Inactive full-length p53 mutants lacking dominating wild-type p53 inhibition highlight loss of heterozygosity as an important aspect of p53 status in human cancers

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Over 1000 different mutants of the tumor suppressor protein p53 with one amino acid change in the core domain have been reported in human cancers. In mouse knock-in models, two frequent mutants displayed loss of wild-type (wt) p53 function, inhibition of wt p53 and wt p53-independent gain of function. The remaining mutants have been systematically characterized for loss of wt p53 function, but not other phenotypes. We report the concomitant assessment of loss of function and interference with wt p53 using URA3-based p53 yeast and confirmatory mammalian assays. We studied 76 mutants representing 54% of over 15 000 reported missense core domain mutations. The majority showed the expected complete loss of wt p53 function and dominant p53 inhibition. A few infrequent p53 mutants had wt p53-like activity. Remarkably, one-third showed no interference with wt p53 despite loss of wt p53 function at 37 °C. Half of this group consisted of temperature-sensitive p53 mutants, but the other half was surprisingly made up of mutants with complete loss of wt p53 function. Our findings illustrate the diverse behavior of p53 mutants and mechanisms of malignant transformation by p53 mutants. The identification of full-length p53 mutants without dominant inhibition of wt p53 highlights the importance of determining the status of the wt p53 allele in human cancers, in particular in the context of clinical studies. In the case of p53 mutants with no or weak dominant p53 inhibition, presence of the wt allele may indicate a good prognosis cancer, whereas loss of heterozygosity may spell an aggressive, therapy-resistant cancer.

Introduction

p53 exerts its tumor suppressor activity mainly as a transcription factor that induces cell cycle arrest, apoptosis, DNA repair and/or senescence after DNA damage and other cellular stresses. The 393 amino acid protein has an N-terminal transactivation domain, a core domain that recognizes p53 DNA binding sites (DBS), and a C-terminal tetramerization domain (1,2). In unstressed cells, p53 has a short half-life owing to negative feedback loops (3). These must be overcome for p53 to activate hundreds of downstream target genes [4] and references therein. p53 also represses genes, and for most of them the mechanism of p53-mediated repression is unknown (5). Lastly, p53 uses non-transcriptional mechanisms as part of its tumor suppressor activity. Cytoplasmic p53 interacts with Bcl-2 family members, regulators of apoptosis, and results in mitochondrial cytochrome c release and apoptosis (6).

Approximately half of all human cancers carry p53 gene mutations (7,8). The International Agency for Research on Cancer (IARC) TP53 Mutation Database contains 21 588 somatic p53 mutations (R10, http://www-p53.iarc.fr/index.html) (8). A total of 15 387, or 71% of these, result in full-length protein with a single amino acid change in the core domain (amino acids 96–292). A total of 1004 different amino acid changes have been reported for this region, yielding an enormous variety of full-length p53 mutants. The top eight account for 30%, and the top 50 for 54%, of p53 mutants with one amino acid change in the core domain (8). Relatively little data are available on p53 loss of heterozygosity in human cancers, but it is estimated that 40% of human cancers still carry the wild-type (wt) p53 allele (M. Olivier and P. Hainaut, personal communication).

The structure of full-length p53 is not known, but the crystal structure of the wt core domain (9), in conjunction with biophysical and NMR studies, has yielded significant insights (10). A beta-sandwich is the scaffold for two loops and a loop–sheet–helix motif, which form the DNA-binding surface and contact the DNA backbone and bases. Cancer missense mutations may have at least one of several structural consequences: a crucial DNA contact is lost, a local structure of the core domain is perturbed or the entire core domain is unfolded.

The functional consequences of p53 mutants are quite complex (11,12). Numerous studies have clearly shown that p53 mutants are unable to activate target genes of wt p53 (13–15). Initial studies suggested a complete loss of sequence-specific transcriptional activation by p53 mutants, but subsequent investigations have also described p53 mutants, usually infrequent, with a partial loss of function (16–21). The intact tetramerization domain allows p53 mutants to form hetero-tetramers with wt p53, and some p53 mutants have been shown to induce a mutant conformation of the hetero-tetramer resulting in inability to bind to p53 DBS in vitro (22). Expression of p53 mutants in mammalian cells with wt p53 leads to suppression of wt p53 functions. These studies often relied on overexpression of p53 mutants and thus may not have adequately reflected the
ratio of mutant and wt p53 that is found in human cancer cells heterozygous for p53 (11,12). However, in yeast assays for p53 with equal expression of wt and mutant p53, p53 mutants clearly inhibited wt p53 activity (23). Use of a bidirectional expression vector ensuring equal expression levels of mutant and wt p53 in mammalian cells also documented interference with wt p53 and showed that the effect can be promoter-dependent (24). Two models of knock-in embryonic stem cells that express p53 mutants under the control of the endogenous p53 promoter further documented interference with wt p53 activities (25). p53 mutants also have wt p53-independent gain of functions that lead to increased proliferation, resistance to chemotherapy drugs and more aggressive tumors in animal models (11,12).

Even though the described in vitro assay systems can be considered quite artificial, conclusions derived from them have been confirmed for two of the most frequent p53 mutants using the stringent genetic approach of mouse knock-in models that express p53 mutants under the control of the endogenous p53 promoter. Two mouse models for p53 mutant R172H (26,27) and one for R270H (27) (corresponding to human R175H and R273H, respectively) displayed phenotypes consistent with loss of function, interference with wt p53 (if present) and gain of function in the absence of wt p53.

The discussed studies, reaching from biochemical experiments to mouse models, have in common that only a few p53 mutants were studied at a time. Yet, considering the wide variety of p53 mutants, it is likely that each of the ~1000 reported p53 mutants shows a unique combination of these characteristics. Functional assays in yeast have defined some aspects of larger numbers of p53 mutants. Once full-length characteristics, Functional assays in yeast have defined some reported p53 mutants shows a unique combination of these.

Materials and methods

Plasmids and plasmid constructions

Yeast expression plasmids for p53 mutants were mainly derived from our previous studies that expressed p53 mutants in the context of a natural p53 cDNA (23,36) or an engineered open reading frame (37), all encoding for a proline at position 72. An additional seven mutants each were obtained from Jo Milner (University of York, York, England; K132R, M246V, N239S, F270C, A276P, M237R and T256A) and Richard Igo (ISREC, Epalinges, Switzerland; H193R, C238F, H179L, R249W, P177L, R267W and R283H). Plasmid cDNA fragments with these p53 mutations were cloned into the yeast p53 expression plasmid pBR16 (38). The codon 72 status was not determined for these 14 p53 mutants. The URA3 reporter gene plasmids for p53 DBS were described previously (39). Mammalian p53 expression plasmids were from previous studies (36,37) or were cloned by transferring SgrA I/SA plasmid I cDNA fragments from the yeast plasmids into pCMV V143A (40,41). All p53 proteins studied in mammalian cells for p53 mutant-mediated inhibition of wt p53 carried a proline at position 72.

p53 assays in Saccharomyces cerevisiae

All yeast assays were performed as described previously (23,36–39).

Cell culture and transfection

Human H1299 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 μg/ml). Transient transfections were performed with Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Reporter gene assays

All experiments were done in triplicate and repeated at least twice. p53 cancer mutants were evaluated in transient reporter gene assays using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) in H1299 cells as described previously (23,36,37,39). Briefly, wt and mutant p53 expression plasmids were co-transfected with 500 ng of reporter gene plasmids with DBS of different p53 downstream target genes. The total amount of DNA transfected was made equal for all samples by adding an empty parental control vector. Cells were exposed to the transfection mixture for 5 h and harvested 24 h after initiation of transfection. Lysates were analyzed for Firefly Luciferase activity after normalization to Renilla Luciferase activity. Total p53 protein levels were determined by immunoblotting using 10 μg of cell lysate.

Yeast protein extraction

Overnight yeast cultures (10 ml) were grown to log phase (OD600 of ~0.5–1.0), centrifuged (1500 r.p.m. for 5 min) and washed in 1 ml of ddH2O. One hundred microliters of lysis buffer [50 mM Tris, pH 7.5, 5 mM EDTA, pH 8.0, 1% sodium dodecyl sulfate (SDS), 1x Complete Protease Inhibitors (Roche Molecular Biochemicals), 0.1% beta-mercapto-ethanol] and 120 μl of glass beads (0.5 mm diameter) were added to yeast pellets, followed by vigorous vortexing for 10 min at 4°C. Samples were then transferred to a 100°C block for 3 min followed by centrifugation for 20 min at 4°C. The supernatant was collected, quantified and analyzed by standard immunoblotting.

Immunoblotting

Proteins were detected using standard methods and the following specific antibodies: rabbit polyclonal anti-p53 (FL-393 HRP, Santa Cruz, CA), mouse monoclonal anti-alpha tubulin (Sigma, MO) and mouse monoclonal anti-topoisomerase II alpha, beta (StressGen, Victoria, BC, Canada). The preparation of nuclear and cytoplasmic extracts was performed as described (42).

Results

In the original design of our p53 yeast assay, p53 is expressed from a CEN-based plasmid (one copy per cell) under control of the constitutive ADH1 yeast promoter, p53 binds to a consensus DBS located in the tightly repressed SPO13 promoter upstream of the URA3 reporter gene. Transcriptional activity of p53 results in growth on plates lacking uracil (synthetic complete or SC-Ura, Urua phenotype) and no growth on plates selecting for the p53 plasmid and containing 5-fluoroorotic acid (Foa; the URA3 gene product is involved in converting Foa into a toxic substance, Foa-sensitive or Foa phenotype) (38). Common p53 mutants interfere with wt p53 activity in this yeast assay and change the phenotype from Foa-sensitive to Foa-resistant (23). Building on this first assay, we studied the activity of wt p53 with isolated p53 DBS of p53 target genes (39). Unexpectedly, p53 DBS of only 22 out of 57 p53 target genes (39%) were positive. Almost all p53 target genes related to cell cycle arrest, DNA repair and the death receptor.
## Table I. p53 cancer mutants—loss of function and interference with wt p53

The table summarizes data for 11 p53 DBS that give a Ura^+ Foa^- with wt p53 in yeast. The left panel shows the activity of p53 mutants alone (Supplemental Figure 1A, left diploid). ‘–’ indicates no activity (= Ura^- phenotype); lavender, green and blue boxes stand for activity at 25, 30 and 37°C, respectively. One diagonal line signifies activity only at the highest temperature shown (either 30 or 37°C); two diagonal lines indicate activity at 30 and 37°C, but not at 25°C. The right panel shows interference of p53 mutants with wt p53 (Supplemental Figure 1A, right diploid). ‘+’ indicates inhibition (= Foa^- phenotype). Blue boxes indicate lack of inhibition that correlates with activity of p53 mutants alone with the particular p53 DBS. Black boxes with ‘+’ identify cases where p53 mutants inhibited wt p53 despite activity with the p53 DBS alone. Red boxes stand for lack of wt p53 inhibition, even though the p53 mutants alone showed no activity with the particular p53 DBS. The same color code is used in the first column to highlight p53 mutants with unique activity patterns. Rank, Entries and Percentages are based on the R10 version of the IARC TP53 Mutation Database. '% (core domain)' refers to percentage of all reported mutations that result in a single amino acid change in the p53 core domain. '% (total)' indicates percentage of entire database.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Rank</th>
<th>Entries</th>
<th>% (total)</th>
<th>loss of wt p53 function</th>
<th>interference with wt p53</th>
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<td></td>
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<td></td>
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Total: 2918. 53.93. 38.44

Importance of loss of heterozygosity in assessing p53 status of human cancers
Table II. Classes of p53 mutants

<table>
<thead>
<tr>
<th>Class</th>
<th>Examples</th>
<th>Color code</th>
<th>Localization</th>
<th>Wt p53 function</th>
<th>Nuclear wt p53 inhibition</th>
<th>No. of mutants</th>
<th>% of core domain mutants</th>
<th>% of our collection</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>R175H</td>
<td>White</td>
<td>nuclear or equally distributed</td>
<td>none</td>
<td>strong</td>
<td>47</td>
<td>36.1</td>
<td>66.8</td>
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<tr>
<td>2</td>
<td>R273C</td>
<td>Red</td>
<td>nuclear or equally distributed</td>
<td>none</td>
<td>modest to minimal</td>
<td>14</td>
<td>7.5</td>
<td>13.9</td>
</tr>
<tr>
<td>3</td>
<td>R282W</td>
<td>Lavender or green</td>
<td>nuclear or equally distributed</td>
<td>temperature sensitive, 25° or 30°C</td>
<td>modest to minimal</td>
<td>9</td>
<td>7.6</td>
<td>14.1</td>
</tr>
<tr>
<td>4</td>
<td>R283H</td>
<td>Blue</td>
<td>nuclear or equally distributed</td>
<td>wt p53-like</td>
<td>minimal to none</td>
<td>4</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>5</td>
<td>Y220C</td>
<td>C</td>
<td>cytoplasmic</td>
<td>none or temperature sensitive</td>
<td>modest to minimal</td>
<td>2</td>
<td>2.5</td>
<td>4.6</td>
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</table>

Our results in yeast assays identified four different phenotypes of p53 mutants (Classes 1–4). A fifth unique class was defined on the basis of predominant cellular localization in human cells (Supplemental Table II). Because of their unique cytoplasmic localization, Y220C and Y163C were not included in the count for Classes 1–4 that are based on loss of function and interference with wt p53 in yeast and mammalian assays. ‘% of core domain mutants’ specifies what percentage of 15387 reported core domain missense mutations is represented by mutants tested in our study. ‘% of our collection’ indicates what percentage of 8298 reported mutations, represented by our collection of p53 mutants, is captured in one particular class.

Yeast assays for loss of function separate p53 mutants into three groups

We decided to use this experimental approach to analyze a collection of 76 p53 mutants that comprise 54% of all core domain single missense mutants reported in the IARC TP53 Mutation Database (8) and include 35 of the top 50 mutants and 50 of the top 100 mutants. We combined the p53 mutants with reporter plasmids for p53 DBS through mating of haploid yeast (Supplemental Figure 1A, left). Diploid yeast were selected using the marker genes of the plasmids and analyzed for expression of the URA3 reporter gene on SC-Ura plates after incubation at 25, 30 and 37°C for 1–2 days. As expected, p53 DBS known to be negative with wt p53 (39) were also negative with p53 mutants (data not shown). For p53 DBS that are positive with wt p53, 62 mutants showed no activity, but 14 p53 mutants had activity with a significant subset of p53 DBS. Four less frequent mutants showed transcriptional activity at 37°C (R175L, R283H, M237R, T256A). Five, including four of the top 50 mutants, showed significant activity at 30°C (R282W, E285K, Y234C, V272M, H168R), and another five p53 mutants, including four of the top 50 mutants, showed activity at 25°C (Y220C, V157F, C141Y, P278L, F270C). The data for all p53 DBS with a Ura^R phenotype for wt p53 are summarized in Table I (left panel). Supplemental Table I shows data for additional p53 DBS with an intermediate phenotype for wt p53: yeast are Ura^+. But Foa-resistant because URA3 expression is not high enough to lead to Foa-sensitivity. Supplemental Figure 1B shows the actual yeast results for select p53 mutants. Our results are consistent with previous reports that a subset of p53 mutants has wt p53-like activities at 37°C or lower temperatures (11,12). Our data further indicate that this activity is often dependent on specific p53 DBS.

Yeast assays for interference with wt p53 define four classes of p53 mutants

p53 mutants interfere with wt p53 activities in a variety of experimental systems (often referred to as a dominant-negative effect). However, this property has not been systematically assessed for p53 mutants and correlated with the activity of p53 mutants alone. In our yeast assays, eleven p53 DBS showed a Ura^Foa^ phenotype for wt p53 that allows for scoring of an inhibitory effect of p53 mutants through a change of phenotype from Foa^+ to Foa^- owing to decreased wt p53-mediated URA3 expression. For p53 mutants without inhibitory effect, the Foa^+ phenotype of wt p53 does not change. We analyzed the effect of all 76 p53 mutants on wt p53 (Supplemental Figure 1A, right). (i) The majority of p53 mutants showed interference with wt p53 in all yeast assays (Class 1 of Table II; Table I, right panel and Supplemental Figure 1C for actual yeast phenotypes of select p53 mutants). Three additional classes of mutants emerged. (ii) One surprising class of p53 mutants showed complete loss of function, yet did not inhibit wt p53 as predicted (see, for example, R273C and R158L; red boxes in right panel of Table I; Class 2 of Table II). Low p53 mutant levels did not account for this unexpected lack of inhibition, since similar levels for wt p53 and the different mutant classes were confirmed by anti-p53 immunoblotting of yeast lysates (Supplemental Figure 1D). (iii) The class of temperature-sensitive p53 mutants exhibited a strong positive correlation between activity at either 25 or 30°C and lack of interference with wt p53 at 37°C (52 out of 63 or 83% of cases: green or lavender squares in left panel of Table I match with blue squares in right panel; see, for example, R282W and Y234C, Class 3 of Table II). In addition, this class of mutants occasionally showed no interference with wt p53 even though lack of activity with a p53 DBS had predicted it (red boxes in right panel of Table I). (iv) As expected, p53 mutants with wt p53-like activity at 37°C did not interfere with wt p53 (see,
Localization to the cytoplasm defines a unique class of p53 mutants

Subcellular localization of p53 mutants may be an important aspect of their activities, particularly in light of the recent discovery that wt p53 has a direct role in apoptosis induction at the mitochondria (6). To our knowledge, this aspect of p53 mutants has not been assessed systematically. In a previous study, we noticed three patterns of cellular localization of p53 mutants: predominantly nuclear, equally distributed at nucleus and cytoplasm and predominantly cytoplasmic (37). In the current study, we transiently overexpressed all 22 p53 mutants in p53-negative H1299 cells and assessed cellular localization by anti-p53 immunoblotting of nuclear and cytoplasmic extracts (see Supplemental Table II and Supplemental Figure 2). All 22 mutants showed either predominant nuclear localization or equal distribution. Thus, Y220C and Y163C from our previous study (37) continue to be the only two p53 mutants that we found to be uniquely localized to the cytoplasm in H1299 cells.

Mammalian reporter gene assays confirm loss of function phenotypes observed in yeast assays

We confirmed the findings of the yeast assays for p53 mutants alone in mammalian transient luciferase reporter gene assays in H1299 cells for one p53 DBS with a strong wt p53 yeast phenotype (Ura\(^{+}\), p21, 5′ site, Supplemental Figure 3A) and one with an intermediate wt p53 yeast phenotype (Ura\(^{R}\), Noxa, Supplemental Figure 3B) (39). As predicted by the yeast results, several p53 mutants with activity in yeast only at 25 and 30°C showed no activity at 37°C in mammalian assays, whereas two less frequent p53 mutants (R283H and M237R) showed activity at 37°C in both yeast and mammalian assays. When tested at 30°C, the temperature-sensitive p53 mutants showed broader activity in mammalian than in yeast assays (Supplemental Figure 4).

Mammalian reporter gene assays confirm interference with wt p53 phenotypes observed in yeast assays

In the yeast assays, mutant and wt p53 are expressed from CEN plasmids resulting in very similar steady state protein levels (Supplemental Figure 1D). Under these conditions, mutant p53 can clearly interfere with wt p53 (Supplemental Figure 1C). Commonly used mammalian reporter gene assays often require much higher levels of mutant p53 to observe interference with wt p53, a situation unlikely to be the case in human cancers. The need for higher p53 mutant levels is probably due to several artificial aspects of the assays, including significant overexpression of the p53 proteins (11,12). Despite this shortcoming, we chose this assay type, because it allows for the rapid assessment of numerous yeast results in mammalian assays. Furthermore, recent results in mouse knock-in models suggest that these artificial assays nevertheless are reflective of the actual biology in a whole organism (26,27). We used R248Q, a p53 mutant with complete loss of function that interfered with wt p53 in all yeast assays (Table I), to establish conditions in mammalian reporter gene assays suitable for recapitulation of yeast results. Using 10 ng of wt p53 expression plasmid, 50 ng of R248Q plasmid reduced luciferase expression in H1299 cells to <50% of wt p53 alone for two p53 DBS with a strong wt p53 phenotype in yeast (KILLER/DR5 and p21, 5′ site, Ura\(^{+}\)Foa\(^{5}\)) (Figure 1) and for two with an intermediate yeast phenotype (Noxa and PCNA, Ura\(^{+}\)Foa\(^{5}\)) (Supplemental Figure 5). Two hundred and fifty nanograms of R248Q with 10 ng of wt p53

<table>
<thead>
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<th>Class 1 versus other classes</th>
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<tbody>
<tr>
<td><strong>mutant (250 ng)</strong></td>
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<tr>
<td>Relative Activity</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>R248Q</td>
</tr>
<tr>
<td>R273C</td>
</tr>
<tr>
<td>R282W</td>
</tr>
<tr>
<td>Y220C</td>
</tr>
<tr>
<td>V157F</td>
</tr>
<tr>
<td>E285K</td>
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<tr>
<td>Y234C</td>
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<td>Y205C</td>
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<tr>
<td>V272M</td>
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<tr>
<td>R158L</td>
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<td>P151S</td>
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<td>P278L</td>
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**Fig. 1.** Mammalian reporter gene assays confirm interference with wt p53 by p53 mutants observed in yeast. The effects of increasing amounts of R248Q (Class 1; 10–250 ng of expression plasmid) on wt p53-dependent (10 ng) luciferase expression were assessed in transient reporter gene assays in H1299 cells. Two p53 DBS with a strong yeast phenotype (KILLER/DR5 and p21, 5′ site, Table I) were evaluated. All lysates were normalized to Renilla luciferase activity and evaluated for total p53 protein levels by anti-p53 immunoblotting. The inhibitory effects of 12 p53 mutants with unique patterns of interference with wt p53 in yeast were assessed as for R248Q (Class 1 versus other classes). p53 mutants in italics are part of Class 2 in Table II and those in bold are part of Class 3. R248Q served as a control and is an example of Class 1.

Importance of loss of heterozygosity in assessing p53 status of human cancers
expression plasmid reduced luciferase expression to levels similar to 250 ng of R248Q alone. Using the most extreme conditions of 10 ng of wt and 250 ng of mutant plasmid, we tested the most frequent p53 mutants that did not interfere with wt p53 in yeast assays using the p21, 5′ site. Results for all of them were distinctly different from R248Q, since even at these high levels of mutant expression plasmid, luciferase expression was still >20% of wt p53 alone (Figure 1). The only exception was R273C; it did not inhibit wt p53 in yeast assays (Table I), but behaved similarly to R248Q in mammalian cells.

On the basis of these results, we analyzed four mutants in greater detail using the same four p53 DBS as for R248Q. R158L served as an example of p53 mutants with complete loss of function, yet no interference with wt p53 in yeast (Class 2 of Figure 2 and Table II). R273C as a second example of this class was analyzed further because of the discrepancy between yeast and preliminary mammalian results. R282W and Y234C were chosen as examples of temperature-sensitive mutants with minimal interference with wt p53 in yeast (Class 3 of Figure 2 and Table II). As predicted by the results of Figure 1, R158L, R282W and Y234C showed distinctly less interference with wt p53 than R248Q, R158L being the one with least wt p53 inhibition (KILLER/DR5 and p21, 5′ site; Figure 2). In general, slightly more interference was observed with the two weaker p53 DBS with only an intermediate wt p53 phenotype in yeast (Noxa and PCNA; Supplemental Figure 6). R273C behaved similarly to R248Q, further confirming the difference between yeast and mammalian results. Overall, however, these mammalian results were consistent with the findings of the p53 yeast assays and defined two unique classes of p53 mutants (Classes 2 and 3 of Table II).

Cytoplasmic p53 mutants exhibit little interference with wt p53 in mammalian reporter gene assays

Y220C and Y163C are both predominantly localized to the cytoplasm in H1299 cells (Supplemental Table II) (37). In yeast, Y220C showed no interference with six p53 DBS, whereas Y163C always interfered with wt p53 (Table I). We determined whether their unique subcellular localization in H1299 cells affects their activities in mammalian reporter gene assays. Both Y220C and Y163C showed only moderate interference with wt p53 that was very similar to R158L, R282W and Y234C, suggesting that predominant cytoplasmic localization in H1299 cells affects results in assays that assess interference with nuclear activities of wt p53 (Figure 3 and Supplemental Figure 7; Class 5 in Table II).

Discussion

The challenge to functionally characterize all p53 mutants

Cancer researchers are faced with the remarkable complexity of ~1000 different p53 mutants that are expressed in a large fraction of human cancers. These p53 mutants all carry one amino acid change in the core domain. Through the combined efforts of many laboratories and using various experimental systems, it has been established that these single amino acid changes are likely to have one or more of the following functional consequences: loss of function, interference with wt p53 (if still present) and wt p53-independent gain of function (11,12). These conclusions have been confirmed for two of the most frequent p53 mutants in stringent mouse knock-in models (26,27), indicating that previously used functional assays were informative despite their artificial aspects. It remains a considerable task to accurately characterize most, if not all, p53 mutants. This challenge must eventually be met, since full understanding of the functional characteristics of all p53 mutants will allow investigators to better assess the significance of specific p53 mutations for clinical outcomes.

Loss of function of p53 mutants

Our analysis of p53 DBS with p53 mutants alone separated p53 mutants into three groups characterized by (i) complete loss of function, (ii) temperature sensitivity or (iii) wt p53-like activity. These results are in agreement with previous studies (11,12). It has been noted that p53 mutants with few entries in p53 mutation databases often display wt-like activity, suggesting that many of these p53 mutants are not
causally linked to the cancer they are attributed to (30,45). R283H, M237R and T256A are likely to be examples of this type of p53 mutant. Likewise, a number of temperature-sensitive p53 mutants have been reported, and the Ishioka laboratory has systematically assessed their collection of over 2000 p53 mutants for temperature sensitivity (46). They identified 113 p53 mutants with activity at 30°C that represent ~10% of all reported single amino acid changes of the core domain in human cancers (8).

Interference of p53 mutants with wt p53
The focus of our study was the concomitant assessment of loss of function and interference with wt p53 for p53 mutants, since a combined analysis of these two functional aspects has not been performed to date. Our yeast assays are well suited to study both of these phenotypes, since they express wt and mutant p53 at approximately equal levels and have stringent, easy-to-score phenotypes (23). Most p53 mutants behaved as one would have predicted. The biggest

![Graphs showing relative activity of p53 mutants](image)

Fig. 2. Class 2 mutant R158L and Class 3 mutants R282W and Y234C show reduced interference with wt p53 in mammalian reporter gene assays. Class 2 mutants R273C and R158L and Class 3 mutants R282W and Y234C were evaluated in the same reporter gene assays as in Figure 1. Consistent with the results of Figure 1, R273C assays showed interference with wt p53 similar to R248Q, further confirming the discrepancy between yeast and mammalian results. Results for the other three p53 mutants were consistent with the observed phenotypes in yeast.
group of p53 mutants showed complete loss of function and interference with wt p53 (Class 1 in Table II), whereas very few p53 mutants had wt-like activity at 37°C and consequently did not inhibit wt p53 (Class 4 in Table II).

Surprisingly, however, two unique classes of p53 mutants emerged as the most significant discovery of our studies in yeast. We noticed a striking correlation between activity of temperature-sensitive p53 mutants at 25 or 30°C and lack of interference with wt p53 at 37°C. These findings correlated with results in mammalian reporter gene assays (Class 3 in Table II). We observed a second class of p53 mutants that did not interfere with wt p53 in yeast assays, even though they showed complete loss of function. Results in yeast assays for this class were largely confirmed in mammalian assays (Class 2 in Table II). We observed a second class of p53 mutants that did not interfere with wt p53 in yeast assays, even though they showed complete loss of function. Results in yeast assays for this class were largely confirmed in mammalian assays (Class 2 in Table II). The only exception was R273C, and the reasons for this are unclear. The slightly lower steady state protein levels for R273C compared with wt p53 in yeast may be part of the explanation, even though other p53 mutants with similar protein levels in yeast (R282W, C141Y and P151S) did not show this discrepancy between yeast and mammalian results. One possible explanation for the observed lack of interference of p53 mutants with wt p53 is that wt p53 aids mutant p53 to properly fold and maintain the wt p53 conformation in the context of mutant and wt heterotramers. Very likely, additional mechanisms contributed to the observation in mammalian reporter gene assays that some p53 mutants showed little interference with wt p53 even at a 25-fold excess of the mutant expression plasmid. These may include, for example, a prolonged half-life of wt p53 in the presence of mutant p53 as discussed earlier.

Our results suggest that a subset of p53 mutants show unique patterns of activity that are very different from the most commonly observed phenotype of complete loss of function and interference with wt p53. This subset is likely to be quite sizable, considering that temperature-sensitive p53 mutants of Shiroma et al. (46) (tested at 30°C only) represent ~10% of all cancer mutations resulting in a single amino acid core domain change (8) and taking into account that we identified an equally large set of p53 mutants with activity only at 25°C (Tables I and II). On the basis of our results, we estimate that temperature-sensitive mutants (Class 3 in Table II), combined with Class 2 (loss of function, but no interference with wt p53) and Class 4 (wt-like activity) mutants may account for as much as one-third of all p53 mutants found in human cancers (Table II, last column).

p53 mutants with unique cytoplasmic localization

Subcellular localization is another important aspect of the activities of p53 mutants, particularly in light of the recent discovery that wt p53 can induce apoptosis directly at the mitochondria (6). We found that the majority of over-expressed p53 mutants localized either predominantly to the nucleus or equally to nucleus and cytoplasm in H1299 cells. However, two p53 mutants, Y220C and Y163C, were predominantly localized to the cytoplasm (Class 5 in Table II) (37). These findings are unlikely to be artifacts, since we observed clear differences in localization despite overexpression of the p53 mutants, but rather are likely to reflect unique functional aspects of these p53 mutants in human cancers. Consistent with this notion, Y220C and Y163C showed little interference with wt p53 in mammalian reporter gene assays that score for nuclear activities of wt p53.

The importance of analyzing human cancers for loss of heterozygosity for p53

The discovery of p53 mutants that show no or little interference with wt p53 highlights the importance of determining whether human cancers with p53 mutations still carry the wt p53 allele or have lost it (loss of heterozygosity). For Classes 2, 3 and 5 of our collection, presence of the wt allele may
Importance of loss of heterozygosity in assessing p53 status of human cancers

Supplementary material

Supplementary data are available at Carcinogenesis online.

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References


indicate a cancer with good prognosis, because the p53 pathway is likely to still be somewhat functional. On the other hand, loss of heterozygosity may specify a poor prognosis cancer highly resistant to cancer therapy. The data for this central question are surprisingly limited. The IARC TP53 mutation database currently has loss of heterozygosity data for <1% of all reported p53 mutations (not released on the web site). Of 189 entries, 159 result in single amino acid changes in the core domain (90 distinct mutants). Ninety-four show loss of heterozygosity, whereas the remaining 65 cancers (41%) still carry wt p53 (M. Olivier and P. Hainaut, personal communication). Germline p53 mutations account for the large majority of the Li-Fraumeni syndrome, a rare autosomal disorder characterized by familial clustering of tumors (47–48). Similar to the limited data set on somatic cancers, loss of heterozygosity is found in only approximately half of all studied tumors in Li-Fraumeni patients (49–51). The currently available data for loss of heterozygosity are too small to identify meaningful correlations with our classes of p53 mutants, emphasizing the need for large clinical studies that not only identify p53 gene mutations but also assess for loss of heterozygosity. Such studies must overcome a number of challenges, including the problem of tumor heterogeneity, contamination of the sample by cancer-associated normal cells and acquisition of normal control cells for a reliable loss of heterozygosity analysis. Furthermore this type of analysis has to take into account that chromosome 17p deletions can also lead to loss of other tumor suppressor genes [see, for example, refs 52–54].

Outlook

Our strategy of analyzing p53 mutants simultaneously for loss of function and dominant-negative behavior provides a significant improvement compared with previously used systematic functional characterizations, as it is able to identify a unique subset of p53 cancer mutants that lack dominant-negative behavior, despite overexpression and loss of wt p53 function. Additional studies are needed to explore whether interference of mutant p53 with p53 family members p63 and p73 further divides our p53 mutant classes. Our studies were almost exclusively done with p53 proteins that contained a proline at position 72. Future studies involving p53 family members will have to pay even more attention to the amino acid at position 72, because an arginine at this position of p53 mutants leads to a much stronger interaction with and inhibition of p63 and p73 that correlates with poor response to chemotherapy and shorter survival in otherwise similar head and neck cancer patients (55–58). Our data are also useful because they identify p53 mutants that will be particularly informative to study in greater detail in other assay systems, such as mouse knock-in models.

The unique group of p53 core domain missense mutants without dominant-negative effect may account for one-third of all p53 mutants and has to be taken into consideration when analyzing large clinical data sets. For example, two recently published large studies on 3583 colorectal cancer patients and 1794 breast cancer patients could not analyze patients according to p53 hetero- or homozygosity because the data were not available (59,60). Our results suggest that analysis for p53 heterozygosity combined with functional data such as ours may uncover important clinical correlations that are otherwise lost.