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The EMBO Journal Vol.17 No.7 pp.1847–1859, 1998

Genetic selection of intragenic suppressor mutations that reverse the effect of common *p53* cancer mutations

Rainer K.Brachmann[1], Kexin Yu, Yolanda Eby, Nikola P.Pavletich[2] and Jef D.Boeke[3]

Department of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, Baltimore, MD 21205 and [2]Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA

[1]Present address: Division of Molecular Oncology, Departments of Medicine and Pathology, Washington University School of Medicine, St Louis, MO 63110, USA

[3]Corresponding author

Several lines of evidence suggest that the presence of the wild-type tumor suppressor gene *p53* in human cancers correlates well with successful anti-cancer therapy. Restoration of wild-type p53 function to cancer cells that have lost it might therefore improve treatment outcomes. Using a systematic yeast genetic approach, we selected second-site suppressor mutations that can overcome the deleterious effects of common *p53* cancer mutations in human cells. We identified several suppressor mutations for the V143A, G245S and R249S cancer mutations. The beneficial effects of these suppressor mutations were demonstrated using mammalian reporter gene and apoptosis assays. Further experiments showed that these suppressor mutations could override additional *p53* cancer mutations. The mechanisms of such suppressor mutations can be elucidated by structural studies, ultimately leading to a framework for the discovery of small molecules able to stabilize p53 mutants.

Keywords: human/*Saccharomyces cerevisiae*/tumor suppressor/yeast

Introduction

Reduction or elimination of the activity of the tumor suppressor protein p53 is a characteristic of more than half of all human cancers (Hollstein *et al.*, 1991; Caron de Fromentel and Soussi, 1992; Harris and Hollstein, 1993; Greenblatt *et al.*, 1994). Reduced p53 activity can result from the presence of abnormally high levels of host proteins (e.g. mdm-2), or viral proteins (e.g. high-risk human papilloma virus E6) (Vogelstein and Kinzler, 1992; Donehower and Bradley, 1993; Gottlieb and Oren, 1996; Neil *et al.*, 1997). However, in the majority of cancers, p53 inactivation is caused by missense mutations in one *p53* allele with concomitant loss-of-heterozygosity (Michalovitz *et al.*, 1991; Vogelstein and Kinzler, 1992; Donehower and Bradley, 1993; Levine, 1997). The unusually high frequency of *p53* missense mutations in human cancers can be explained by their dominant-negative effect. Interference with the remaining wild-type *p53* allele leads to decreased genetic stability, loss-of-heterozygosity and thus complete abrogation of p53 function (Michalovitz *et al.*, 1991; Vogelstein and Kinzler, 1992; Hann and Lane, 1995; Brachmann *et al.*, 1996; Ko and Prives, 1996). In addition, at least some of the same missense mutations may confer a gain-of-function phenotype (Gottlieb and Oren, 1996; Ko and Prives, 1996; Levine, 1997).

p53 is active as a homotetramer and exerts its tumor suppressor function mainly as a transcription factor by inducing, among others, genes that lead to G_1 arrest or apoptosis (Donehower and Bradley, 1993; Pietenpol *et al.*, 1994; Haffner and Oren, 1995; Gottlieb and Oren, 1996; Ko and Prives, 1996; Hansen and Oren, 1997; Levine, 1997). Apoptosis can also be induced by p53-dependent non-transcriptional mechanisms (Caelles *et al.*, 1994; Haupt *et al.*, 1995; White, 1996; Hansen and Oren, 1997). Reconstitution of wild-type p53 activity to cancers should thus be of substantial therapeutic benefit, an idea that is supported by several lines of evidence. First, cell lines with functional p53 are more sensitive to commonly used anti-cancer agents (Clarke *et al.*, 1993; Lowe *et al.*, 1993a,b, 1994). Cancer types that are rarely associated with *p53* mutations can be successfully treated even at advanced stages (Fisher, 1994; Lowe, 1995; Harris, 1996). Finally, clinical studies show that human cancers without *p53* mutations are more likely to be eradicated (Harris and Hollstein, 1993; Fisher, 1994; Bergh *et al.*, 1995; Lowe, 1995; Harris, 1996).

The restoration of wild-type p53 activity to cancer cells could theoretically be achieved in two ways: one could reintroduce wild-type p53, perhaps by gene therapy (Roth *et al.*, 1996), or one could restore wild-type function to the mutated p53 in tumors (Gibbs and Oliff, 1994; Lowe, 1995; Milner, 1995; Harris, 1996). In the second case, the presence of the mutant p53 protein could be used therapeutically in at least two ways. One approach would be to interfere with the C-terminal regulatory domain of p53 using antibodies (Halazonetis and Kandil, 1993; Hupp *et al.*, 1993; Abarzua *et al.*, 1995; Niewolik *et al.*, 1995) or peptides (Hupp *et al.*, 1995; Abarzua *et al.*, 1996; Selivanova *et al.*, 1997) to relieve the likely negative regulation of p53 activity. This approach could succeed with p53 mutants that retain residual activity so that their up-regulation may allow their activity to exceed the threshold required for biological effects. However, these potentially therapeutic macromolecules may be difficult to use in patients since they could conceivably activate mutant and wild-type p53 proteins indiscriminately, thus leading to unwanted side effects due to inappropriately exuberant apoptosis induced by wild-type p53 activity in normal tissues.

Another approach is pharmacologically to reverse the effects of tumorigenic mutations based on the structure of the p53 core domain. The vast majority of tumor-derived

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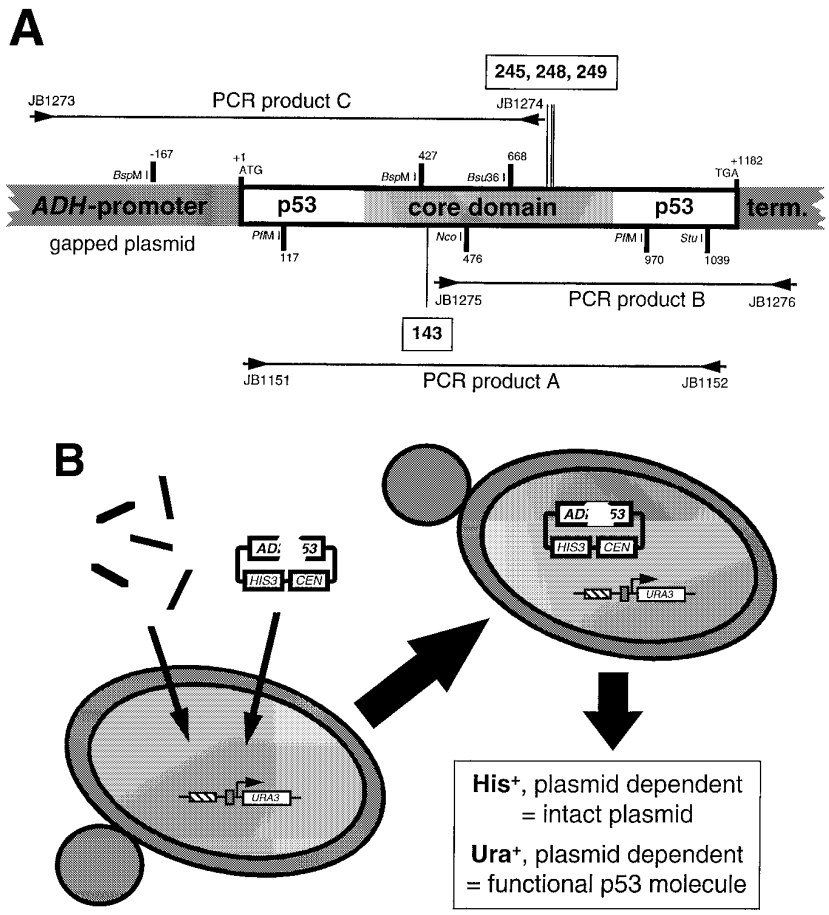

Fig. 1. Design of the PCR mutagenesis and yeast gap repair experiments. (**A**) In the first approach, the entire core domain of the *p53* ORF was mutagenized in several yeast plasmids expressing common cancer missense mutation versions of *p53* using primers JB1151 and JB1152 (PCR product A). The same plasmids were gapped with the restriction enzyme *Pfl*MI. This strategy incorporated the original mutation into the PCR product. In the second approach, regions adjacent to the *p53* mutations were PCR mutagenized with the intent of reducing reversion of the original *p53* mutations to the wild-type codon. For V143A, the downstream region was PCR amplified with primers JB1275 and JB1276 (PCR product B) and the expression plasmid gapped with an *Nco*I–*Stu*I digestion. For G245D, G245S, R248W and R249S the upstream region was PCR amplified with primers JB1273 and JB1274 (PCR product C) and the expression plasmids gapped with *Bsp*MI and *Bsu*36I. (**B**) The PCR products and gapped plasmids were designed to have overlapping segments at both ends (ranging from 38 to 335 nucleotides in length). Co-transformation of both into the yeast reporter strain led to very efficient repair of the gapped plasmids through homologous recombination with PCR products. The yeast transformants could then be easily assessed; those that grew on plates lacking histidine and uracil in a plasmid-dependent fashion had to contain an intact yeast expression plasmid (His^+) with an intact *p53* ORF encoding for a functional p53 molecule (Ura^+), potentially containing the original *p53* missense mutation and a new second-site suppressor mutation.

p53 mutations maps to the core DNA-binding domain (core domain) and invariably these mutations result in reduction or loss of DNA binding. Based on the crystal structure of the p53 core domain–DNA complex and biochemical data (Cho *et al.*, 1994), the tumor-derived mutations can be grouped into two classes; one maps to DNA-contacting residues and eliminates p53–DNA contacts (functional mutations), while the other, larger class affects amino acids important for the structural integrity of the DNA-binding domain. Failure of the latter class of mutants to bind DNA can be attributed to structural defects, ranging from small structural shifts to the global destabilization and unfolding of the p53 core domain (structural mutations). In principle, then, increasing the stability of the folded state of p53 or introducing additional p53–DNA contacts could restore functional activity to subsets of tumor-derived mutants.

Similarly, second-site suppressor mutations that either introduce additional DNA contacts or increase the stability of the folded state of the DNA-binding domain could restore function to mutant p53 molecules. The identification of the latter type of suppressor mutations would be of particular significance, as these may be able to suppress the large structural class of tumor-derived mutations, in effect acting as global suppressors, similar to those identified with model systems such as staphylococcal nuclease (Shortle and Lin, 1985).

An example of a second-site suppressor mutation that presumably introduces a new p53–DNA contact has been provided by Wieczorek *et al.* (1996), who relied on the crystal structure of the p53 core domain (Cho *et al.*, 1994) to predict suppressor mutations. This strategy of rational suppressor mutation design, although promising, could generate false predictions and will almost certainly be incomplete in scope.

We have taken a more systematic genetic approach in

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Table I. Independent intragenic suppressor mutations for the *p53* cancer mutations V143A, G245S and R249S

Original mutation	Identification of suppressor mutations					Confirmatory subcloning			Mammalian activity[a]	
	Suppressor amino acid changes(s)	Human cancer mutation	Suppressor nucleotide changes(s)	Original suppressor plasmid	Original suppressor strain	Yeast plasmid	Yeast strain	Mammalian plasmid	Reporter gene assays	Programmed cell death assay
V143A	N268D	No	AAC→GAC	pRB306	RBy284	pRB334	RBy290	pRB340	20–60	15
V143A	N268D (+A161A)	No	AAC→GAC (+GCC→GCT)	pRB307	RBy285	–	–	–	–	–
G245S	T123P	No	ACT→CCT	pRB284	RBy256	pRB290	RBy262	pRB320	90–260	55
G245S	T123P (+M40I)	No	ACT→CCT (+ATG→ATA)	pRB285	RBy257	–	–	–	–	–
G245S	N239Y	No	AAC→TAC	pRB282	RBy255	pRB301	RBy273	pRB325	15–120	70
G245S	S240N	No	AGT→AAT	pRB286	RBy260	pRB308	RBy286	pRB326	85–120	65
R249S	T123A +H168R	–	ACT→GCT CAC→CGC	pRB280	RBy253	pRB298	RBy270	pRB323	40–50	45
	T123A	No	ACT→GCT	–	–	pRB294	RBy266	–	–	–
	H168R	Yes	CAC→CGC	–	–	pRB296	RBy268	–	–	–

[a]Percentage of wild-type p53.

All yeast plasmids are based on pRB16, the parent strain to all yeast strains is RBy33 (Brachmann *et al.*, 1996; Vidal *et al.*, 1996) and the mammalian plasmids are based on pCMVNeoBam (Kern *et al.*, 1992). The phenotypes of the double and triple p53 mutants in yeast are summarized in Figures 2 and 5. The activity of the p53 mutants in mammalian assays is expressed in percentages of wild-type p53 activity. The range of percentages reflects the results with different combinations of cell lines and reporter constructs. All percentage numbers are rounded off to the nearest 5% increment.

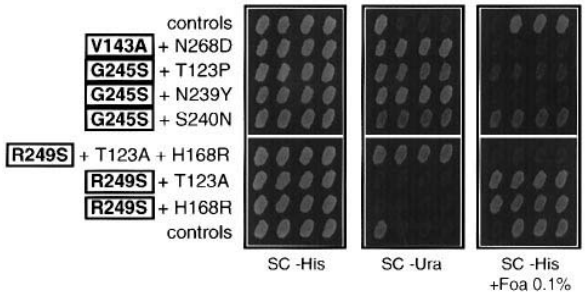

Fig. 2. Phenotypes in yeast of intragenic second-site suppressor mutations with the cancer mutations V143A, G245S and R249S. All subcloned suppressor mutations showed the same phenotype as the initially isolated plasmids. Shown are four patches corresponding to four independent yeast transformants containing the indicated *p53* expression construct. The patches were grown at 30°C on SC His^-, SC Ura^- and SC His^- + Foa 0.1%. V143A+N268D behaved like wild-type p53 in our assay (Ura^+Foa^S). N239Y suppressed G245S completely (Ura^+Foa^S), while T123P and S240N showed only partial suppression (Ura^+Foa^R). T123A+H168R completely suppressed the mutant phenotype of R249S. However, neither T123A nor H168R alone showed any suppression of R249S. The controls for the upper plates were from left to right: RBy41 (reporter strain with wild-type p53), RBy57 (parent strain without p53-dependent reporter gene expressing wild-type p53), RBy287 (reporter strain with V143A) and RBy198 (reporter strain with G245S). The controls for the lower plates were from left to right: RBy41, RBy55 (reporter strain with vector control), RBy57 and RBy235 (reporter strain with R249S).

yeast to identify missense mutations able to suppress common *p53* cancer mutations. This approach should yield the most potent suppressor mutations attainable. We used our previously described p53 yeast assay (Brachmann *et al.*, 1996; Vidal *et al.*, 1996) that assesses p53 function by its ability to bind to a p53 DNA binding site and transactivate the downstream reporter gene *URA3*. Using PCR mutagenesis and gap repair in yeast, we selected for several intragenic missense suppressor mutations that reverse the effects of the *p53* cancer mutations V143A, G245S and R249S. The same suppressor mutations had similar effects on additional *p53* cancer mutations. Remarkably, these suppressor mutations restored p53 function in mammalian cells using both reporter gene and apoptosis induction readouts.

Results

Identification of second-site suppressor mutations for the cancer mutations V143A, G245S and R249S

The yeast assay for p53 uses a tightly regulated reporter gene, *1cUAS53::URA3*, integrated into the yeast genome (Brachmann *et al.*, 1996; Vidal *et al.*, 1996), whose expression relies on p53 interaction with a binding site upstream of *URA3*. Human *p53* is expressed from a yeast *CEN* expression plasmid under the control of a constitutive promoter (Scharer and Iggo, 1992). We used a previously described synthetic binding site that conforms to the p53 binding site consensus (el-Deiry *et al.*, 1992). The activity of wild-type p53 is scored in two ways: *URA3* expression enables the yeast reporter strain to survive on plates without uracil (Ura^+), but also sensitizes yeast cells to 5-fluoroorotic acid (5-Foa). Thus, Foa sensitivity (Foa^S) is the second phenotype of wild-type p53. All p53 mutants tested so far have the opposite phenotype of wild-type p53 in yeast, namely Ura^-Foa^R (Brachmann *et al.*, 1996). The assay can also score an intermediate phenotype (Ura^+Foa^R) that reflects sufficient *URA3* expression for survival on SC Ura^- plates, but insufficient expression to experience the toxic effects of 5-Foa.

We exploited the clear phenotypic differences between wild-type and mutant p53 in this assay to identify second-site suppressor mutations for *p53* cancer mutations. The general strategy relied on PCR mutagenesis and gap repair in yeast (Figure 1). The initial design removed most of the *p53* ORF from the yeast expression plasmid by restriction digestion; the resulting gap was bridged with an overlapping PCR product that contained the original cancer mutation, as well as a potential second-site mutation (Figure 1A, PCR product A). We used relatively high-fidelity standard PCR conditions and *Taq* polymerase, as

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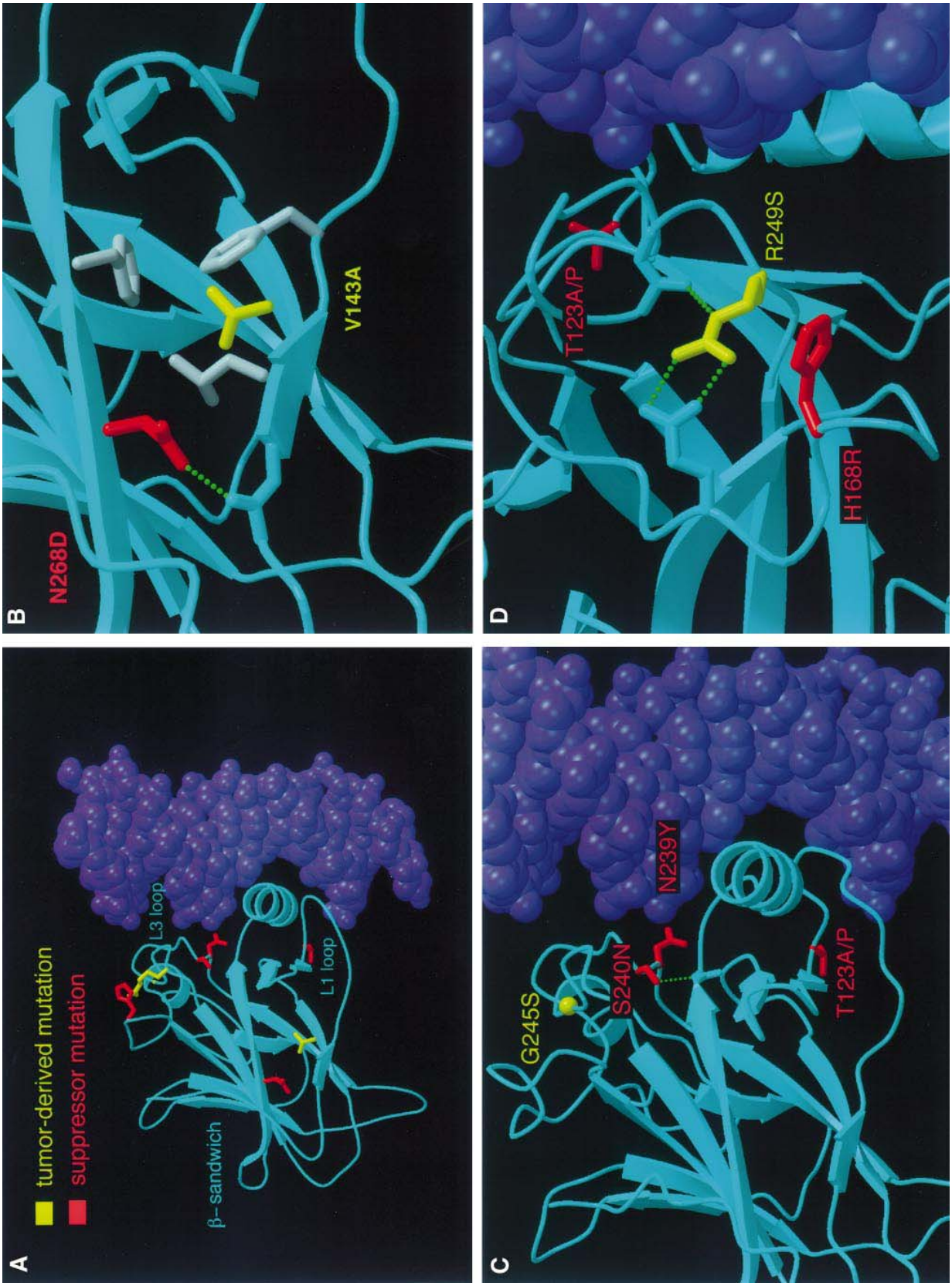

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opposed to intentionally mutagenic conditions, in order to minimize the likelihood of generating confounding multiple missense mutations. Co-transformation of both products into the yeast reporter strain led to very efficient repair of the gapped plasmid through homologous recombination with a PCR product (Muhlrad *et al.*, 1992). The yeast transformants could then be easily assessed; those that grew on plates lacking histidine and uracil in a plasmid-dependent fashion contained an intact yeast expression plasmid (His^+) with a restored *p53* ORF encoding a functional p53 molecule (Ura^+) (Figure 1B). Inclusion of the original cancer mutation in the PCR product resulted in a very high background of reversions of the original mutation to the wild-type amino acid (data not shown).

We therefore changed our strategy and excluded the original cancer mutation from the PCR product (Figure 1A, PCR products B or C). Because the cancer mutations were virtually assured to be present in the resultant isolates, this mutagenesis scheme was clearly superior in terms of the relative frequency of suppressor mutations. In this initial study we concentrated on the regions downstream of V143A (Figure 1A, PCR product B) or upstream of G245D, G245S, R248W and R249S (Figure 1A, PCR product C). Our experiments for G245D (12 Ura^+-conferring plasmids analyzed) and R248W (four Ura^+-conferring plasmids analyzed) yielded no suppressor mutations, for which there may be several explanations: (i) our screen was not sufficiently exhaustive; (ii) very few or no individual suppressor mutations for these two p53 mutants exist; or (iii) we mutagenized the wrong portion of the *p53* coding region. The last possibility is supported by a recent study (Wieczorek *et al.*, 1996). This describes a downstream suppressor mutation, T284R, obtained on the basis of modeling studies, which is able to suppress p53 mutants R248W, R273C and R273H, even though full activity in certain functional assays required further artificial activation of these mutants by removal of the very C-terminal autoregulatory domain.

For V143A, G245S and R249S we analyzed 44, 88 and seven Ura^+-conferring plasmids, of which two, seven and one, respectively, showed persistence of the original mutations. For V143A, the suppressor mutation N268D (two independent clones) was identified. For G245S, three suppressor mutations were isolated: T123P (four total, two independent), N239Y (two total) and S240N (one total) (Table I). For R249S, a single isolate with two missense mutations, T123A and H168R, was identified (Table I). By sequencing and subcloning fragments with putative second-site suppressor mutations, we confirmed that these mutations alone were sufficient to suppress the corresponding cancer mutations (Figure 2). Both the T123A and H168R mutations were required to suppress R249S. Neither mutation individually showed even partial suppression of R249S (Figure 2). Within the detection levels of our assay, all suppressor mutations, except T123P and S240N (phenotype Ura^+Foa^R instead of Ura^+Foa^S), led to complete restoration of wild-type p53 activity in yeast at 30°C.

The Ura and Foa phenotypes of all strains were also tested at 25°C and 37°C since the suppressor phenotype could be temperature-dependent (data not shown). V143A+N268D at 37°C was very weakly Ura^+, but still Foa^S. This probably reflects a partial loss of function which is masked on Foa plates by the increased innate sensitivity of yeast to 5-Foa resulting from growth at 37°C. All suppressor mutations for G245S were Ura^+Foa^S at 37°C, consistent with the same explanation or an improved function of these second-site mutations at the higher temperature. G245S+T123P and G245S+S240N were inactive (Ura^-Foa^R) at 25°C.

Modeling the basis of suppression

The structure of the core domain p53 complex (Cho *et al.*, 1994) suggested that tumorigenic mutations inactivate p53 by either altering amino acids that contact the DNA (functional mutations), or in the more frequent case, by affecting the structural integrity and stability of the DNA-binding surface or of the β-sandwich (structural mutations). The structure of the p53 core domain (Cho *et al.*, 1994) consists of a β-sandwich that serves as a scaffold for two large loops (termed L2 and L3) and a loop–sheet–helix motif (Figure 3A). The loops and the loop–sheet–helix motif form the DNA-binding surface of p53 and provide contacts to the DNA backbone and the edges of the bases.

In our screen for second-site suppressors, we started with scaffolding mutants as they could lead to global suppressor mutations that increase the overall stability of the folded state of the core domain. The mutants we used as a basis for isolating suppressors, V143A, G245S and R249S represent a wide range of structural defects. Val143 is involved in the packing of the hydrophobic core of the β-sandwich, and its mutation to alanine probably reduces the stability of the folded state, resulting in denaturation of the core domain (Cho *et al.*, 1994) (Figure 3B). Gly245 and Arg249 are in the L3 loop that provides the critical

Fig. 3. Suppressor mechanisms are suggested by the structure of the wild-type p53–DNA complex. (**A**) Schematic representation of the wild-type p53 core domain (cyan) bound to DNA (blue) (Cho *et al.*, 1994) highlighting in yellow the residues that are mutated in cancer that were used to select for suppressors and in red the residues where the isolated second-site suppressor mutations map. (**B**) The mutation of Val143 (shown in yellow) to Ala in cancer disrupts the packing of this side chain with the hydrophobic residues shown in gray and may destabilize the hydrophobic core. This mutation can be rescued by the mutation of Asn268 (colored red) to Asp. In the wild-type structure, Asn268 makes a hydrogen bond, indicated with a dotted green line, that bridges the two sheets of the β-sandwich and may contribute to the stability of the β-sandwich. Additional hydrogen bonding interactions afforded by the carboxylate on N268D is expected to further stabilize this region. (**C**) Gly245, whose C_α atom is shown as a yellow sphere, occurs in the L3 loop that provides one of the critical DNA contacts. This region has little space for an amino acid other than a glycine, and the G245S tumorigenic mutation thus affects the structure and stability of the L3 loop and of its DNA contact. The residues where second-site mutations suppress the G245S and several other L3 loop mutations are shown in red. In the wild-type structure, Asn239 is near the DNA, but several angstroms too far away to be making a contact; its mutation to a Tyr can readily bring this residue within contact distance to the phosphodiester backbone of the DNA. In the wild-type structure, Ser240 makes a hydrogen bond (green dotted line) with a backbone amide of the β-sandwich; its mutation to Asp may improve this hydrogen bond or allow formation of an additional one. In the wild-type structure, Thr123 is mostly solvent-exposed with no apparent structure-stabilizing role; however, the L1 loop appears to have a somewhat flexible conformation and T123A/P mutations may stabilize a conformation favorable for L1 loop–DNA contact. (**D**) The mutation of Arg249 (shown in yellow) to Ser would deprive the L3 loop of the hydrogen bond network (green dotted lines) that bridges the L2 and L3 loops. The mutation of the neighboring His168 on the L2 loop, shown in red, to Arg may restore components of this hydrogen bond network.

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Arg248–DNA contact (Cho *et al.*, 1994). Gly245 is in a region that has little space for an amino acid other than glycine; hence its substitution by serine is predicted to affect the conformation of this loop (Cho *et al.*, 1994; Figure 3C). The Arg249 side chain is central to a hydrogen bond network that stabilizes the L3 loop, and its mutation to serine would again be expected to affect the conformation of the L3 loop (Cho *et al.*, 1994; Figure 3C). Both the G245S and R249S mutations, in addition to affecting indirectly the critical DNA contact made by Arg248, may also reduce the overall stability of the core domain (Cho *et al.*, 1994).

A possible basis for second-site suppression is suggested by the wild-type p53 structure. Among the suppressor mutants identified, only N268D maps to the β-sandwich, and it probably increases the stability of the β-sandwich. In the wild-type structure, Asn268 occurs on the edge of the β-sandwich, in the general vicinity but not within contact distance of Val143. Its side chain makes a backbone hydrogen bond to the other sheet of the β-sandwich (Figure 3B) and its mutation to Asp may allow it to make alternate hydrogen bonds that help keep the two sheets of the β-sandwich together.

Three suppressor mutations were identified in the L3 loop and its immediate vicinity. The N239Y mutation likely introduces an additional DNA contact in the critical area where the L3 loop makes its primary DNA contacts (Cho *et al.*, 1994). In the wild-type structure, Asn239 is solvent-exposed on the DNA binding surface of p53, but does not make any DNA contacts as it is several angstroms too far away from the DNA. Its mutation to a Tyr could position the hydroxyl group to within hydrogen bonding distance of the phosphodiester backbone of the DNA, and may thus result in an additional DNA contact. The S240N mutation probably introduces additional stabilizing interactions between the L3 loop and its scaffold, the β-sandwich. In the wild-type structure, Ser240 makes a hydrogen bond to a backbone amide group of the β-sandwich, and its mutation to an Asn may allow for an additional hydrogen bond to be made to a backbone carbonyl group of the β-sandwich (Figure 3C). In contrast to the N239Y and S240N suppressor mutations, whose proposed mechanism of action may allow them to suppress multiple tumor-derived mutations, the mechanism of action of H168R is likely to be specific for the R249S tumor-derived mutant. R249S is predicted to eliminate a guanidinium group that bridges the L2 and L3 loops via hydrogen bonds, and the H168R suppressor mutation would introduce a guanidinium group in the immediate vicinity that may restore some of these interactions (Figure 3D). This proposal is consistent with the observation that in the absence of the R249S mutation, H168R interferes with p53 activity in our assay (data not shown). Furthermore, only the H168R mutation has been found in tumors, whereas none of the other suppressor mutations, as expected, has been associated with tumors (Cariello *et al.*, 1996; Hollstein *et al.*, 1996).

The remaining two suppressor mutations both map to Thr123 at the L1 loop of the loop–sheet–helix motif. In the wild-type structure, the L1 loop provides a single DNA contact (Lys120) although its structure appears to be significantly more flexible than the L3 loop (Cho *et al.*, 1994). Thr123 is solvent-exposed, and it does not seem to be involved in any stabilizing interactions with other portions of p53 (Cho *et al.*, 1994). Therefore it is not clear how its mutation to Ala or Pro could improve the DNA-binding activity of p53, although a likely mechanism is that these substitutions stabilize a loop conformation that is more favorable for the Lys120–DNA contact. It is noteworthy that T123A was previously identified as a mutation that singly increases the DNA-binding affinity of p53 (Freeman *et al.*, 1994).

Evaluation of V143A, G245S and R249S with their suppressor mutations for transcriptional activity in human cell lines

To validate that second-site suppressors isolated in yeast function in human cells, all *p53* cancer mutations—with and without their respective suppressor mutations—were evaluated in mammalian reporter gene assays. The transient transfection experiments were initially performed in the osteosarcoma cell line Saos-2 and the lung cancer cell line H1299, both of which are p53-null (Haffner and Oren, 1995). A luciferase reporter was used with the following upstream DNA binding sites: a tandem array of 13 p53 binding sites (PG13-Luc; Kern *et al.*, 1992; el-Deiry *et al.*, 1993), an array of 15 mutated p53 binding sites (MG15-Luc; Kern *et al.*, 1992; el-Deiry *et al.*, 1993) or the p53 response element of *p21/WAF1* (WWP-Luc; el-Deiry *et al.*, 1993). The latter downstream gene is required for p53-mediated G_1 arrest. All transcriptional activity readouts were normalized by co-transfection with a *lacZ* expression plasmid to account for variable factors such as transfection efficiency and cell death.

Using PG13-Luc as the reporter plasmid, V143A and R249S showed no transcriptional activity as expected; G245S showed ~25% residual activity as compared with wild-type p53 (Figure 4A, PG13-Luc). Consistent with the findings in yeast at 37°C, V143A+N268D showed increased but not wild-type levels of transcriptional activity as compared with V143A. T123P and S240N clearly suppressed the effect of the G245S cancer mutation. Surprisingly, G245S+N239Y showed transcriptional activity comparable with that of G245S alone (Figure 4A, PG13-Luc). T123A+H168R showed suppression of R249S, resulting in 40% of wild-type p53 transcriptional activity. None of the double and triple mutants transactivated the array of mutated p53 binding sites (Figure 4A, MG15-Luc), indicating that their interaction with p53 binding sites was specific. Results with the physiological p53 response element of *p21/WAF1* (el-Deiry *et al.*, 1993) were similar to those with PG13-Luc, except that N239Y was able to suppress G245S (Figure 4B).

These results in human cells mostly reproduced the findings of the p53 yeast assay. Notably however, G245S—the seventh most common *p53* cancer mutation (Cariello *et al.*, 1996; Hollstein *et al.*, 1996)—showed residual transcriptional activity in this assay, and N239Y did not suppress G245S when PG13-Luc was used. Several explanations for this anomaly were considered. G245S+N239Y was selected with a p53 consensus DNA binding site in yeast which is much more similar to the p53 response element of *p21/WAF1* in the organization of its binding half sites than to the artificial tandem array of 13 binding sites. The particular structure of PG13-Luc may therefore prevent certain p53 molecules, such as

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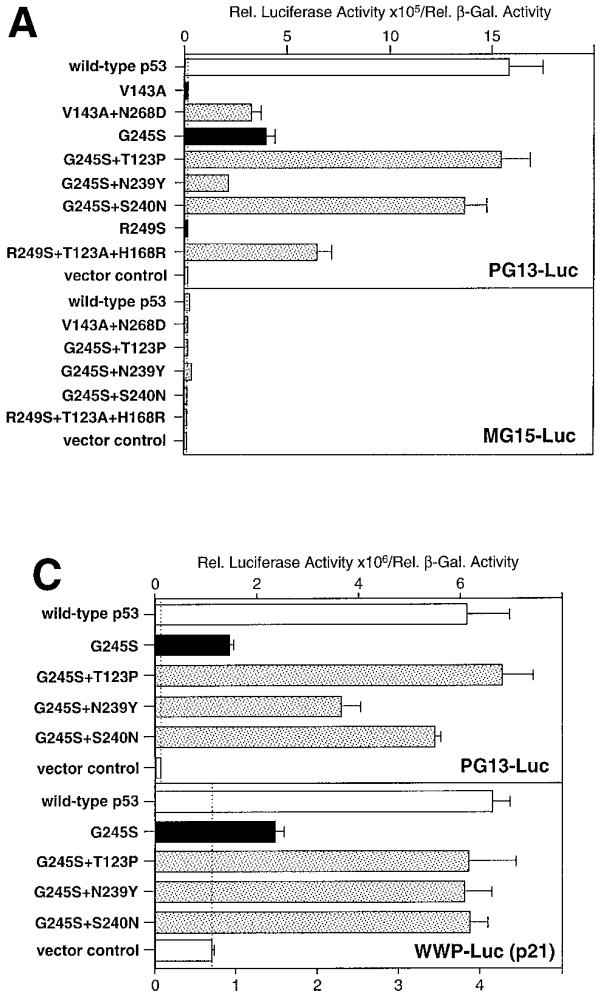

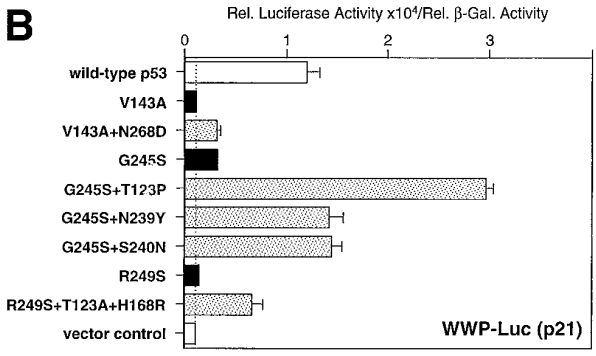

Fig. 4. Evaluation of suppressor mutations with their original *p53* cancer mutations in human reporter gene assays. (**A**) Transient transfection assays in the p53-null cell line Saos-2 were used to assess the transcriptional activity of the p53 double and triple mutants as compared with the cancer mutations alone. With the PG13-Luc reporter gene plasmid (containing a tandem array of 13 p53 binding sites upstream of the reporter gene Luciferase), the results from yeast at 37°C could be confirmed except that N239Y failed to restore function to the cancer mutation G245S. Experiments with MG15-Luc (containing an array of 15 mutated p53 binding sites) established that the binding of the double and triple mutants was specific for p53 binding sites. (**B**) The reporter gene plasmid WWP-Luc contains the native p53 response element of *p21/WAF1*, the essential downstream gene for p53-mediated G_1 arrest. Thus, it probably provides a better assessment of the true biological activity of the p53 mutants than PG13-Luc. Indeed, using the WWP-Luc reporter in Saos-2 cells, all three suppressor mutations identified for G245S in yeast were able to override G245S. Results for V143A and R249S were again consistent with the yeast results at 37°C. (**C**) The failure of N239Y to suppress the cancer mutation G245S observed in (A) could be due to the particular genetic background of the Saos-2 cell line. We therefore tested two cell lines with G245S mutations which showed similar results to Saos-2 (data not shown). A second p53-null cell line, H1299, differed in that G245S+N239Y also showed transcriptional activity when PG13-Luc was used. This suggests that the observed differences between yeast and mammalian assays are due (at least in part) to the chosen cell lines.

G245S+N239Y, from binding to it. Alternatively, G245S+N239Y, and the cancer mutation G245S alone, may require a particular genetic background to behave exactly as predicted by the p53 yeast assay or, in the case of G245S, by epidemiological data. To address this possibility we performed the same set of experiments in another p53-null cell line (H1299, a lung cancer cell line; Haffner and Oren, 1995) and two cell lines with a G245S *p53* allele [SK-LMS-1, a leiomyosarcoma cell line (Stratton *et al.*, 1990) and Su.86.86, a pancreatic carcinoma cell line (Redston *et al.*, 1994)]. The activity patterns with SK-LMS-1 and Su.86.86 were similar to those of Saos-2 (data not shown). Experiments in H1299 with either PG13-Luc or WWP-Luc showed again residual activity of G245S, but good suppression of G245S with N239Y in both cases, suggesting that the genetic backgrounds of cell lines may indeed account for some differences between yeast and mammalian assays (Figure 4C). We also considered an alternative explanation for some of these results: the differences between the yeast reporter results versus the mammalian transfection experiments (e.g. G245S N239Y with PG13 Luc is considerably less active than G245S alone in Saos-2 cells; Figure 4A) could in principle be attributable to simple differences in mutant p53 protein stability between yeast and mammalian cells. This does not appear to be the case, because the WWP-Luc reporter in Saos-2 cells had significantly more activity with G245S N239Y than with G245S (Figure 4B). Therefore the G245S N239Y protein is expressed in mammalian cells at physiologically significant levels.

Allele-specificity of suppressor mutations

Given the thousands of p53 mutations identified in tumors to date, it was of great interest to determine whether the above second-site mutations suppressed other *p53* cancer mutations. We used our collection of dominant-negative p53 cancer mutants selected in yeast (Brachmann *et al.*, 1996) to address this question. A comprehensive analysis has been hampered by the limited availability of restriction sites and often by the close proximity of the suppressor and the cancer mutations to be tested. This has made testing of all combinations of suppressor and cancer mutations very laborious. In this study we therefore focused on mutation pairs that were relatively easily recombined by subcloning (Figure 5).

The double mutants were first evaluated in yeast at 25°C, 30°C and 37°C (Figure 5). All double mutations that showed some suppression in yeast were then subcloned into mammalian expression vectors and analyzed in H1299 cells with transient transfection assays using PG13-Luc and WWP-Luc as reporter gene plasmids (Figure 6). The mutations C135F, P151H, R175H and P278S were not

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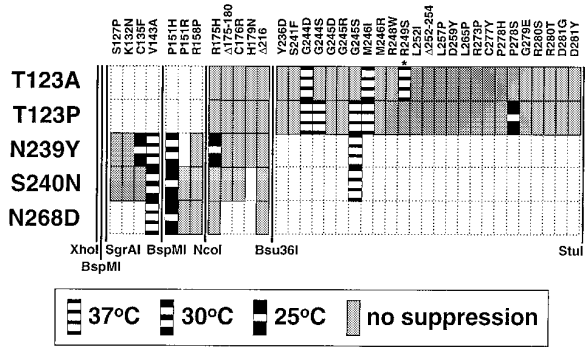

Fig. 5. Evaluation of suppressor mutations with other *p53* cancer mutations in yeast. Simple subcloning steps allowed the suppressor mutations to be evaluated with a small set of other *p53* cancer mutations. All suppressor mutations suppressed their original cancer mutations to some degree at 37°C (three white stripes). As the diagram indicates, several other cancer mutations were suppressed as well, even though not necessarily at all three temperatures tested. The figure also includes the relative location of restriction sites that were used in this study. The box intersecting T123A and R249S is marked with an asterisk since R249S also requires H168R for suppression.

suppressed in mammalian assays (data not shown), consistent with the yeast results that showed suppression of these alleles only at 25°C or 30°C. The same was true for V143A+S240N. A single cancer mutant, M246I, failed to show a defect in transcriptional activation in the chosen mammalian assays, and suppressor mutations T123A and T123P in combination with M246I did not lead to a clear increase in its transcriptional activity (Figure 6).

N239Y showed minimal suppression of V143A, consistent with the weakly $Ura^{+}Foa^{S}$ phenotype in yeast at 37°C. Similar to G245S, G244D and G244S had some residual transcriptional activity. In both cases the suppressor mutations T123A and/or T123P restored transactivation to wild-type p53 levels (Figure 6). Again, the overall concordance between the results obtained in yeast and in mammalian cells was excellent. These results suggest, not surprisingly, that the suppressor mutations are more likely to restore wild-type function to p53 mutants that still have some residual activity.

Evaluation of all functional combinations of cancer and suppressor mutations for induction of apoptosis

An independent and physiologically highly relevant way of assessing the functional activity of p53 double and triple mutants is an assay for apoptosis. This assay is so significant because it evaluates that activity of p53 that appears to be of central importance in its positive role in successful anti-cancer therapy (Clarke *et al.*, 1993; Lowe *et al.*, 1993a,b, 1994; Fisher, 1994; Lowe, 1995). Such experiments may reflect the overall biological effect of a given p53 expression plasmid regardless of its ability or inability to activate an artificial reporter gene.

BHK cells were transfected with the above-described p53 expression constructs along with a *lacZ* reporter plasmid. Transfected cells were scored for apoptotic cell death by morphology (cell shrinkage and membrane blebbing) (Figure 7). In this assay, wild-type p53 induced cell death in 21% of the transfected cells (above a background of 6.5%). All p53 cancer mutants, including

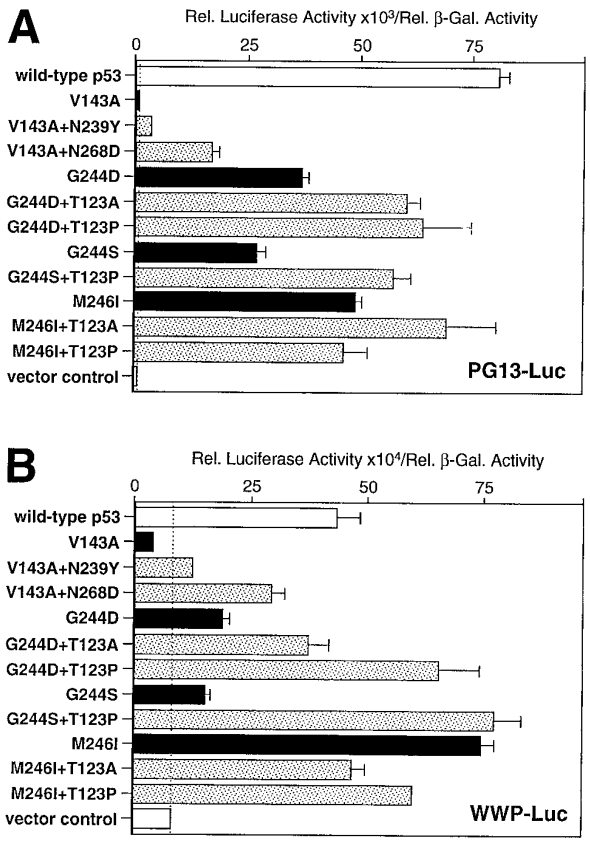

Fig. 6. Evaluation of suppressor mutations with other *p53* cancer mutations in human reporter gene assays. All combinations of suppressor and cancer mutations that showed some extent of suppression in the yeast assay were tested in mammalian reporter gene assays. (**A**) Results with H1299 cells and PG13-Luc. (**B**) Results for H1299 and WWP-Luc. Consistent with the yeast results, V143A, G244D and G244S were suppressed in human cells at 37°C. N239Y showed minimal suppression of V143A which correlates with the weakly $Ura^{+}Foa^{S}$ phenotype in yeast at 37°C. V143A+N268D was included for comparison purposes. Similar to G245S, G244D and G244S had some residual transcriptional activity. In both cases the suppressor mutations T123A and/or T123P led to restoration of transcriptional activity to wild-type p53 levels. The double mutants for M246I were also functional. However, M246I by itself showed substantial transcriptional activity, so that the results with M246I are not meaningful.

those that had shown residual activity in the reporter gene assays, were unable to induce apoptosis above the vector control background. The results for the p53 double and triple mutants were entirely consistent with the data obtained in the p53 yeast assay. The suppressor mutations reconstituted activity to 45–85% of wild-type p53 activity. V143A+N268D showed only minimal activity, as was also noted in the above-described transcriptional activation assays.

Discussion

Suppressors of p53 cancer mutations selected in yeast function in mammalian cells

We have used a genetic assay for functional p53 in yeast to identify several second-site mutations that suppress the effects of common *p53* cancer mutations. The strength of this genetic approach in yeast is that it selects for functional

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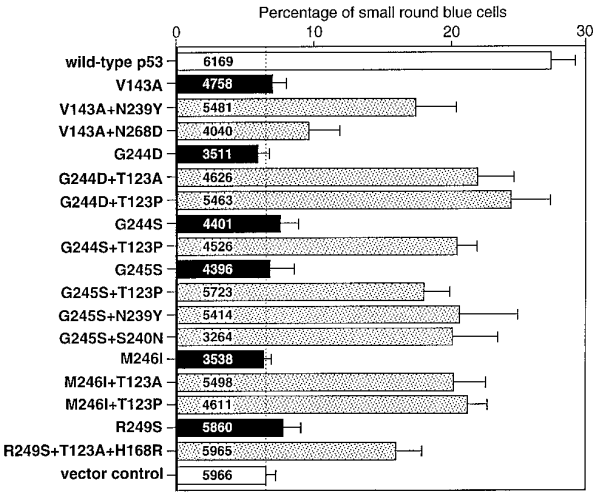

Fig. 7. Evaluation of all functional combinations of cancer and suppressor mutations in an apoptosis assay. All combinations of suppressor and cancer mutations that showed transcriptional activity in the mammalian reporter gene assays were tested for their ability to induce apoptosis. BHK cells were co-transfected with a *lacZ* expression plasmid and the indicated p53 expression plasmids. After 24 h the cells were fixed and stained with X-Gal. The columns represent the percentage of small round blue cells (= apoptotic morphology) among total number of blue cells counted. The data were collected from two independent experiments done in triplicate. The total number of blue cells counted is shown within the columns. This assay confirmed the yeast results in all cases. All p53 cancer mutants, including those with varying activities in the reporter gene assays, behaved as expected and were unable to induce apoptosis above the background of vector control. All double and triple p53 mutants showed activity ranging from 45 to 85% of wild-type p53 except V143A+N268D, a double mutant that had only minimal function in the p53 yeast assay at 37°C.

suppressors in an unbiased way, and allows for huge numbers of candidates to be screened in a biologically relevant manner. This method contrasts with rational second-site suppressor design based on the crystallographic p53 structure (Wieczorek *et al.*, 1996), and thus can lead to the identification of unexpected suppressor mechanisms.

The results in yeast showed an excellent correlation with subsequent experiments in mammalian cells, strongly validating this approach (see Tables I and II for summary). We evaluated the mammalian transcriptional activities of the cancer mutations with and without their suppressors using several reporter gene constructs in a variety of cell lines. We also assessed the biological activity of these p53 double and triple mutants in a second, more biologically meaningful way, using an assay for one of the endpoints of wild-type p53 activity, induction of apoptosis. This may be particularly important, since the ability of wild-type p53 to induce apoptosis is a crucial mechanism for radiation-induced or chemotherapy-induced cell death (Clarke *et al.*, 1993; Lowe *et al.*, 1993a,b, 1994; Fisher, 1994; Lowe, 1995).

We found that, of the three cancer mutations for which we initially identified second-site suppressors, two were unable to activate transcription using various mammalian reporters, thus behaving as expected for mutant p53 proteins. However, the third mutation, G245S, was a weak activator of both PG13-Luc and WWP-Luc. Interestingly, Bae *et al.* (1996) provided evidence that G245S activated a p53 promoter in yeast, whereas we isolated it as a mutant unable to activate *1cUAS53::URA3* (Brachmann *et al.*, 1996). A probable explanation for these conflicting results in yeast is that different p53 DNA binding sites in the different reporter constructs could subtly distinguish between mutant p53 molecules, as has been described for S121F (Freeman *et al.*, 1994). These results suggest that G245S has not completely lost DNA-binding activity and may still transactivate certain promoters. The G245S mutant is the seventh most common in cancer (Cariello *et al.*, 1996; Hollstein *et al.*, 1996) and thus is unlikely to have preserved biologically meaningful wild-type p53 activity. Consistent with this hypothesis, G245S was completely unable to induce cell death in our apoptosis assay. G245S has therefore probably lost the important ability to transactivate downstream genes responsible for apoptosis or to activate another mechanism that ultimately leads to apoptosis. We conclude that mammalian reporter gene assays are not completely accurate in predicting complex p53 functions. Paradoxically, our yeast reporter gene system appears to be more predictive of apoptotic function than the mammalian reporter assays.

Suppressor mutations suppress multiple cancer mutations

Suppressor mutations become of even greater interest when they suppress not only the cancer mutation used to select them, but also other additional cancer mutations. We have shown that most of the suppressor mutations described here can suppress additional cancer mutations. Those cancer mutations that are suppressed showed residual transcriptional activity in the mammalian reporter gene assays, like G245S. This result is not surprising since it should be easier to restore wild-type p53 function to a cancer mutant that has only a subtle defect. However, none of these cancer mutants was active in the apoptosis assay, whereas combination of the cancer mutations with the suppressor mutations in all cases led to a significant restoration of apoptosis induction.

Our current analysis was limited to a small set of cancer mutations and focused on cancer and suppressor mutation pairs that could be easily subcloned. It is therefore likely that the suppressor mutations will suppress a much broader range of cancer mutations. Future studies will evaluate the effect of these suppressor mutations together with all of the most common cancer mutations.

Modeling structural effects of the suppressor mutations

Based on the structure of the wild-type p53–DNA complex (Cho *et al.*, 1994), the predicted basis for second-site suppressor mutation action involves either the introduction of new contacts to the DNA backbone (N239Y) or the stabilization of portions of the core domain. N268D appears to stabilize the β-sandwich, whereas S240N and T123A/P appear to stabilize the DNA-contacting L3 and L1 loops, respectively. Although none of the second-site mutations identified thus far can be described as global suppressors, they clearly increase the activity of discrete subsets of tumor-derived mutants, in particular those with mutations in the structural element on which the suppressor mutation acts. This suggests that, although a single small

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Table II. Suppressor mutations combined with other *p53* cancer mutations

p53 Cancer + suppressor mutation	Yeast plasmid	Yeast strain	Mammalian plasmid	Mammalian reporter gene assays[a]	Mammalian programmed cell death assays[a]
C135F+N239Y	pRB365	RBy296	pRB460	0	–
V143A+N239Y	pRB362	RBy297	pRB461	5–10	55
V143A+S240N	pRB348	RBy298	pRB464	0	–
P151H+N239Y	pRB366	RBy299	pRB462	0	–
P151H+S240N	pRB354	RBy300	pRB465	0	–
P151H+N268D	pRB329	RBy301	pRB466	0	–
R175H+N239Y	pRB368	RBy302	pRB463	0	–
G244D+T123A	pRB379	RBy303	pRB452	75–80	75
G244D+T123P	pRB411	RBy304	pRB455	80–160	85
G244S+T123P	pRB412	RBy305	pRB456	70–195	65
M246I+T123A	pRB384	RBy306	pRB453	85–110	65
M246I+T123P	pRB416	RBy307	pRB457	55–145	70
P278S+T123P	pRB428	RBy308	pRB459	0	–

[a]Percentage of wild-type p53.
Plasmids and strains used are the same as in Table I. The phenotypes of the double p53 mutants in yeast are summarized in Figure 5. The range of percentages reflects the results with the two different reporter constructs, PG13-Luc and WWP-Luc. All percentage numbers are rounded off to the nearest 5% increment. The combinations of suppressor mutations with M246I showed transcriptional activity in the reporter gene assays, but so did M246I by itself (see Figure 6). In the assay for programmed cell death however, both M246I double mutants were active while M246I showed no activity (see Figure 7).

molecule compound may not be able to restore activity to all of the structural mutants, compounds targeting specific regions, such as the β-sandwich, L3 and L1 loops, may have activity against subsets of tumor-derived mutants.

Implications of suppressor mutations for future anti-cancer therapies

We have demonstrated that suppressor mutations can significantly restore the ability of p53 cancer mutants to induce apoptosis. Such induction is an important mechanism by which wild-type p53 contributes to the successful treatment of cancer patients with radiation or chemotherapy; thus, reconstitution of wild-type p53 activity is clearly a desirable goal in anti-cancer therapy (Fisher, 1994; Gibbs and Oliff, 1994; Kinzler and Vogelstein, 1994; Lowe, 1995; Milner, 1995; Harris, 1996). Promising studies have shown that the residual activity of mutant p53 protein present in cancer cells can be increased by antibodies against the putative C-terminal negative-regulatory domain of p53 (Abarzua *et al.*, 1995) or by peptides derived from that region (Abarzua *et al.*, 1996; Selivanova *et al.*, 1997). However, as this strategy primarily relieves the negative regulation of p53, it may require the p53 mutant to have significant residual wild-type activity. It could theoretically also lead to an indiscriminate activation of both wild-type and mutant protein and thus could result in significant side effects due to inappropriate wild-type p53-induced apoptosis in normal tissues. A long-term therapeutic goal is to identify small molecules that can specifically target activity against broad subsets of p53 mutants. Since the wild-type protein structure is inherently stable, such small molecules should not lead to the same type of activation of wild-type p53 as achieved by interference with the C-terminal autoregulatory domain. Although this approach has not yet been successfully applied to p53, similar small-molecule searches in other systems have resulted in some preliminary success. Small-molecule compounds that bind the central cavity of the hemoglobin tetramer can act as allosteric effectors and stabilize the R state of hemoglobin over the T state (Abraham *et al.*, 1992); and small-molecule compounds that stabilize the transthyretin tetramer against dissociation can prevent amyloid fibril formation *in vitro* (Miroy *et al.*, 1996). Our demonstration that second-site suppressors can be selected using a powerful functional selection scheme in yeast points to the long-term feasibility of this approach with p53 and brings this goal one step closer. The successful isolation of functional suppressors in yeast cells suggests that screens for small molecules, chosen randomly or rationally, may identify lead compounds able to reverse the phenotype of yeast cells containing the *1cUAS53:: URA3* reporter gene and might thereby be a simple and effective method for identifying novel therapeutic agents against cancer.

Future prospects

The current study was by no means exhaustive. Expanded screens for suppressor mutations of other common p53 mutants will provide additional insights and may lead to new targets for drug design. Based on our experience, the selection scheme can be improved in at least two ways. (i) There was a surprisingly high background of reversions to wild-type in the original mutations despite exclusion of these codons from the PCR product. This problem could be overcome by starting with an altered parental *p53* gene containing codons for the mutant amino acid which are very unlikely to revert back to wild-type (i.e. TCG for serine requires two base-pair changes for reversion to the wild-type codon arginine whereas the frequently found cancer mutation for R249S, AGT, requires only one). One could also analyze the upstream or downstream regions of in-frame deletions which cannot revert. (ii) So far, we have intentionally used standard PCR conditions, thus limiting our results to those amino acid changes which are readily achieved by single base-pair changes. A broader spectrum of suppressor mutations could be obtained by using 'mutagenic' PCR conditions.

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Allowing multiple base-pair substitutions may lead to the isolation of even more potent suppressors.

It is intriguing that none of the suppressor mutations (except H168R) has been reported in human cancers. This presumably reflects their ability to function as well as wild-type p53 in protecting against cancer. The fact that they can override the effect of *p53* cancer mutations, the result of one previous study regarding T123A (Freeman *et al.*, 1994), and our own preliminary data suggest that these suppressor mutants alone may in fact function better than wild-type p53. We are currently defining these differences in trying to explore these suppressor mutants as research and therapeutic tools.

Materials and methods

Primers

The following primers were used for DNA sequencing and PCR amplifications (see also Figure 1A): JB990, JB991, JB1052 (Brachmann *et al.*, 1996), JB1084 (5′-TTGTTGAGGGCAGGGGAGT-3′), JB1151 (5′-GAGGAGCCGCAGTCAGAT-3′), JB1152 (5′-TTTATGGCGGGAGGTAGA-3′), JB1273 (5′-CATTTGCCATCTATTGAA-3′), JB1274 (5′-GCCCATGCAGGAACTGTT-3′), JB1275 (5′-GTTGATTCCACACCCCCG-3′), JB1276 (5′-AGACCCAAAACCCAAAAT-3′), JB1348 (5′-CAGTGCTCGCTTAGTGCTCC-3′), JB1514 (5′-CATGCAGGAACTGTAACACATGTAGTTGTAGTGGAT-3′), JB1515 (5′-CACTACAACTACATGTGTTACAGTTCCTGCATGGGC-3′), JB1550 (5′-CAACTACATGTGTAACAATTCCTGCATGGGCGGCATG-3′) and JB1551 (5′-GCCGCCCATGCAGGAATTGTTACACATGTAGTTGTAG-3′).

Plasmid constructs

For the identification of intragenic suppressor mutations, the following plasmids were used which were identical to pRB16 (*ADH-p53* expression cassette in a *HIS3/CEN* plasmid) (Brachmann *et al.*, 1996; Vidal *et al.*, 1996) except for the indicated missense mutations: pRB212 (R175H), pRB217 (G245D), pRB28 (G245S), pRB218 (R248W), pRB214 (R249S) and pRB219 (R273P) (Brachmann *et al.*, 1996; Vidal *et al.*, 1996). Restriction sites and fragments used for the plasmid constructs are indicated in Figures 1A and 5. To create pRB255 (V143A), the *Pfl*MI fragment of pC53-SCX3 containing the mutation (Baker *et al.*, 1990; Kern *et al.*, 1992) was cloned into pRB16. Successful cloning was verified by restriction digestion with *Bsg*I (which recognizes the wild-type codon 143) and sequencing with primer JB990.

Initially isolated plasmids with a cancer and a suppressor mutation and those resulting from subsequent subcloning are listed in Table I. The *Bsu*36I–*Stu*I fragment of pRB306 with the suppressor mutation N268D was subcloned into pRB255 (V143A). Successful cloning was verified by sequencing with JB1348. For R249S, the double suppressor mutations T123A and H168R (pRB280) were cloned together (using the unique *Sgr*AI–*Bsu*36I sites) and separately (using the following sites: *Bsp*MI for T123A, *Nco*I–*Bsu*36I for H168R) into pRB214 with R249S (resulting in pRB298, pRB294 and pRB296, respectively). Successful subcloning was confirmed by digestion with *Bst*XI (which recognizes T123A) for T123A and T123A+H168R and sequencing with JB1274, JB1348 and/or JB990 for all three subcloning steps. For G245S, the suppressor mutation T123P (pRB284) was subcloned into pRB28 (G245S) as described for T123A (resulting in pRB290) and sequenced with JB1084, JB1274 and JB1348. N239Y and S240N could not be separated from the original mutation G245S by restriction digestion. Therefore, *Bsu*36I–*Stu*I fragments containing both G245S and N239Y or S240N were cloned into pRB16 (resulting in pRB301 and pRB308, respectively). These cloning steps were confirmed by digestion with *Bbv*I (which recognizes G245S) and sequencing with JB1275 and JB1348.

For the evaluation of suppressor mutations with other *p53* cancer mutations, T123A and T123P were subcloned into pRB16 (wild-type p53) as described above (pRB295 and pRB291, respectively). Because of the proximity to the suppressed cancer mutation G245S, N239Y and S240N had to be recreated by site-directed mutagenesis using the 'fusion PCR' approach (Higuchi *et al.*, 1988). In brief, using *Pfu* polymerase and an outer and inner primer each, two PCR products were made in which both inner primers encode the desired mutation. The final PCR product was obtained by using the two outer primers and the two purified PCR products as template. For N239Y, the first set consisted of outer primer JB1151 and inner primer JB1514 and the second set of outer primer JB1152 and inner primer JB1515. For S240N, the outer primers were the same, and the inner primers were JB1551 and JB1550, respectively. *Bsu*36I–*Stu*I fragments of the final PCR products were cloned into pRB16. Successful cloning was screened for with *Alw*NI for S240N (which recognizes the wild-type codon 240). For both cloning steps (pRB309 for N239Y, pRB335 for S240N), the subcloned PCR fragments were sequenced in their entirety using JB1052, JB1152, JB1275 and JB1348 to exclude the possibility of new mutations introduced by PCR.

For all subclonings regarding T123A and T123P, the *Nco*I–*Sac*I fragment of pRB335 containing S240N was subcloned into pRB295 (T123A) and pRB291 (T123P). *Nco*I–*Sac*I fragments with the different cancer mutations (see Figure 5) were then cloned into the resulting plasmids (pRB359 and pRB360, respectively). All cloning steps were confirmed with *Alw*NI (which recognizes the wild-type codon 240). For N239Y, the *Bsu*36I–*Stu*I fragment of pRB309 with N239Y was cloned into pRB255, resulting in a V143A+N239Y double mutant (pRB362). Cloning was checked by sequencing with JB1348. For S240N, the *Xho*I–*Bsu*36I fragment of pRB255 was cloned into pRB335, resulting in a V143A+S240N double mutant (pRB348). This and the subsequent cloning steps were confirmed by digestion with *Bsg*I (which recognizes the wild-type codon 143). *Xho*I–*Bsu*36I fragments with p53 cancer mutations (see Figure 5) were then cloned into plasmids pRB362 and pRB348. For N268D, *Xho*I–*Bsu*36I fragments with cancer mutations (see Figure 5) were cloned into pRB306 (V143A+N268D) and confirmed with *Bsg*I.

For the mammalian assays, the pCMV*neo*-based plasmids pC53-SN3 (wild-type p53) and pC53-SCX3 (V143A) were used (Baker *et al.*, 1990; Kern *et al.*, 1992). *Sgr*AI–*Stu*I fragments of the above mutants were cloned into the wild-type or V143A plasmid, depending on whether a V143A mutation was or was not introduced (see Tables I and II). Cloning was again confirmed with *Bsg*I. For the initial set of mutants (Table I), successful cloning was also confirmed by additional restriction digestion with *Bst*XI (which recognizes T123A), *Bbv*I (which recognizes G245S) and *Alw*NI (which recognizes wild-type codon 240), as well as by sequencing with either JB990 or JB1348.

Yeast assays to identify and evaluate intragenic suppressor mutations

Our initial strategy was PCR-mediated mutagenesis of most of the core domain, followed by gap repair in yeast (Muhlrad *et al.*, 1992). All of the above plasmids except pRB255 (V143A) were gapped with *Pfl*MI. Conventional PCR conditions using *Taq* polymerase and primers JB1151 and JB1152 were used to obtain PCR products for the same intact mutant plasmids which extended from base-pair 4 to base-pair 1143 of the *p53* ORF (+1 corresponds to the A of the start codon) which overlapped with both ends of the gapped plasmids (114 nucleotides at the 5′ and 173 at the 3′ overlap; see Figure 1A, PCR product A).

Since this strategy primarily yielded true intra-codon revertants, we developed a new strategy which selectively mutagenized regions of the *p53* ORF adjacent to, but not including, the starting mutations. For V143A, we mutagenized the region downstream of codon 143 by gapping the plasmid with restriction enzymes *Nco*I and *Stu*I (Figure 1A, PCR product B). Conventional PCR conditions using *Taq* polymerase and primers JB1275 and JB1276 were used to obtain a PCR product extending from base-pair 439 of the *p53* ORF to base-pair 78 after the stop codon which overlapped with both ends of the gapped plasmid (335 nucleotides at the 5′ and 64 at the 3′ overlap). Taking advantage of *Saccharomyces cerevisiae*'s high-efficiency homologous recombination, the PCR product and the gapped plasmid were co-transformed into RBy33 (containing one integrated copy of *1cUAS53::URA3*, the p53-dependent reporter gene; Brachmann *et al.*, 1996; Vidal *et al.*, 1996) (Figure 1B). 1.2 μg of PCR product and 0.6 μg of gapped plasmid were co-transformed (resulting in 3.8×10^5 His^+ colonies), directly plated onto SC His^- Ura^- plates (except for the controls) and incubated at 30°C, thus selecting for repaired, functional plasmids (His^+) and a (possibly) functional p53 molecule (Ura^+). His^+Ura^+ clones were single-colony-purified, grown non-selectively on YPD plates, streaked for single colonies on YPD plates and replica-plated to SC His^- and SC Ura^- plates to confirm plasmid dependency of the Ura^+ phenotype by co-segregation of the Ura^+ and His^+ phenotypes. These plasmids were rescued (Robzyk and Kassir, 1992) and transformed into RBy33 to recheck their phenotypes. We studied a subset of 720 Ura^+ colonies of which 580 were plasmid-dependent. 44 clones were sequenced with JB1274, 42 of which were wild-type. The *p53* ORFs of the other two plasmids (pRB306 and 307) were sequenced in their entirety using

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JB990, JB1052, JB1152, JB1275 and JB1348 in order to identify the second-site suppressor mutation. After confirmatory subcloning of the suppressor mutations (see Plasmid constructs), four independent transformants of these plasmids into RBy33 were patched onto SC His^-, replica-plated to SC Ura^- and SC His^- + Foa 0.1%, incubated at 25°C, 30°C and 37°C, and pictures taken after 2–4 days. The control strains were RBy41 (RBy33 with pRB16), RBy57 (BY385 with pRB16), RBy287 (pRB255 with V143A in RBy33) and RBy198 (pRB28 with G245S in RBy33) (Brachmann *et al.*, 1996; Vidal *et al.*, 1996) (Figure 2).

A similar gap repair strategy was used for the upstream regions of G245D, G245S, R248W and R249S. The plasmids were gapped using restriction enzymes *Bsp*MI and *Bsu*36I. The overlapping PCR products were prepared using primers JB1273 and JB1274 (38 nucleotides at the 5′ and 221 at the 3′ overlap; Figure 1A, PCR product C). 1.2 µg of PCR product and 0.6 µg of gapped plasmid were co-transformed, leading on average to 8.0×10^5 His^+ colonies. Subsequently, 68, 308, 26 and 221 His^+Ura^+ colonies were isolated of which 12, 93, 4 and 7 were plasmid-dependent, respectively. The presence of the original mutation was checked by sequencing with JB1348 and/or JB991. All 12 plasmids for G245D and all four for R248W showed reversion to the wild-type amino acid. This was also the case for 81 of 88 G245S and six of seven R249S plasmids. Seven plasmids for G245S and one for R249S were sequenced entirely using JB990, JB1052, JB1084, JB1274 and JB1275. After confirmatory subcloning, the phenotypes of the plasmids were determined for four transformants into RBy33 as described above (Figure 2). The controls for all suppressor mutations of G245S were identical to the one for V143A, the controls for the R249S suppressor mutations were RBy41, RBy55 (RBy33 with pRS413; Sikorski and Hieter, 1989), RBy57 and RBy235 (pRB214 with R249S in RBy33; Vidal *et al.*, 1996).

Yeast expression plasmids with new combinations of suppressor and p53 cancer mutations were evaluated in the same fashion at 25°C, 30°C and 37°C. Controls were as above and in addition included expression plasmids with the corresponding original mutations alone (Brachmann *et al.*, 1996). As compared with wild-type p53 (Ura^+Foa^S), all p53 cancer mutants tested were Ura^-Foa^R at the three chosen temperatures. Combinations that showed any extent of suppression were analyzed further in mammalian assays.

Cell culture, transfection and reporter assays

BHK (gift of J.M.Hardwick), H1299 and Saos-2 cell lines (gifts of M.Kastan) were grown in high-glucose DMEM with 10% fetal bovine serum; SK-LMS-1 (obtained from ATCC) and Su.86.86 (gift of S.Kern) cell lines were grown according to ATCC specifications. All transfections were done with Lipofectamine (Life Technologies, Inc., Gaithersburg, MD) according to the manufacturer's protocol. For all reporter gene assays, 5×10^5 cells were plated in 35 mm dishes 24 h prior to the transfection. The transfection mix contained (per 35 mm dish) 10 µl Lipofectamine and a total of 3 µg plasmid DNA that had been Qiagen-purified (Qiagen, Santa Clarita, CA). For each Qiagen preparation, 1 µg plasmid was separately linearized using unique restriction sites and run on an agarose gel prior to transfections to ensure the integrity and concentration of the plasmid preparations.

All experiments were done in triplicate and in general repeated twice. p53-dependent reporter activity values were normalized to β-galactosidase activity by co-transfection of 1 µg pIC400, an expression plasmid carrying the *lacZ* gene under the control of the CMV promoter (kindly provided by J.Nathans). pIC400 was constructed by cloning a *Bam*HI fragment with the *lacZ* ORF into pRK5 (Gorman *et al.*, 1990). The 3 µg plasmid DNA per transfection mix consisted of 1 µg pIC400, 1 µg reporter gene plasmid (either PG13-Luc, MG15-Luc or WWP-Luc) and 1 µg of the pCMV*neo* expression plasmids expressing the indicated p53 mutant. Control experiments used empty pCMV*neo* plasmid to a total of 3 µg per transfection. Cells were exposed to the transfection mix for 5 h and harvested 28–48 h after initiation of transfection. The cells were lysed in 100 µl Reporter Lysis Buffer (Promega, Madison, WI) per 35 mm dish according to the manufacturer's protocol. Luciferase reporter gene assays with 0.2–20 µl cell lysate were performed with the Promega luciferase assay System. β-galactosidase assays with 0.03–5 µl cell lysate were performed with the Luminescent β-galactosidase Genetic Reporter System II (Clontech, Palo Alto, CA). Light emission was detected with the Monolight 2010 luminometer from Analytical Luminescence Laboratory, San Diego, CA. The amount of cell lysate analyzed was adjusted depending on cell line and lysis time point so that the measurements occurred in the linear range. For the figures, all luciferase activities were adjusted to the average β-galactosidase activity of the three wild-type p53 samples in a particular experiment.

Apoptosis assay

The *lacZ* transfection viability assays were performed as previously described (Miura *et al.*, 1993). 3×10^3 BHK cells were plated per 35 mm dish 24 h prior to the transfection. Transfections were performed in a manner similar to that used for the reporter gene assays except that the transfection time was 2 h. 1 µg of pIC400 and 2 µg of either the various p53 expression plasmids or the vector control pCMV*neo* were used per transfection. The cells were fixed and stained with X-Gal 24 h after transfection. Cell viability was scored in a double-blind fashion by counting small round blue cells (= apoptotic morphology) among total number of blue cells in four high-power fields per 35 mm dish.

Acknowledgements

We thank the Boeke laboratory and S.Tugendreich for fruitful discussions, J.M.Hardwick, M.Kastan, S.Kern, J.Nathans and B.Vogelstein for gifts of plasmids and cell lines, J.M.Hardwick, R.Clem and the Hardwick laboratory for advice with the *lacZ* transfection viability assay, David Symer for helpful criticism of the manuscript, and Philip D.Jeffrey for the figures of the p53 structure. This work was supported by grants to J.D.B. from the NIH (CA16519) and the W.W.Smith Charitable Trust.

References

Abarzua,P., LoSardo,J.E., Gubler,M.L. and Neri,A. (1995) Microinjection of monoclonal antibody PAb421 into human SW480 colorectal carcinoma cells restores the transcription activation function to mutant p53. *Cancer Res.*, **55**, 3490–3494.

Abarzua,P., LoSardo,J.E., Gubler,M.L., Spathis,R., Lu,Y.A., Felix,A. and Neri,A. (1996) Restoration of the transcription activation function to mutant p53 in human cancer cells. *Oncogene*, **13**, 2477–2482.

Abraham,D.J., Wireko,F.C., Randad,R.S., Poyart,C., Kister,J., Bohn,B., Liard,J.F. and Kunert,M.P. (1992) Allosteric modifiers of hemoglobin: 2-[4-[[(3,5-disubstituted anilino)carbonyl]methyl]phenoxy]-2-methylpropionic acid derivatives that lower the oxygen affinity of hemoglobin in red cell suspensions, in whole blood, and *in vivo* in rats. *Biochemistry*, **31**, 9141–9149.

Bae,I., Smith,M.L., Sheikh,M.S., Zhan,Q., Scudiero,D.A., Friend,S.H., O'Connor,P.M. and Fornace,A.J.,Jr (1996) An abnormality in the p53 pathway following gamma-irradiation in many wild-type p53 human melanoma lines. *Cancer Res.*, **56**, 840–847.

Baker,S.J., Markowitz,S., Fearon,E.R., Willson,J.K. and Vogelstein,B. (1990) Suppression of human colorectal carcinoma cell growth by wild-type p53. *Science*, **249**, 912–915.

Bergh,J., Norberg,T., Sjogren,S., Lindgren,A. and Holmberg,L. (1995) Complete sequencing of the p53 gene provides prognostic information in breast cancer patients, particularly in relation to adjuvant systemic therapy and radiotherapy. *Nature Med.*, **1**, 1029–1034.

Brachmann,R.K., Vidal,M. and Boeke,J.D. (1996) Dominant-negative p53 mutations selected in yeast hit cancer hot spots. *Proc. Natl Acad. Sci. USA*, **93**, 4091–4095.

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.

Cariello,N.F., Douglas,G.R. and Soussi,T. (1996) Databases and software for the analysis of mutations in the human p53 gene, the human hprt gene and the lacZ gene in transgenic rodents. *Nucleic Acids Res.*, **24**, 119–120.

Caron de Fromentel,C. and Soussi,T. (1992) TP53 tumor suppressor gene: a model for investigating human mutagenesis. *Genes Chromosomes Cancer*, **4**, 1–15.

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.

Clarke,A.R., Purdie,C.A., Harrison,D.J., Morris,R.G., Bird,C.C., Hooper,M.L. and Wyllie,A.H. (1993) Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature*, **362**, 849–852.

Donehower,L.A. and Bradley,A. (1993) The tumor suppressor p53. *Biochim. Biophys. Acta*, **1155**, 181–205.

el-Deiry,W.S., Kern,S.E., Pietenpol,J.A., Kinzler,K.W. and Vogelstein,B. (1992) Definition of a consensus binding site for p53. *Nature Genet.*, **1**, 45–49.

el-Deiry,W.S. *et al*. (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825.

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

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Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00042

Freeman,J., Schmidt,S., Scharer,E. and Iggo,R. (1994) Mutation of conserved domain II alters the sequence specificity of DNA binding by the p53 protein. *EMBO J.*, **13**, 5393–5400.
Gibbs,J.B. and Oliff,A. (1994) Pharmaceutical research in molecular oncology. *Cell*, **79**, 193–198.
Gorman,C.M., Geis,D.R. and McCray,G. (1990) Transient production of proteins using an adenovirus transformed cell line. *DNA Protein Eng. Tech.*, **2**, 3–10.
Gottlieb,T.M. and Oren,M. (1996) p53 in growth control and neoplasia. *Biochim. Biophys. Acta*, **1287**, 77–102.
Greenblatt,M.S., Bennett,W.P., Hollstein,M. and Harris,C.C. (1994) Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res.*, **54**, 4855–4878.
Haffner,R. and Oren,M. (1995) Biochemical properties and biological effects of p53. *Curr. Opin. Genet. Dev.*, **5**, 84–90.
Halazonetis,T.D. and Kandil,A.N. (1993) Conformational shifts propagate from the oligomerization domain of p53 to its tetrameric DNA binding domain and restore DNA binding to select p53 mutants. *EMBO J.*, **12**, 5057–5064.
Hann,B.C. and Lane,D.P. (1995) The dominating effect of mutant p53. *Nature Genet.*, **9**, 221–222.
Hansen,R. and Oren,M. (1997) p53; from inductive signal to cellular effect. *Curr. Opin. Genet. Dev.*, **7**, 46–51.
Harris,C.C. (1996) Structure and function of the p53 tumor suppressor gene: clues for rational cancer therapeutic strategies. *J. Natl Cancer Inst.*, **88**, 1442–1454.
Harris,C.C. and Hollstein,M. (1993) Clinical implications of the p53 tumor-suppressor gene. *N. Engl. J. Med.*, **329**, 1318–1327.
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.
Higuchi,R., Krummel,B. and Saiki,R.K. (1988) A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res.*, **16**, 7351–7367.
Hollstein,M., Sidransky,D., Vogelstein,B. and Harris,C.C. (1991) p53 mutations in human cancers. *Science*, **253**, 49–53.
Hollstein,M., Shomer,B., Greenblatt,M., Soussi,T., Hovig,E., Montesano,R. and Harris,C.C. (1996) Somatic point mutations in the p53 gene of human tumors and cell lines: updated compilation. *Nucleic Acids Res.*, **24**, 141–146.
Hupp,T.R., Meek,D.W., Midgley,C.A. and Lane,D.P. (1993) Activation of the cryptic DNA binding function of mutant forms of p53. *Nucleic Acids Res.*, **21**, 3167–3174.
Hupp,T.R., Sparks,A. and Lane,D.P. (1995) Small peptides activate the latent sequence-specific DNA binding function of p53. *Cell*, **83**, 237–245.
Kern,S.E., Pietenpol,J.A., Thiagalingam,S., Seymour,A., Kinzler,K.W. and Vogelstein,B. (1992) Oncogenic forms of p53 inhibit p53-regulated gene expression. *Science*, **256**, 827–830.
Kinzler,K.W. and Vogelstein,B. (1994) Cancer therapy meets p53. *N. Engl. J. Med.*, **331**, 49–50.
Ko,L.J. and Prives,C. (1996) p53: puzzle and paradigm. *Genes Dev.*, **10**, 1054–1072.
Levine,A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323–331.
Lowe,S.W. (1995) Cancer therapy and p53. *Curr. Opin. Oncol.*, **7**, 547–553.
Lowe,S.W., Ruley,H.E., Jacks,T. and Housman,D.E. (1993a) p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell*, **74**, 957–967.
Lowe,S.W., Schmitt,E.M., Smith,S.W., Osborne,B.A. and Jacks,T. (1993b) p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*, **362**, 847–849.
Lowe,S.W., Bodis,S., McClatchey,A., Remington,L., Ruley,H.E., Fisher,D.E., Housman,D.E. and Jacks,T. (1994) p53 status and the efficacy of cancer therapy *in vivo*. *Science*, **266**, 807–810.
Michalovitz,D., Halevy,O. and Oren,M. (1991) p53 mutations: gains or losses? *J. Cell. Biochem.*, **45**, 22–29.
Milner,J. (1995) DNA damage, p53 and anticancer therapies. *Nature Med.*, **1**, 879–880.
Miroy,G.J., Lai,Z., Lashuel,H.A., Peterson,S.A., Strang,C. and Kelly,J.W. (1996) Inhibiting transthyretin amyloid fibril formation via protein stabilization. *Proc. Natl Acad. Sci. USA*, **93**, 15051–15056.
Miura,M., Zhu,H., Rotello,R., Hartwieg,E.A. and Yuan,J. (1993) Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene ced-3. *Cell*, **75**, 653–660.
Muhlrad,D., Hunter,R. and Parker,R. (1992) A rapid method for localized mutagenesis of yeast genes. *Yeast*, **8**, 79–82.
Neil,J.C., Cameron,E.R. and Baxter,E.W. (1997) p53 and tumour viruses: catching the guardian off-guard. *Trends Microbiol.*, **5**, 115–120.
Niewolik,D., Vojtesek,B. and Kovarik,J. (1995) p53 derived from human tumour cell lines and containing distinct point mutations can be activated to bind its consensus target sequence. *Oncogene*, **10**, 881–890.
Pietenpol,J.A., Tokino,T., Thiagalingam,S., el-Deiry,W.S., 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.
Redston,M.S. *et al*. (1994) p53 mutations in pancreatic carcinoma and evidence of common involvement of homocopolymer tracts in DNA microdeletions. *Cancer Res.*, **54**, 3025–3033.
Robzyk,K. and Kassir,Y. (1992) A simple and highly efficient procedure for rescuing autonomous plasmids from yeast. *Nucleic Acids Res.*, **20**, 3790.
Roth,J.A. *et al*. (1996) Retrovirus-mediated wild-type p53 gene transfer to tumors of patients with lung cancer (see comments). *Nature Med.*, **2**, 985–991.
Scharer,E. and Iggo,R.D. (1992) Mammalian p53 can function as a transcription factor in yeast. *Nucleic Acids Res.*, **11**, 1539–1545.
Selivanova,G., Iotsova,V., Okan,I., Fritsche,M., Strom,M., Groner,B., Grafstrom,R.C. and Wiman,K.G. (1997) Restoration of the growth suppression function of mutant p53 by a synthetic peptide derived from the p53 C-terminal domain. *Nature Med.*, **3**, 632–638.
Shortle,D. and Lin,B. (1985) Genetic analysis of staphylococcal nuclease: identification of three intragenic 'global' suppressors of nuclease-minus mutations. *Genetics*, **110**, 539–555.
Sikorski,R.S. and Hieter,P. (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*, **122**, 19–27.
Stratton,M.R. *et al*. (1990) Mutation of the p53 gene in human soft tissue sarcomas: association with abnormalities of the RB1 gene. *Oncogene*, **5**, 1297–1301.
Vidal,M., Brachmann,R.K., Fattaey,A., Harlow,E. and Boeke,J.D. (1996) Reverse two-hybrid and one-hybrid systems to detect dissociation of protein–protein and DNA–protein interactions. *Proc. Natl Acad. Sci. USA*, **93**, 10315–10320.
Vogelstein,B. and Kinzler,K.W. (1992) p53 function and dysfunction. *Cell*, **70**, 523–526.
White,E. (1996) Life, death, and the pursuit of apoptosis. *Genes Dev.*, **10**, 1–15.
Wieczorek,A.M., Waterman,J.L., Waterman,M.J. and Halazonetis,T.D. (1996) Structure-based rescue of common tumor-derived p53 mutants. *Nature Med.*, **2**, 1143–1146.

Received December 15, 1997; revised and accepted January 26, 1998

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