Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 4091–4095, April 1996
Genetics

Dominant-negative p53 mutations selected in yeast hit cancer hot spots

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Communicated by Bert Vogelstein, Johns Hopkins Oncology Center, Baltimore, MD, January 4, 1996 (received for review December 29, 1995)

ABSTRACT **Clinically important mutant p53 proteins may be tumorigenic through a dominant-negative mechanism or due to a gain-of-function. Examples for both hypotheses have been described; however, it remains unclear to what extent they apply to *TP53* mutations in general. Here it is shown that the mutational spectrum of dominant-negative p53 mutants selected in a novel yeast assay correlates tightly with p53 mutations in cancer. Two classes of dominant-negative mutations are described; the more dominant one affects codons that are essential for the stabilization of the DNA-binding surface of the p53 core domain and for the direct interaction of p53 with its DNA binding sites. These results predict that the vast majority of *TP53* mutations leading to cancer do so in a dominant-negative fashion.**

More than half of all human cancers are associated with one or more alterations in the tumor suppressor gene *TP53* (1–4). Many premalignant lesions, a subset of malignant clones and germ lines of families prone to cancer are characterized by the presence of one wild-type and one mutant allele of *TP53* (5–9). In this situation the mutant p53 protein may act in a dominant-negative fashion, ultimately leading to loss of heterozygosity and thus a further growth advantage for the malignant cells. Alternatively, the mutant p53 protein may have acquired a new tumor-promoting activity which is independent of wild-type p53. These hypotheses are based on the analysis of only a few *TP53* mutations, usually in the setting of overexpression of the mutant protein, and their relevance to *TP53* mutations in general has not been proven (8, 10–13). We decided to use a novel yeast assay for p53 and its consensus DNA binding site to screen for and analyse spontaneous dominant-negative p53 mutations. We show that such mutations cluster in the mutational hot spots of human cancers. We demonstrate different degrees of dominance, the most dominant mutations localizing to codons 179, 241–248, and 277–281. These results are fully consistent with a dominant-negative mode of action for the large majority of tumorigenic *TP53* alleles.

MATERIALS AND METHODS

Yeast Strains, Plasmids, and Isolation of p53 Mutants. All of the yeast media used here (e.g., −His) were dropout media based on synthetic complete medium (SC) (14) lacking the indicated nutrient(s). The yeast strains and plasmids used are described in Table 1.

For isolation of independent p53 mutations, patches of single colonies from RBy41 [containing an *ADH-p53 HIS3* expression vector (pRB16) and the integrated reporter gene *lcUAS53*::*URA3*; M.V., R.K.B., A. Fattaey, E. Harlow, and J.D.B., unpublished data] were grown on SC −His plates, replica-plated to SC −His +0.15% 5-fluoroorotic acid (Foa) plates, and incubated for 2–4 days at 37°C until Foa^R papillae emerged. Only a single Foa^R colony was isolated from each parental patch. These Foa^R clones were (*i*) mated to RBy159 (*MATα*, isogenic to RBy41, but lacking an *ADH-p53* expression vector) and replica-plated to SC −Ura plates and (*ii*) mated to RBy160 [RBy159 with the *ADH-p53 LEU2* plasmid pLS76 (15)] followed by replica-plating to SC −His −Leu plates to select for diploids and then SC −His −Leu +0.15% Foa plates to evaluate the dominance/recessivity of the Foa^R phenotype. Clones which were Ura$^+$ in mating assay *i* and Foa^S in assay *ii* were recessive and were not due to p53 plasmid-dependent mutations. Most of these clones represent recessive mutations that knock out *lcUAS53*::*URA3*. Clones which were Ura$^-$ in assay *i* and Foa^S in assay *ii* were p53 plasmid-dependent recessive mutations. Only clones which were Foa^R in assay *ii* potentially contained a dominant-negative p53 plasmid-dependent mutation; these were further characterized by growing them nonselectively and isolating strains which had lost the (potentially mutated) pRB16. A wild-type p53 expression plasmid was then introduced into these strains as follows. The plasmid-free strains were mated to RBy162 (*MATα ura3-52* and containing pLS76), replica-plated to SC −Ade −Leu plates to select for diploids, followed by replica-plating of the diploids to SC −Leu +0.15% Foa plates. Foa^R clones which regained their Foa^S phenotype as a result of these manipulations were judged to contain dominant-negative p53 plasmid-dependent mutations.

Table 1. Yeast strains

Strain	Relevant genotype*	Plasmids (markers)†
RBy33	*MATa lcUAS53*::*URA3*	—
RBy41	*MATa lcUAS53*::*URA3*	pRB16 (*ADH-p53 HIS3 CEN*)
RBy159	*MATα lcUAS53*::*URA3*	—
RBy160	*MATα lcUAS53*::*URA3*	pLS76 (*ADH-p53 LEU2 CEN*)
RBy161	*MATα lcUAS53*::*URA3*	pLS76 (*ADH-p53 LEU2 CEN*)
		pRB17 (*ADH-p53 TRP1 CEN*)
RBy162	*MATα ura3-52*	pLS76 (*ADH-p53 LEU2 CEN*)

*All strains listed (except RBy162) are also *lys2Δ202 trp1Δ63 his3Δ200 leu2Δ1*. RBy162 is also *lys2Δ202 trp1Δ63 his3Δ200 leu2Δ1 ade2Δ*.
†pRB16 and pRB17 were derived from pLS76 (15) by subcloning the *Xho* I–*Sac* I fragment containing *ADH-p53* (including the *CYC1* transcription terminator) into *CEN* vectors pRS413 and pRS414 (16), respectively.

Characterization of p53 Mutants. The mutant pRB16 plasmids from all identified dominant-negative p53 plasmid dependent clones were recovered in bacteria (17) and retransformed into RBy33 (RBy41 without pRB16), and the phenotypes were rechecked. The dominant-negative phenotypes were then further classified by testing the degree of dominance over one or two doses of wild-type *ADH-p53* as follows. The retransformed strains bearing mutant pRB16 derivatives were mated to RBy160 and RBy161 [RBy159 containing two *ADH-p53* expression plasmids, pLS76 (15) and pRB17, which is identical to pRB16 except for the selectable marker *TRP1*

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Abbreviations: Foa, 5-fluoroorotic acid; SC, synthetic complete medium.

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Table 2. Properties of independent p53 mutations selected in yeast

Total number of Foa^R clones	717
No. of p53 plasmid-dependent mutants	111*
Recessive mutants	67 (9%)
Dominant mutants	31 (4%)†
Class 1	13
Class 2	18

*Thirteen plasmid-dependent mutants could not be classified because they did not show consistent phenotypes before and after plasmid recovery and retransformation.

†Eighteen additional independent dominant-negative mutants were obtained as false positives in a cDNA library screen. These mutants are independent by virtue of a unique mutation and are identified by * in Table 3.

(16)]; replica-plating them to SC −His −Leu and SC −His −Leu −Trp plates, respectively; replica-plating them to the same selective plates with 0.15% Foa, and incubating them at 30°C for 2–4 days.

Some of the dominant-negative p53 mutants were isolated as false positives from a cDNA library screen that is irrelevant to this paper; these mutants were characterized in the same fashion (Table 2). Due to the fact that this subset of the mutants studied did not necessarily represent independent isolates, a numerical analysis of mutation frequencies within this subset would be meaningless.

The recessive plasmid-dependent p53 mutants were also recovered into bacteria and retransformed. These isolates (as well as the dominant isolates) were evaluated by immunoblotting with anti-p53 antibody PAb 1801, performed as described (M.V., R.K.B., A. Fattaey, E. Harlow, and J.D.B., unpublished data). RBy50 [pRS413 (16) in RBy33] was used as the negative control.

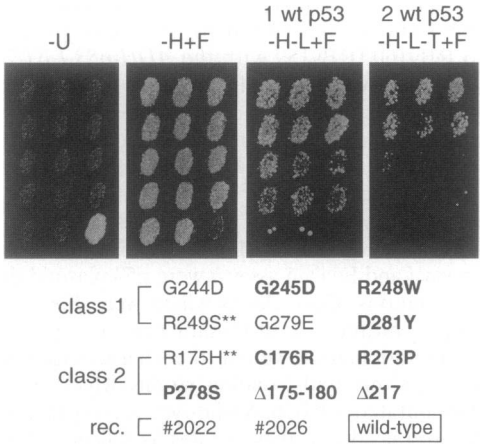

FIG. 1. Phenotypes of p53 mutants selected in yeast. In contrast to wild-type p53 (phenotype Ura$^+$ Foa^S), all dominant-negative as well as recessive mutants are Ura$^-$ Foa^R. Upon mating to strains with one or two wild-type *ADH-p53* expression vectors the dominant-negative mutants can be classified by their degree of dominance over wild-type p53. The stronger class 1 interferes with one and two copies of wild-type *ADH-p53* and thus survives on Foa plates. The weaker class 2 is only dominant over one wild-type copy. For the p53 mutants in boldface letters, *Nco* I/*Stu* I fragments with the mutations were recloned into the wild-type *ADH-p53* plasmid pRB16. The mutants with ** represent hot spot codons which were not identified by our screen (1, 2, 4) (see Table 3). Mutations #2022 and #2026 are recessive mutations leading to the expression of truncated p53 proteins. The media used were SC −Ura (−U), SC −His +Foa (−H+F), SC −His −Leu +Foa (−H−L+F), and SC −His −Leu −Trp +Foa (−H−L−T+F). The −U and −H+F media test for p53 function (wild type grows on −U and fails to grow on −H+F). The −H−L+F medium tests for the ability of mutant p53 to interfere with the function of a single wild-type copy of p53 (present on a *LEU2* plasmid); dominant-negative mutants will grow on this medium. The −H−L−T+F medium tests for the ability of mutant p53 to interfere with the function of two wild-type copies of p53 (present on *LEU2* and *TRP1* plasmids).

Sequencing of the Dominant-Negative p53 Mutants. Miniprep DNA (17) for the plasmids was RNase A treated (7 μg/ml for 10 min at 37°C), extracted with phenol/chloroform and sequenced with *Taq* polymerase (Perkin–Elmer) using Prizm kit dye-terminator cycle sequencing on an Applied Biosystems model 373A stretch automated sequencer. Sequences were analyzed using SEQUENCHER software (Gene Codes, Ann Arbor, MI) for the Macintosh. The core domains of *ADH-p53* were sequenced using primers JB990 (5′-ACCAGCAGCTC-CTACACC-3′) and JB991 (5′-GAGGAGCTGGTGTT-GTTG-3′). Eight dominant-negative clones (boldface numbers in Table 3) were further analyzed by ligating *Nco* I/*Stu* I fragments with the mutations (base pairs 477–1039) into pRB16 using standard methods (18). Wild-type sequence for the C-terminal parts of these fragments was verified by sequencing with primers JB1052 (5′-CCATCCTCACCATCAT-CAC-3′) and JB1091 (5′-GCAGGGGAGGGAGAGATGG-3′). The hotspot mutations for codons 175 and 249 (¶ symbol in Table 3) were cloned into pRB16 using the same strategy (M.V., R.K.B., A. Fattaey, E. Harlow, and J.D.B., unpublished data). Phenotypes were checked as described above.

RESULTS

The Assay for p53 and Its Consensus DNA Binding Site. Our p53 assay is based on the principles of yeast systems designed

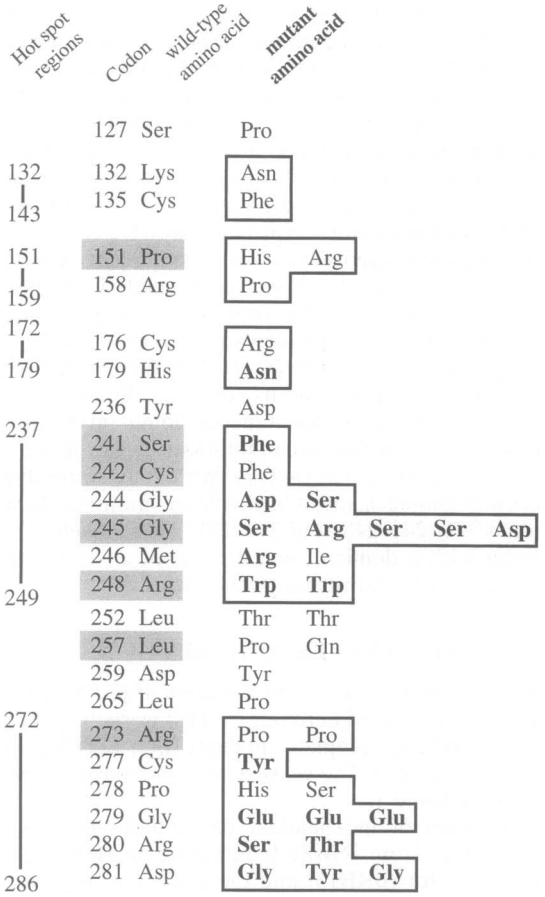

FIG. 2. Comparison of the dominant-negative *ADH-p53* mutations selected in yeast to the five hot spot regions of human cancer mutations and to reported germ-line mutations (Li-Fraumeni syndrome and others). The boxed yeast mutations hit the hot spot regions (2, 5). For codons with shaded background, germ-line mutations have been reported (7, 27, 28). The figure shows the clustering of the strongest dominant mutations to codons 179, 241–248, and 277–281. Mutations of class 1 are in boldface type and of class 2 in plain text.

by Fields and others, which allow the study of macromolecular interactions by simple phenotypic readouts (19–23). An important difference is the use of *URA3* as the reporter gene, which allows screening for and against p53 expression (M.V., R.K.B., A. Fattaey, E. Harlow, and J.D.B., unpublished data). Activation of *URA3* leads to survival on medium lacking uracil, but prevents growth on plates containing Foa due to the conversion of Foa to a toxic product (resulting in a Ura^+ Foa^S phenotype) (24). In our assay, *URA3* activation depends upon the site-specific binding of p53 to its consensus DNA binding site (25) placed upstream of *URA3*; p53 expression is driven by the *ADH1* promoter from a *CEN* (centromeric) plasmid (*ADH-p53*), which is maintained at approximately one copy per cell. Our prediction was that coexpression of a dominant-negative (26) p53 mutant should be able to interfere with wild-type p53, giving rise to an Foa^R phenotype.

Identification and Classification of Dominant-Negative p53 Mutants. We isolated a total of 49 independent spontaneous

Table 3. Sequence data on dominant-negative p53 mutations selected in yeast

Mutation no.	Codon	Mutation nucleotide	Amino acid	Class	Described in cancer (29, 30)
32*	127	TCC → CCC	Ser → Pro	2	No
27*	132	AAG → AAC	Lys → Asn	2	Yes
26*	135	TGC → TTC	Cys → Phe	2	Yes
43*	151	CCC → CGC	Pro → Arg	2	Yes
67	151	CCC → CAC	Pro → His	2	Yes
30*	158	CGC → CCC	Arg → Pro	2	Yes
76	176	TGC → CGC	Cys → Arg	2	Yes
17*	179	CAT → AAT	His → Asn	1	Yes
50*	236	TAC → GAC	Tyr → Asp	2	Yes
64	241	TCC → TTC	Ser → Phe	1	Yes
70	242	TGC → TTC	Cys → Phe	2	Yes
13*	244	GGC → GAC	Gly → Asp	1	Yes
14*	244	GGC → AGC	Gly → Ser	1	Yes
12*	245	GGC → AGC	Gly → Ser	1	Yes
16*	245	GGC → CGC	Gly → Arg	1	Yes
55	245	GGC → AGC	Gly → Ser	1	Yes
57	245	GGC → AGC	Gly → Ser	1	Yes
101*	245	GGC → GAC	Gly → Asp	1	Yes
41*	246	ATG → ATT	Met → Ile	2	Yes
62	246	ATG → AGG	Met → Arg	1	Yes
1*	248	CGG → TGG	Arg → Trp	1	Yes
63	248	CGG → TGG	Arg → Trp	1	Yes
48*	252	CTC → ATC	Leu → Ile	2	No
65	252	CTC → ATC	Leu → Ile	2	No
20*	257	CTG → CCG	Leu → Pro	2	Yes
37*	257	CTG → CAG	Leu → Gln	2	Yes
36*	259	GAC → TAC	Asp → Tyr	2	Yes
29*	265	CTG → CCG	Leu → Pro	2	Yes
69	273	CGT → CCT	Arg → Pro	2	Yes
74	273	CGT → CCT	Arg → Pro	2	Yes
7*	277	TGT → TAT	Cys → Tyr	1	Yes
28*	278	CCT → CAT	Pro → His	2	Yes
38*	278	CCT → TCT	Pro → Ser	2	Yes
10*	279	GGG → GAG	Gly → Glu	1	Yes
53	279	GGG → GAG	Gly → Glu	1	Yes
61	279	GGG → GAG	Gly → Glu	1	Yes
8*	280	AGA → ACA	Arg → Thr	1	Yes
58	280	AGA → AGC	Arg → Ser	1	No
3*	281	GAC → GGC	Asp → Gly	1	Yes
5*	281	GAC → TAC	Asp → Tyr	1	Yes
56	281	GAC → GGC	Asp → Gly	1	Yes
18*, **68**, 71, 72, 73, 75	Δ175–180 (or 176–181 or 177–182)†			2	Yes
35*	Δ216 (or 217 or 218)‡			2	Yes
42*	Δ252–254 (or 251–253)§			2	Yes
	175¶	CGC → CAC	Arg → His	2	Yes
	249¶	AGG → AGT	Arg → Ser	1	Yes

Clones in boldface type were characterized further by cloning the mutation into wild-type *ADH-p53* and rechecking the phenotypes.

*These dominant-negative mutations were obtained as false positives in a cDNA library screen.

†This deletion presumably arises frequently because of the direct repeat GCGCTGC present at codons 175–176 and 181–182.

‡Deletion of one of three tandem GTG codons.

§Direct repeat of ATC flanks the deleted nucleotides.

¶These hot spot mutations were cloned into wild-type *ADH-p53* since our screen did not identify mutations of these codons.

p53 mutants that behaved in a dominant-negative fashion. These mutants were identified using a two-step selection procedure. In the first step, haploid yeast colonies deficient in *URA3* expression were selected on plates containing Foa. In the second step, these colonies were mated to strains containing either the wild-type reporter gene or one copy of wild-type *ADH-p53* and subsequently transferred to plates containing Foa. Dominant-negative alleles of p53 showed an Foa^R phenotype in both cases. Recessive alleles of p53 or *cis*-acting reporter-linked mutations exhibited an Foa^S phenotype in the presence of an additional copy of wild-type *ADH-p53* or the wild-type reporter gene, respectively. Recessive mutations in the reporter gene were found in 87% and in the p53 gene in 9% of all mutants. Four percent of the Foa^R colonies contained dominant-negative p53 mutations (Table 2). Once the dominant-negative p53 mutants had been identified, the p53 plasmids were recovered and transformed into a fresh reporter strain (RBy33) to exclude artifacts of the original strain. In all cases the same dominant-negative phenotype could be reproduced. The dominant-negative mutants could be further classified by mating them to a strain with two wild-type *ADH-p53* plasmids, thus characterizing the dominance of the mutant proteins in the presence of two doses of the wild-type *ADH-p53* gene. The most dominant mutants were able to interfere with one and two copies of wild-type *ADH-p53* (class 1). Less dominant p53 mutants could only override the activity of a single wild-type allele (class 2) (Fig. 1). These classes represented 43% and 57% of the dominant-negative p53 mutants, respectively.

Sequences of the Dominant-Negative p53 Mutants. We then sequenced the core domains (codons 102–292) of the 49 dominant-negative mutants. Forty-one mutants had a single missense mutation and eight had an in-frame deletion. Very strikingly, the mutations clustered around five of the six known hot spot codons in the *TP53* gene: 245, 248, 249, 273, and 282 (1, 2, 4). We identified five mutations in codon 245, two in codon 248, and two in codon 273. Eighty-eight percent of the missense mutations hit the five hot spot regions for mutations (132–143, 151–159, 172–179, 237–249, and 272–286) or codons for which germ-line mutations have been described (Fig. 2) (2, 5, 7, 27, 28). Ninety-six percent of the mutations we recovered in yeast have been described in human cancers or cancer cell lines (Table 3) (4, 29, 30). Our screen hit five of the seven amino acids important in direct DNA binding (codons 241, 248, 273, 277, and 280) and three of the four amino acids involved in zinc atom contact (codons 176, 179, and 242) (31–33).

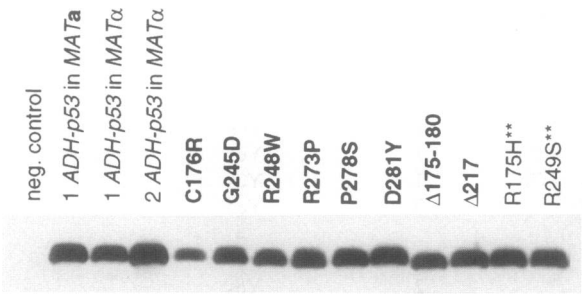

FIG. 3. Western blot analysis with PAb 1801 (34) for p53 protein expression in yeast strains with wild-type and mutant *ADH-p53* expression vectors. Protein levels for the dominant-negative mutants are similar to that of wild-type p53. The yeast strain with two expression vectors for wild-type *ADH-p53* shows ≈2-fold more p53 protein than all other strains, indicating that the strongest dominant p53 mutants of class 1 can in fact override higher levels of wild-type protein. For the p53 mutants in boldface letters *Nco* I/*Stu* I fragments with the mutations were cloned into wild-type *ADH-p53*. The mutants with ** represent hot spot codons that were not identified by our screen (1, 2, 4).

With the exception of H179N, all of the most dominant mutations (class 1) localized to codons 241–248 and 277–281. Eighty-three percent of the mutations in these two regions had the class 1 phenotype (Fig. 2), indicating a strong correlation between the location of mutations and their degree of dominance.

To exclude that second mutations up- or downstream of the core domain contributed to the described phenotypes, we subcloned *Nco* I/*Stu* I fragments (codons 159–347 encoding only the mutation of interest as confirmed by sequencing) into a wild-type *ADH-p53* plasmid for the following mutants: C176R, Δ175–180, Δ217, G245D, R248W, R273P, P278S, and D281Y. In all cases, the same dominant-negative phenotype was reproduced (Fig. 1, Table 3).

Our screen hit 3 hot spot amino acids (codons 245, 248, and 273) but failed to identify mutations in the other 3 (codons 175, 249, and 282). These hot spots in human cancers are due in large part to methylation of the CpG dinucleotides present in codons 175 and 282 and exposure to aflatoxin B_1 for codon 249 (1–4, 8); neither situation applies to our yeast system. Two amino acid substitutions for these hot spots, R175H and R249S, were subcloned into wild-type *ADH-p53* and shown to prevent *UAS53*::*URA3* transcription (M.V., R.K.B., A. Fattaey, E. Harlow, and J.D.B., unpublished data). These mutants were also found to be dominant over wild type (Fig. 1, Table 3).

Protein Expression Levels of Dominant-Negative p53 Mutants. The wild-type and the mutant *ADH-p53* genes are expressed from the same promoter in our system. To investigate whether the dominant-negative phenotypes were partially caused by an increased stability of the mutant protein, we analyzed protein levels by immunoblotting with anti-p53 antibody PAb 1801 (34). Fig. 3 shows that protein levels for the mutant p53 proteins were similar to that of wild type.

Analysis of Recessive p53 Mutants. We also analyzed the more abundant recessive p53 mutants. Since we considered the likelihood of nonmissense mutations high, we immunoblotted protein extracts from the 67 independently obtained recessive p53 mutants. None of these clones showed full-length protein. Four mutants expressed shorter proteins consistent with C-terminal truncation since PAb 1801 recognizes the N terminus (ref. 34; data not shown).

DISCUSSION

Based on our work in yeast, where recessive p53 mutations outnumbered dominant ones by about two to one, we believe that recessive p53 mutations probably occur at a higher rate in human cells than dominant mutations, but that the recessive mutations are much less likely to lead to cancer (and therefore to be sequenced) because the remaining wild-type allele continues to exert its important functions. Our selection in yeast for dominant-negative *TP53* mutations has identified a variety of missense mutations and in-frame deletions whose locations show a striking correlation with the hot spot regions of human cancer mutations. This suggests that the high frequency of human cancer mutations in these hot spot regions is in large part due to their dominant-negative effect on the wild-type p53 protein. Our data show that the dominant-negative mutants interfere with the wild-type protein to varying degrees; thus the amount of residual p53 activity in cells heterozygous for different p53 mutations is likely to be different. However, even for the strongest dominant-negative mutants, there is likely to be some residual p53 function. The dominant-negative interference with the function of wild-type p53 should lead to elevated rates of DNA damage, chromosome loss, and other forms of loss of heterozygosity of the *TP53* locus. Loss of heterozygosity would eliminate the residual activity provided by the wild-type *TP53* allele and provide the (pre-)malignant clone with further growth advantages.

Class 1 p53 mutants in our assay are more proficient than class 2 mutants in interfering with wild-type p53 function. The

locations of all class 1 mutations correspond closely to areas of the core domain which are essential for the structure of the DNA binding surface of p53 (L2 loop, codons 163–195 and L3 loop, codons 236–251), for major groove contacts in the pentamer sequence of the consensus DNA binding site (H2 α helix of the loop-sheet-helix motif, codons 278–286) and for minor groove contacts in the A·T-rich region of the binding site (L3 loop) (31–33). These mutations may be more efficient in destabilizing a heterotetramer of mutant and wild-type p53. Assuming (*i*) a single mutant subunit can poison a p53 tetramer, (*ii*) equal size pools of mutant and wild-type protein and (*iii*) unbiased mixing of mutant and wild-type subunits, heterozygous dominant mutations should lower p53 activity 16-fold. Thus, overexpression of a dominant-negative mutant relative to wild type is theoretically not required for abrogation of wild-type p53 function, and our experiments in yeast confirm this. These data suggest that the mutant p53 overexpression observed in human cancers represents an additional level of complexity in p53 deregulation.

We thank L. Hedrick for pointing out the trees in the forest; S. Friend and N. Pavletich for gifts of plasmids; and K. Cho, B. Vogelstein, and M. Kastan for their advice. R.K.B. thanks C. Brachmann for her continuous support. This work was supported in part by grants from the National Institutes of Health and the W.W. Smith foundation (J.D.B.), an American Cancer Society Faculty Research Award (J.D.B.), and Earnest H. Winkler Memorial Fellowship (R.K.B.), the Belgian Fonds National de la Recherche Scientifique, the Massachusetts General Hospital Discovery Fund, and an American Cancer Society Senior Fellowship (M.V.). M.V. thanks E. Harlow for his support.

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