

# The DNA-binding and Transcription-Activation Abilities of p53 Are Necessary but not Sufficient for Its Antiproliferation Function<sup>1</sup>

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## Abstract

**Normal p53 protein suppresses cell proliferation and *ras* oncogene-induced cell transformation. Missense mutations in the middle conserved conformational domain of p53 decrease its antiproliferation function. In this work, we studied the requirement of the NH<sub>2</sub>- and COOH-terminal regions of p53 in its antiproliferation function using two independent assays, growth of chronic myelogenous leukemia K562 cells on methylcellulose semisolid medium and *ras* oncogene-induced focus formation of rat fibroblast cells (Rat-1). We found that deletion of 80 or 159 amino acids from the NH<sub>2</sub>-terminus and deletion of 67 amino acids from the COOH-terminus of p53 drastically reduced the antiproliferation function of p53. However, the COOH-terminal deletion mutant is capable of binding to a p53 DNA-binding element, p53CON (GGACATGCC-GGGCATGTCC), and of activating p53CON-mediated transcription. These results suggest that p53's abilities to bind p53CON and activate transcription are not sufficient for its antiproliferation function and that p53CON-regulated genes may not be growth suppressive.**

## Introduction

Among the most common genetic changes identified in tumor cells are the p53 missense mutations (1). Cells containing mutated p53 lose the ability to restrict cell growth at G<sub>1</sub> when exposed to radiation or DNA-damaging drugs (2). Thus, damaged genes, which confer upon the cells a selective growth advantage, may not have sufficient time for repair before DNA synthesis, causing the damage to be propagated and accumulated in the cells. Inactivation of tumor suppressor genes like the *p53* gene and activation of oncogenes have been shown to correlate with tumorigenesis. The function of p53 as an antiproliferation factor has been illustrated in experiments in which the introduction of wild-type *p53* into tumor cells resulted in the restoration

of cell cycle arrest (3, 4), inhibition of cell proliferation (5–7), and inhibition of activated *ras* oncogene-induced cell transformation (7, 8).

Recent evidence demonstrates that the p53 protein is a transcriptional factor (9–12). A simple hypothesis which explains the molecular basis of the antiproliferative function of p53 is that p53 activates genes in antiproliferation pathways and/or inactivates genes in proliferation pathways. Consistent with this hypothesis is the finding that the p53 protein can activate the transcription of the DNA damage-inducible gene *gadd45*. A failure in or delay of p53 induction may in turn cause failure of *gadd45* gene activation, which may be responsible for the radiation sensitivity of patients with ataxia-telangiectasia (13). p53 also activates the expression of the *WAF1* (or *Cip1*) gene, the product of which forms complexes with cyclin and cyclin-associated kinases and inhibits cdk 2 kinase, thus inhibiting cell proliferation (14, 15). However, p53-regulated genes are not only involved in the inhibition of cell growth. For example, p53 activates the expression of *mdm2*, a transforming oncogene which in turn negatively regulates the transcription activity of p53 (16–18).

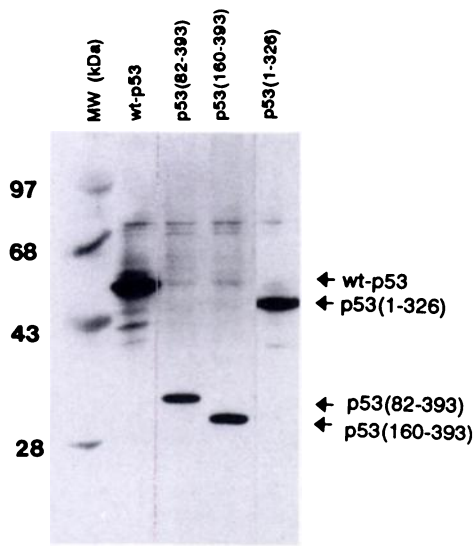
The p53 protein has three functional domains. The NH<sub>2</sub>-terminus contains the transcription activation region (9, 19). The COOH-terminus is required for both specific (20) and nonspecific (21) DNA binding. The COOH-terminus also contains two oligomerization motifs responsible for forming p53 oligomers (22). The central conformational region of the p53 protein is hydrophobic, and mutations in this region often expose a unique epitope which is recognized by the monoclonal antibody PAb240. Concomitantly, an epitope normally exposed in wild-type p53 is lost (23, 24). Recently, this region was found to bind DNA directly (25–28).

The great majority of missense mutations of p53 in human tumor cells are localized within the central conformational domain of p53 (1). Mutations in this region produce a protein defective in the antiproliferation functions of p53 (5–8). In addition, mutations in this region often abolish or weaken the DNA-binding and transactivation activities of p53 (9, 11, 12, 29–33). In this study, we examined in two assays whether the NH<sub>2</sub>- and COOH-termini of the p53 protein are required for its antiproliferation function. We attempted to correlate the DNA-binding and transactivation abilities of p53 with its antiproliferation function. Our results showed that NH<sub>2</sub>-terminal and COOH-terminal deletion mutants abolished or weakened the ability of p53 to suppress cell proliferation and oncogene-induced focus formation. These data suggest that the two termini of p53 are required for its antiproliferation function. The data further show that the COOH-terminal deletion mutant can bind to the p53-binding DNA element p53CON and can activate the transcription of a reporter gene through p53CON. These results indicate that the DNA-binding of p53 and transcription activation abilities are not sufficient for its

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**Fig. 1.** Expression of wild-type p53 (wt-p53) and deletion mutants in transfected K562 cells. K562 cells were transfected with expression vectors encoding wild-type p53, the COOH-terminal deletion mutant p53(1-326), and the NH<sub>2</sub>-terminal deletion mutants p53(82-393) and p53(160-393). After 48 h, the cells were labeled with [<sup>35</sup>S]methionine, and protein was extracted. The expression of p53 was analyzed by immunoprecipitation with anti-p53 monoclonal antibody PAb421 (for wild-type p53 and the two NH<sub>2</sub>-terminal deletion mutants) or PAb1801 (for the COOH-terminal deletion mutant). Molecular weight markers of the indicated masses are shown in the left column.

antiproliferation function and that p53 may also activate genes outside the growth-suppression pathway.

## Results

**Both the NH<sub>2</sub>-Terminus and COOH-Terminus of p53 Are Required for Its Antiproliferative Function.** Mutations of p53 in the central conformational region result in a mutant p53 which is defective in suppressing cell proliferation; therefore, we began this work by asking the question: Are the NH<sub>2</sub>-terminus and COOH-terminus needed for the antiproliferation function of p53?

One COOH-terminal deletion mutant, p53(1-326), which has 67 amino acids deleted from the COOH-terminus, and two NH<sub>2</sub>-terminal deletion mutants, p53(82-393) and p53(160-393), which, respectively, have 80 and 159 amino acids deleted from the NH<sub>2</sub>-terminus, were analyzed for their ability to suppress cell proliferation. Two assays were used: the methylcellulose colony assay of K562 cells and the focus-formation assay of Rat-1 fibroblasts. Wild-type p53 and several missense mutants were used as controls.

In the colony formation assay, each p53 expression vector was transfected into p53-null K562 cells. The expression of p53 proteins was confirmed 2 days after transfection by immunoprecipitation (Fig. 1). Monoclonal antibody PAb421, which recognizes an epitope on the COOH-terminus of p53, was used in the precipitation of the two NH<sub>2</sub>-terminal deletion mutants, p53(82-393) and p53(160-393). PAb1801, which recognizes an epitope on the NH<sub>2</sub>-terminal region of p53, was used to precipitate the COOH-terminal deletion mutant, p53(1-326). The mutant p53(1-326) but not p53(82-393) or

**Table 1** Colony formation of transfected K562 cells

Transfectant	Colonies/dish
Wild-type p53	10, 11, 17
175His	149, 164, 244
248Trp	248, 190
CMV.neo	245, 235
p53(1-326) + CMV.neo	187, 130
p53(160-393) + CMV.neo	227, 204

**Table 2** Focus formation of transfected Rat-1 cells

Transfectant	Foci/dish
<i>ras</i>	394, 277, 294, 262, 353
Wild-type p53	0, 0, 0, 0, 0
248Trp	0, 1, 2, 0
p53(1-326)	0, 0, 0, 0, 0
p53(82-393)	0, 0, 0, 2, 3
p53(160-393)	0, 0, 2, 2, 3
Wild-type p53 + <i>ras</i>	29, 33, 28, 28
248Trp + <i>ras</i>	120, 132, 113, 91
p53(1-326) + <i>ras</i>	212, 265, 121, 109
p53(82-393) + <i>ras</i>	303, 356, 367, 348, 336
p53(160-393) + <i>ras</i>	413, 491, 569, 578

p53(160-393) can also be detected by anti-p53 monoclonal antibody DO-1 in Western blotting analyses (see below).

The transfected cells were mixed with methylcellulose and cultured in the presence of G418 (0.8 mg/ml) for 2 weeks. Only colonies that consisted of more than 50 cells were counted (Table 1). Very few colonies were produced when wild-type p53 was the transfectant, whereas many colonies grew when transfectants of missense mutants and the three deletion mutants were used. These results suggest that both the NH<sub>2</sub>- and COOH-termini of p53 are required in order for p53 to suppress the proliferation of K562 cells. It is interesting that the single-point mutations had deleterious effects as strong as or stronger than those of the terminal deletion mutations.

The ability of the deletion mutants to inhibit cell proliferation was also analyzed by the use of focus formation assays (Table 2). In these assays, each p53 vector was transfected into Rat-1 fibroblast cells either with or without the activated H-*ras* oncogene. The Rat-1 cells transfected with the activated H-*ras* oncogene alone lost contact inhibition and grew as multilayered foci (Fig. 2A). The wild-type and deletion mutants by themselves did not produce much focus formation. When wild-type p53 was cotransfected with the *ras* oncogene, p53 inhibited the *ras*-induced focus formation (Fig. 2B). In contrast, the deletion mutants had little effect on the colony formation induced by *ras*. Cotransfection of the NH<sub>2</sub>-terminal deletion mutants with *ras* either had no effect or increased the focus formation relative to that induced by *ras* alone (Fig. 2, C and D). Similarly, the COOH-terminal deletion mutant p53(1-326) did not inhibit focus formation markedly (Fig. 2E). However, the missense mutant 248Trp, when cotransfected with activated *ras* oncogene, allowed fewer foci than the three deletion p53 mutants. This result suggests that the deletion of either of the two termini affects the transformation suppressor function more than a point mutation does. Among the deletion mutants, the NH<sub>2</sub>-terminal deletion p53(160-

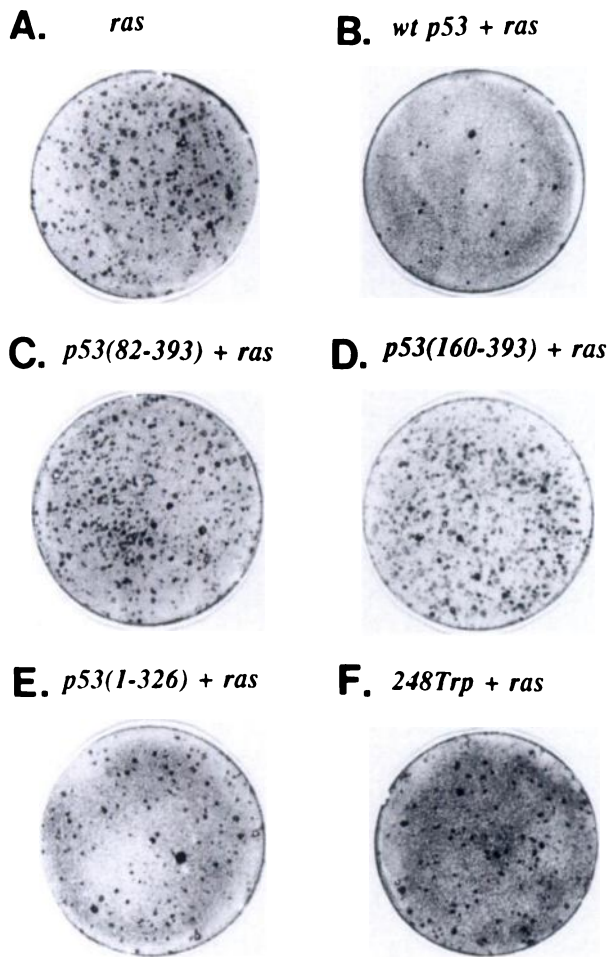


Fig. 2. Focus formation of Rat-1 fibroblasts in the presence of wild-type (wt) or mutant p53 and activated human H-ras oncogene.

393) affected the function most severely. That mutant had dominant transforming activity as evidenced by its producing more foci than *ras* itself.

**The COOH-terminal Deletion Mutant p53(1-326) Is Capable of Binding to DNA and Activating Transcription.** Since the NH<sub>2</sub>-terminal and COOH-terminal deletion mutants failed to inhibit cell proliferation, we anticipated that they would not bind DNA and activate transcription. To test this hypothesis, we studied the ability of the mutants to bind two previously identified p53-binding DNA elements, p53CON (12, 31) and RGC<sup>1</sup> (11, 12), by the mobility shift assay. As expected, no mutant bound the RGC element, and the NH<sub>2</sub>-terminal deletion mutants did not bind the p53CON element (data not shown). However, p53(1-326) did bind to p53CON in the presence of the anti-p53 monoclonal antibody PAb1801 (Fig. 3A).

We then analyzed the ability of p53(1-326) to activate p53CON-mediated transcription. The expression vector encoding p53(1-326) and the p53CON-Luc reporter gene

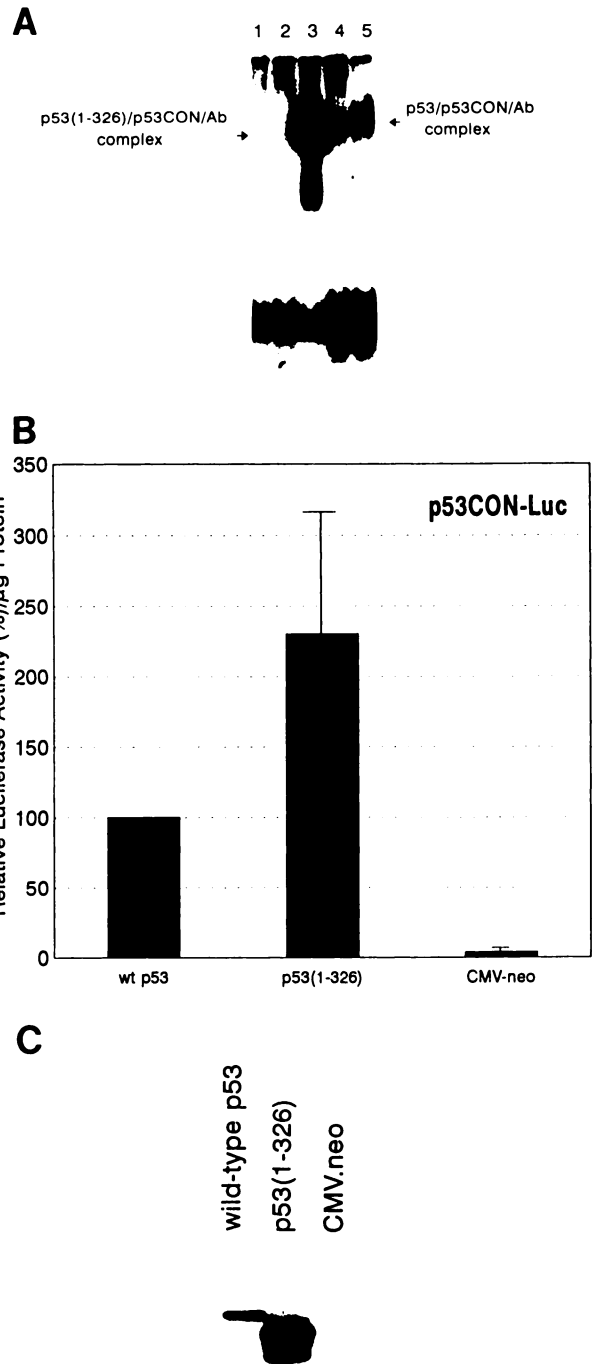


Fig. 3. The COOH-terminal deletion mutant p53(1-326) binds the p53CON DNA element and activates p53CON-mediated transcription. **A**, protein extract from cells transfected with p53(1-326) was mixed with p53CON probe in the presence of control antibody (Ab) PAb419 (Lane 1) or anti-p53 antibody PAb421 (Lane 2) or PAb1801 (Lane 3). Lane 4 is similar to Lane 3 except that 25 times excess unlabeled p53CON oligonucleotide was included as specific competitor. Lane 5 shows the positive control of wild-type (wt) p53 in the presence of PAb421. **B**, luciferase activity relative to that for wild-type p53 (results are the average for three independent transfections). **C**, Western blotting of wild-type p53 and mutant p53 proteins in the transfected cells.

<sup>1</sup> The abbreviations used are: RGC, ribosomal gene cluster; DMEM, Dulbecco's modified Eagle's medium.

were cotransfected into p53-null K562 cells. Luciferase activity (indicating transcription activation) was analyzed 48 h after transfection. Consistent with its DNA-binding ability, p53(1–326) also activated p53CON-mediated transcription (Fig. 3B). The p53 protein levels in the transfected cells were determined by Western blotting (Fig. 3C). Since we showed that p53(1–326) did not suppress cell proliferation, these results suggest that the ability of the COOH-terminal deletion mutant to bind to p53CON and to activate p53CON-mediated transcription are not sufficient to suppress cell proliferation.

## Discussion

Several conclusions can be derived from these data: (a) both the NH<sub>2</sub>-terminal and the COOH-terminal regions are required for the antiproliferation function of p53. Deletion of either domain dramatically reduces or totally abolishes this function; and (b) the deletion mutations abrogate the focus suppression function of p53 more than any p53 missense mutation tested. This finding may indicate that the two terminal regions are the effectors of p53; the middle conformational domain may be a regulator for the effectors on the NH<sub>2</sub>- or COOH-terminus. Therefore, it is not surprising that different point mutations in the conformational domain affect the activities of p53 in different manners, since each mutation may affect the two terminal regions differently. In addition, the effect of a point mutation on a terminus may be limited. Missense mutants may retain some activities of wild-type p53, such as DNA binding, transcription activation, binding to SV40 T-antigen, and even proliferation suppression. As a matter of fact, decreasing the level of a mutant p53 protein by expressing antisense p53 increases the growth rate and tumorigenicity of a human non-small cell lung cancer cell line (34), indicating that some missense p53 mutants may partially retain the tumor suppressor function.

The NH<sub>2</sub>-terminal effector region of p53 is believed to be the transcription activation domain, and this transcription activation may be required for suppressing cell proliferation. This hypothesis is supported by our findings that the two NH<sub>2</sub>-terminal deletion mutants failed to activate transcription mediated by the p53 DNA-binding elements p53CON and RGC.

In contrast, the COOH-terminal deletion mutant was capable of binding to p53CON and activating p53CON-mediated transcription. It appears that this COOH-terminal deletion mutant differentially activates transcription because of a second DNA-binding domain in the middle conserved region of p53 (25). Although the COOH-terminal deletion mutant retained some transactivation function, it was impaired compared with wild-type p53. The partial retention of the transactivation ability of p53(1–326) may explain why it produced fewer foci in collaboration with *ras* than the two NH<sub>2</sub>-terminal deletion mutants, in which the transactivation capacity was abolished. Thus, intact transcription activation function of p53 may be required for its growth-suppression function.

These data suggest that p53 may regulate genes in the growth-suppression pathway and in other pathways. Genes that contain the RGC element may contribute to growth inhibition; mutations of p53 have almost invariably inactivated RGC-mediated transcription, and these p53 mutants have a dominant negative effect on wild-type p53 in activating RGC (11, 29, 30, 35). Nevertheless, some p53 mis-

sense mutants and the COOH-terminal deletion mutant retain the ability to bind to the p53CON element and to activate p53CON-mediated transcription (31). Some p53 missense mutants show a dominant positive effect on wild-type p53 in transactivating p53CON-mediated transcription (35). p53CON thus may regulate genes in pathways other than those causing growth suppression. This hypothesis is strongly supported by our finding of a p53CON-like element in the first intron of the human *H-ras* oncogene. p53 can bind to it and activate the expression of a reporter gene under its control.<sup>4</sup>

It is an intriguing hypothesis that p53 may be involved in the regulation of diverse cellular pathways, especially both antiproliferation and proliferation pathways. This hypothesis is consistent with the fact that p53 can be a tumor suppressor gene (when it is of the wild-type) and an oncogene (when it is a mutant).

## Materials and Methods

**Cells and Plasmids.** K562 chronic myelogenous leukemia cells were obtained from the American Type Culture Collection and maintained in RPMI 1640 supplemented with 10% fetal calf serum. K562 cells do not express either p53 mRNA or p53 protein (31). The Rat-1 fibroblast cells were generously provided by M. Weil (The University of Texas M. D. Anderson Cancer Center) and maintained in DMEM supplemented with 10% fetal calf serum. Both cell lines were cultured in a 37°C incubator containing 5% CO<sub>2</sub>. p53 deletion mutants (36) were generously provided by Y. Shiio (The University of Tokyo). Mutant p53(1–326) had 67 amino acids deleted from the COOH-terminal, mutant p53(82–393) had 80 amino acids deleted from the NH<sub>2</sub>-terminus (the first amino acid, methionine, was retained), and mutant p53(160–393) had 159 amino acids deleted from the NH<sub>2</sub>-terminus. The *neo* gene is not present in these three vectors. Human p53 expression vectors were generously provided by B. Vogelstein (Johns Hopkins University School of Medicine). Each missense mutant is named according to the position of the mutated codon and the mutant amino acid. The expression vectors for human wild-type and missense p53 mutants are under the control of cytomegalovirus enhancer and contain a *neo* gene, conferring on them resistance to G418. The luciferase reporter plasmids Luc [containing basic heat shock protein 70 (HSP70) promoter] and p53CON-Luc (containing basic HSP70 promoter and one copy of the p53CON element) were provided by J. Shay (The University of Texas Southwestern Medical Center at Dallas; Ref. 12).

**Electroporation and Luciferase Assays.** Ten million exponentially growing K562 cells were mixed with 10 µg of p53 expression vector plus 15 µg of luciferase reporter plasmid in RPMI 1640 (no serum) at room temperature for 10 min and then were pulsed (600 µF, 420 V) using a BTX600 transfecter as described previously (35).

**Mobility Shift Assay.** Twenty µg of total protein from the luciferase assay was mixed with 1 ng of <sup>32</sup>P-end-labeled p53CON oligonucleotide for 20 min at room temperature as described previously (35). When indicated, 100 ng of anti-p53 antibody PAb421 or DO-1 (Oncogene Science,

<sup>4</sup> W. Zhang, G. S. Randhawa, J. Gau, C. D. McClain, X-Y. Guo, J. W. Shay, and A. B. Deisseroth. The first intron of human *H-ras* is regulated by p53, submitted for publication.

Inc., Manhasset, NY) was added to each mixture. All oligonucleotides were synthesized using a Cruachem PS240 automated DNA synthesizer. Equal amounts of sense and antisense were mixed in 0.1 M NaCl solution, heated to 85°C for 10 min, and then gradually cooled to room temperature to allow annealing before use. The end labeling of oligonucleotides was done according to the standard procedure. In competition experiments, a 50-fold excess of unlabeled p53CON or a *c-myc*-binding oligonucleotide (5'-CCCCACCACGTGGTGCCTGA-3') was included.

**Western Blotting.** Forty  $\mu$ g of protein extract was boiled in sample buffer (125 mM Tris-HCl (pH 6.8), 1% sodium dodecyl sulfate, 2%  $\beta$ -mercaptoethanol, and 0.01% bromophenol blue) for 5 min and loaded onto a 10% sodium dodecyl sulfate polyacrylamide gel. After electrophoresis overnight at 45 V, the protein was transferred to an Immobilon membrane (Millipore Corp., Bedford, MA), blocked using a blocking solution [50 mM Tris-HCl (pH 7.5), 0.9% NaCl, 3% nonfat milk, 0.2% bovine albumin, and 0.05% Tween 20] for 4 h, and then incubated overnight with anti-p53 antibody DO-1 in blocking solution (0.5  $\mu$ g/ml). The levels of p53 were measured using the enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL) according to the manufacturer's instructions.

**Methylcellulose Assay.** After electroporation, the cells were allowed to recover for 16–24 h. Viable cells ( $2 \times 10^4$ ) were mixed with 3 ml of liquid methylcellulose (Terry Fox Laboratory, Vancouver, British Columbia, Canada) containing 0.8 mg/ml G418 (GIBCO-BRL, Grand Island, NY). The cells were cultured in a humidified 5% CO<sub>2</sub> incubator at 37.5°C. After 2 weeks, colonies that consisted of more than 50 cells were counted.

**Focus Formation Assay.** The Rat-1 cells were seeded in 100-mm dishes containing DMEM supplemented with 10% fetal calf serum. When the cells reached 60–80% confluency, 5  $\mu$ g of activated *ras* expression vector (generously provided by M. Tainsky at M. D. Anderson Cancer Center) or 5  $\mu$ g of activated *ras* vector plus 5  $\mu$ g of either wild-type *p53* or missense *p53* mutant expression vector was transfected using the Lipofectin kit (BRL) according to the manufacturer's instructions. The medium was replaced every 5 days. After 2 weeks, the cells were stained for 30–60 s using 1% crystal violet in 20% ethanol and then were rinsed with water. The foci then were counted.

**Immunoprecipitation.** Forty-eight h after transfection, cells were washed twice with phosphate-buffered saline and then metabolically labeled for 2 h in a solution of 150  $\mu$ Ci/ml [<sup>35</sup>S]methionine, 10% dialyzed fetal calf serum, and 1% glutamine in methionine-free DMEM at 37.5°C in a 5% CO<sub>2</sub> incubator. The cells then were washed three times with phosphate-buffered saline, lysed, precleared, and immunoprecipitated using anti-p53 antibody PAb421 or PAb1801 (Oncogene Science) as described previously (37), except that the salt concentration was maintained at 100 mM.

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