibitors were incompletely effective at blocking transcription and translation. These findings were supported by the results of Haupt *et al.* (1995) who showed that a truncation of p53 (dl214) as well as the p53(22–23) mutant described here, both of which are transcriptionally inactive, can nonetheless induce apoptosis in HeLa cells, albeit with slower kinetics. For these experiments, it is difficult to be sure that the observed effect was not due to endogenous p53 protein in HeLa cells. It is possible that rather than measuring the direct effect of a mutant, these assays assessed the ability of the mutant p53 to compete for p53 binding proteins and thus indirectly activate wild-type p53. For example, the mutants used could have bound the human papilloma virus E6 protein, which normally inhibits p53 in HeLa cells, and thus their overexpression would indirectly have activated the wild-type protein. Moreover, neither of these studies suggested an alternative mechanism for p53 action. Finally, Sabbatini *et al.* (1995) used the p53(22–23) mutant described here and showed that apoptosis was compromised but not abolished. In this case, endogenous p53 was also present, which again potentially clouds the interpretation of the data. Additionally, as this study focused only on the loss of the cell death function and not on how it could be restored, it was unclear whether activation, repression or some other amino-terminal function of p53 was involved in apoptosis.

The system we have used is highly physiological. E1A-expressing fibroblasts have been shown to undergo apoptosis *in vitro*, in a p53-dependent manner, upon serum starvation or treatment with chemotherapeutic agents (Lowe *et al.*, 1993, 1994b). Furthermore, these cells form tumors when transplanted into nude mice, and these tumors regress upon treatment with ionizing radiation, due to p53-mediated cell death (Lowe *et al.*, 1994a). Thus, this system represents a model system for cancer development and therapy. Using this system, we have found that transcriptional transactivation, rather than repression or other unspecified activities of p53, is important for cell death. In addition, our data are consistent with the spectrum of mutations in p53 alleles derived from tumors (Hollstein *et al.*, 1991). Since both G_1 arrest and apoptotic activities are involved in tumor suppression, and transcription is required for these functions, it is clear why disruption of sequence-specific DNA binding is selected for during tumorigenesis.

In addition, our experiments show no evidence for transcription-independent mechanisms. For example, the 273 R-H mutant has been shown by a number of criteria

to retain its native structure. Furthermore, the crystal structure of p53 indicates that this residue is involved in a critical phosphate backbone contact, rather than overall stability of the protein, as is residue 175 (Cho *et al.*, 1994). If there were a transcription-independent mechanism, this mutant and quite likely some of the engineered point mutants would show some apoptosis-inducing activity.

The p53-dependent apoptosis induced by oncogenes such as E1A has been proposed to result from a clash in signals simultaneously instructing the cell to grow and arrest in G_1 (reviewed in Fisher, 1994). Support for this idea has come from experiments which show that blocking the apoptotic pathway, either by providing survival factors or by expressing the bcl-2 apoptosis inhibitor, unmasks a p53-dependent G_1 arrest (Canman *et al.*, 1995; Guillouf *et al.*, 1995). Also, the requirement for p53-mediated transactivation in apoptosis is consistent with p53 acting to perform its usual G_1 arrest function. As it is known that p21 is an important component of irradiation-induced p53-dependent G_1 arrest (Brugarolas *et al.*, 1995; Deng *et al.*, 1995; Waldman *et al.*, 1995), we sought to determine if E1A-induced apoptosis also depended on p21. We demonstrate that p21 is completely dispensable for p53-dependent apoptosis, which implies that p53 is not simply acting to arrest cells during apoptosis, but is performing some function distinct from that which is required for G_1 arrest. That there are different target genes for growth arrest and apoptosis is supported by the finding that the p53(1–343) oligomerization-defective mutant can function in G_1 arrest, but is inactive in apoptosis. These data suggest that genes involved in G_1 arrest can be activated by monomeric p53, while genes involved in apoptosis cannot. An obvious next step is to identify novel targets of p53 involved in cell death in this system. One potential candidate for a mediator of cell death is the Bax protein, which is both directly activated by p53 and known to induce apoptosis (Oltvai *et al.*, 1993; Miyashita and Reed, 1995). However, Bax has been found to be dispensable for p53-mediated cell death in irradiated thymocytes (Knudson *et al.*, 1995). Given the importance of p53-mediated apoptosis in tumor suppression, it is clear that understanding the mechanisms of p53-mediated apoptosis and determining the targets of p53 in cell death will ultimately be important for the design of novel cancer therapies.

Materials and methods

G_1 arrest assays

MEFs were prepared from *p53–/–* embryos, as described (Livingstone *et al.*, 1992). *p53–/–* MEFs (passage 3–6) were plated at ~50% density on glass coverslips and allowed to adhere overnight. Cells were then serum starved by rinsing with phosphate-buffered saline (PBS) and transferring to medium containing 0.25% fetal calf serum (FCS) for 24–48 h. Expression plasmids encoding the various p53 mutants in the pCEP4 vector (Pietenpol *et al.*, 1994) at 25 μg/ml and cytomegalovirus (CMV) β-galactosidase at 5 μg/ml in PBS were then microinjected into the nuclei of cells using an Eppendorf microinjector and micromanipulator. Immediately after injection, cells were placed in DME–HEPES containing 10% FCS, 3 μg/ml BrdU and 0.3 μg/ml fluorodeoxyuridine (FdU) (Sigma). After ~18 h, cells were processed for immunofluorescence as follows. Cells were fixed in 2–4% paraformaldehyde for 15 min, washed with PBS, permeabilized with acetone:methanol (1:1) for 2 min, and washed again with PBS. Blocking solution consisting of 5% goat serum and 0.1% Triton X-100 in PBS was added to cells for at least 15 min. Cells were incubated with rabbit anti-β-galactosidase antibodies (dilution 1:50, 5 Prime-3 Prime, Inc.) for 30–60 min at 37°C, washed twice with PBS and then incubated with rhodamine-conjugated donkey anti-rabbit antibodies (1:250 dilution, Jackson Immunoresearch Laboratories) for 30–60 min at 37°C. Cells were washed with PBS, incubated with DAPI (0.1 μg/ml) for 5 min, and washed with PBS again. Cells were post-fixed with 2% paraformaldehyde, washed with PBS and then incubated for 10 min in 1.5 M HCl. After several washes with PBS, cells were incubated with mouse anti-BrdU antibodies (1:50, Becton-Dickinson) for ~60 min at 37°C, and washed again with PBS. Cells were incubated with FITC-conjugated donkey anti-mouse antibodies (1:250, Jackson Immunoresearch Laboratories) for 30–60 min at 37°C, washed several times with PBS and mounted on glass slides with Mowiol.

p21 immunostaining

To determine if various p53 mutants could induce p21, *p53–/–* MEFs were injected with 25 μg/ml of p53 expression constructs and 5 μg/ml of CMV β-galactosidase. At 6–7 h after injection, cells were processed for immunofluorescence, as described above, using mouse anti-p53 and rabbit anti-p21 (1:60, Brugarolas *et al.*, 1995). To detect p53 proteins containing the amino-terminus, a cocktail of antibodies 1801 and 1620 (1:25 each, Oncogene Science) was used. To detect p53 proteins lacking the amino-terminus, a cocktail of antibodies 240 (1:25, Oncogene Science), 122 (1:25, Boehringer-Mannheim) and G59-12 (1:25, Pharmingen) was used.

Cell death assays

E1A-expressing MEFs were prepared by infecting *p53–/–* MEFs with a retrovirus containing the E1A 12S gene product. The details of this procedure will be described elsewhere (S.W.Lowe, in preparation). For microinjections, cells were plated on poly-D-lysine-coated coverslips. Cells were injected at ~50% confluence with p53 expression plasmids at 25 μg/ml, as described above. After injection, cells were starved by placing them in DME–HEPES with 0.25% FCS. Cells were processed for immunofluorescence and p53 detected as described above. In addition to p53 antibodies, a GAL4-VP16 polyclonal antiserum was used to detect VP16 fusions (1:25, M.Carey and I.Sadowski, unpublished data). In a few cases, cells were injected with p53 expression plasmids at 25 μg/ml and CMV β-galactosidase at 5 μg/ml, and identified by anti-β-galactosidase immunostaining as described above. Expression of p53 mutants co-injected with a β-galactosidase marker was confirmed by direct p53 immunostaining. TUNEL analysis was performed by fixing cells for 15 min in 4% paraformaldehyde, permeabilizing with 0.1% Triton X-100, washing with PBS and incubating cells in 1× terminal deoxynucleotidyltransferase (TdT) buffer, 300 U/ml TdT (Gibco-BRL) and 40 μm biotin-dUTP (Boehringer-Mannheim) for 60 min at 37°C. Cells were then washed with PBS. p53 was detected as described above, and TUNEL-positive cells were detected by incubating cells with rhodamine-conjugated streptavidin (1:140, Jackson Immunoresearch Laboratories) for 30 min at 37°C.

p21–/– fibroblasts were infected with the E1A 12S-expressing retrovirus (to be described, S.W.Lowe, in preparation). Infected cells represent a population of cells, not a clone, and were used within a few days of infection. Two separate populations of *p21–/–* fibroblasts were tested. Cells were serum starved for 24–72 h or treated with varying doses of adriamycin (dissolved in water, Sigma) for 18 h. Cell viability was assessed by trypan blue exclusion, with at least 200 cells counted for each determination.

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References

Brugarolas,J., Chandrasekaran,C., Gordon,J.I., Beach,D., Jacks,T. and Hannon,G. (1995) Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature*, **377**, 552–557.

Caelles,C., Helmberg,A. and Karin,M. (1994) p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. *Nature*, **370**, 220–223.

Canman,C.E., Gilmer,T.M., Coutts,S.B. and Kastan,M.B. (1995) Growth factor modulation of p53-mediated growth arrest versus apoptosis. *Genes Dev.*, **9**, 600–611.

Cho,Y., Gorina,S., Jeffrey,P.D. and Pavletich,N.P. (1994) Crystal structure of a p53 tumor suppressor–DNA complex: understanding tumorigenic mutations. *Science*, **265**, 346–355.

Debbas,M. and White,E. (1993) Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes Dev.*, **7**, 546–554.

Deng,C., Zhang,P., Harper,J.W., Elledge,S. and Leder,P. (1995) Mice lacking $p21^{CIP1/WAF1}$ undergo normal development, but are defective in G1 checkpoint control. *Cell*, **82**, 675–684.

Donehower,L.A., Harvey,M., Slagle,B.L., McArthur,M.J., Montgomery, C.A., Butel,J.S. and Bradley,A. (1992) Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature*, **356**, 215–221.

Dulic,V., Kaufmann,W.K., Wilson,S.J., Tlsty,T.D., Lees,E., Harper,J.W., Elledge,S.J. and Reed,S.I. (1994) p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell*, **76**, 1013–1023.

El-Deiry,W.S., Tokino,T., Velculescu,V.E., Levy,D.B., Parsons,R., Trent, J.M., Lin,D., Mercer,W.E., Kinzler,K. and Vogelstein,B. (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825.

Fisher,D. (1994) Apoptosis in cancer therapy: crossing the threshold. *Cell*, **78**, 539–542.

Friend,S. (1994) p53: a glimpse at the puppet behind the shadow play. *Science*, **265**, 334–335.

Guillouf,C., Grana,X., Selvakumaran,M., De Luca,A., Giordano,A., Hoffman,B. and Liebermann,D.A. (1995) Dissection of the genetic programs of p53-mediated G1 growth arrest and apoptosis: blocking p53-induced apoptosis unmasks G1 arrest. *Blood*, **85**, 2691–2698.

Haffner,R. and Oren,M. (1995) Biochemical properties and biological effects of p53. *Curr. Opin. Genet. Dev.*, **5**, 84–90.

Harper,J.W., Adami,G.R., Wei,N., Keyomarsi,K. and Elledge,S.J. (1993) The p21 Cdk-interacting protein CIP1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, **75**, 805–816.

Haupt,Y., Rowan,S., Shaulian,E., Vousden,K.H. and Oren,M. (1995) Induction of apoptosis in HeLa cells by *trans*-activation-deficient p53. *Genes Dev.*, **9**, 2170–2183.

Hollstein,M., Sidranksy,D., Vogelstein,B. and Harris,C.C. (1991) p53 mutations in human cancers. *Science*, **253**, 49–53.

Jacks,T., Remington,L., Williams,B.O., Schmitt,E.M., Halachmi,S., Bronson,R.T. and Weinberg,R.A. (1994) Tumor spectrum analysis in p53-mutant mice. *Curr. Biol.*, **4**, 1–7.

Kastan,M., Zhan,Q., El-Deiry,W.S., Carrier,F., Jacks,T., Walsh,W.V., Plunkett,B.S., Vogelstein,B. and Fornace,A.J.,Jr (1992) A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell*, **71**, 587–597.

Knudson,C.M., Tung,K.S.K., Tourtellotte,W.G., Brown,G.A.J. and Korsmeyer,S.J. (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science*, **270**, 96–99.

Levine,A.J. (1993) The tumor suppressor genes. *Annu. Rev. Biochem.*, **62**, 623–651.

Lin,J., Chen,J., Elenbaas,B. and Levine,A.J. (1994) Several hydrophobic amino acids in the p53 amino-terminal domain are required for transcriptional activation, binding to mdm-2, and the adenovirus 5 E1B 55-kD protein. *Genes Dev.*, **8**, 1235–1246.

Livingstone,L.R., White,A., Sprouse,J., Livanos,E., Jacks,T. and Tlsty,T.D. (1992) Altered cell cycle arrest and gene amplification potential accompany loss of wild-type p53. *Cell*, **70**, 923–935.

Lowe,S.W. (1995) Cancer therapy and p53. *Curr. Opin. Oncol.*, **7**, 547–553.

Lowe,S.W. and Ruley,H.E. (1993) Stabilization of the p53 tumor suppressor is induced by adenovirus-5 E1A and accompanies apoptosis. *Genes Dev.*, **7**, 535–545.

Lowe,S.W., Ruley,H.E., Jacks,T. and Housman,D.E. (1993) p53-dependent apoptosis modulates the cytoxicity of anticancer agents. *Cell*, **74**, 957–967.

Lowe,S.W., Bodis,S., McClatchey,A., Remington,L., Ruley,H.E., Fisher,D.E., Housman,D.E. and Jacks,T. (1994a) p53 status and the efficacy of cancer therapy *in vivo*. *Science*, **266**, 807–810.

Lowe,S.W., Jacks,T., Housman,D.E. and Ruley,H.E. (1994b) Abrogation of oncogene-associated apoptosis allows transformation of p53-deficient cells. *Proc. Natl Acad. Sci. USA*, **91**, 2026–2030.

Malkin,D. (1993) p53 and the Li–Fraumeni syndrome. *Cancer Genet. Cytogenet.*, **66**, 83–92.

Miyashita,T. and Reed,J.C. (1995) Tumor suppressor p53 is a direct transcriptional activator of the human *bax* gene. *Cell*, **80**, 293–299.

Oliner,J.D. (1993) Discerning the function of p53 by examining its molecular interactions. *BioEssays*, **15**, 703–707.

Oltvai,Z.N., Milliman,C.L. and Korsmeyer,S.J. (1993) Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerate programmed cell death. *Cell*, **74**, 609–619.

Pietenpol,J.A., Tokino,T., Thiagalingam,S., El-Deiry,W., Kinzler,K.W. and Vogelstein,B. (1994) Sequence-specific transcriptional activation is essential for growth suppression by p53. *Proc. Natl Acad. Sci. USA*, **91**, 1998–2002.

Prives,C. (1994) How loops, β sheets, and α helices help us to understand p53. *Cell*, **78**, 543–546.

Purdie,C.A., Harrison,D.J., Peter,A., Dobbie,L., White,S., Howie,S.E.M., Salter,D.M., Bird,C.C., Wyllie,A.H., Hooper,M.L. and Clarke,A.R. (1994) Tumour incidence, spectrum and ploidy in mice with a large deletion in the p53 gene. *Oncogene*, **9**, 603–609.

Sabbatini,P., Lin,J., Levine,A.J. and White,E. (1995) Essential role for p53-mediated transcription in E1A-induced apoptosis. *Genes Dev.*, **9**, 2184–2192.

Smith,M.L., Chen,I.-T., Zhan,Q., Bae,I., Chen,C.-Y., Gilmer,T.M., Kastan,M.B., O'Connor,P.M. and Fornace,A.J.,Jr (1994) Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. *Science*, **266**, 1376–1380.

Stenger,J.E., Tegtmeyer,P., Mayr,G.A., Reed,M., Wang,Y., Wang,P., Hough,P.V.C. and Mastrangelo,I.A. (1994) p53 oligomerization and DNA looping are linked with transcriptional activation. *EMBO J.*, **13**, 6011–6020.

Subler,M.A., Martin,D.W. and Deb,S. (1994) Overlapping domains on the p53 protein regulate its transcriptional activation and repression functions. *Oncogene*, **9**, 1351–1359.

Tsukada,T., Tomooka,Y., Takai,S., Ueda,Y., Nishikawa,S., Yagi,T., Tokunaga,T., Takeda,N., Suda,Y., Abe,S., Matsuo,I., Ikawa,Y. and Aizawa,S. (1994) Enhanced cell proliferative potential in culture of cells from p53-deficient mice. *Oncogene*, **9**, 603–609.

Wagner,A.J., Kokontis,J.M. and Hay,N. (1994) Myc-mediated apoptosis requires wild-type p53 in a manner independent of cell cycle arrest and the ability of p53 to induce p21 WAF1/CIP1. *Genes Dev.*, **8**, 2817–2830.

Waldman,T., Kinzler,K.W. and Vogelstein,B. (1995) p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res.*, **55**, 5187–5190.

Wang,X.W., Yeh,H., Schaeffer,L., Roy,R., Moncollin,V., Egly,J.-M., Wang,Z., Friedberg,E.C., Evans,M.K., Taffe,B.G., Bohr,V.A., Weeda,G., Hoeijmakers,J.H.J., Forrester,K. and Harris,C.C. (1995) p53 modulation of TFIIH-associated nucleotide excision repair activity. *Nature Genet.*, **10**, 188–195.

Xiong,Y., Hannon,G.J., Zhang,H., Casso,D., Kobayashi,R. and Beach,D. (1993) p21 is a universal inhibitor of cyclin kinases. *Science*, **366**, 701–704.

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