Cell, Vol. 119, 847–860, December 17, 2004, Copyright ©2004 by Cell Press

Mutant *p53* Gain of Function in Two Mouse Models of Li-Fraumeni Syndrome

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Summary

The *p53* tumor suppressor gene is commonly altered in human tumors, predominantly through missense mutations that result in accumulation of mutant p53 protein. These mutations may confer dominant-negative or gain-of-function properties to *p53*. To ascertain the physiological effects of *p53* point mutation, the structural mutant $p53^{R172H}$ and the contact mutant $p53^{R270H}$ (codons 175 and 273 in humans) were engineered into the endogenous *p53* locus in mice. $p53^{R270H/+}$ and $p53^{R172H/+}$ mice are models of Li-Fraumeni Syndrome; they developed allele-specific tumor spectra distinct from $p53^{+/-}$ mice. In addition, $p53^{R270H/-}$ and $p53^{R172H/-}$ mice developed novel tumors compared to $p53^{-/-}$ mice, including a variety of carcinomas and more frequent endothelial tumors. Dominant effects that varied by allele and function were observed in primary cells derived from $p53^{R270H/+}$ and $p53^{R172H/+}$ mice. These results demonstrate that point mutant *p53* alleles expressed under physiological control have enhanced oncogenic potential beyond the simple loss of *p53* function.

Introduction

The *p53* tumor suppressor gene encodes a transcription factor that responds to cellular stresses by regulating genes involved in cell cycle arrest, apoptosis, and other pathways (reviewed in Levine [1997]). The p53 protein contains a core sequence-specific DNA binding domain (DBD) that directly binds to sequences in target gene promoters. Missense mutations in the *p53* DBD occur in a large fraction of human tumors and have been found in over 50 different types of cancer. Large deletions, though common in other tumor suppressor genes, occur rarely in *p53* (Olivier et al., 2002). Tumor-derived point mutations in *p53* fall into two general classes: contact mutations affect p53 residues that make direct contact with DNA, while structural mutations affect the global structure of the p53 DBD. Both classes of mutant p53 proteins commonly accumulate to high levels in tumor cells and are defective for wild-type p53 function. Furthermore, *p53* mutations have been linked to poor prognosis in a variety of tumor types and have been shown to affect cellular responses to chemotherapeutics (Cho et al., 1994; Peller, 1998). In addition to sporadic tumors, *p53* point mutations also occur in the germline of patients with Li-Fraumeni syndrome (LFS), a familial cancer predisposition syndrome in which patients develop a broad spectrum of malignancies (Kleihues et al., 1997).

Two prominent models have been proposed to explain the effects of *p53* missense mutation in cancer. The first suggests that hetero-oligomerization between mutant and wild-type p53 polypeptides results in dominant-negative effects on wild-type *p53* function. Considerable biochemical and cell-based data support the ability of different point mutant alleles to inhibit wild-type *p53* function, but the effects can be complex. For example, some mutant alleles of p53 can inhibit the ability of wild-type p53 to transactivate target genes involved in apoptosis, but not those involved in cell cycle arrest (Aurelio et al., 2000). Furthermore, the extent to which tumor-derived mutations in *p53* confer dominant-negative effects under physiological conditions is unclear. The second model suggests that *p53* mutations actively promote tumorigenesis independent of wild-type *p53* function. This "gain-of-function" model is supported by several studies in which *p53*-null cell lines were transformed with mutant *p53* constructs, resulting in increased tumorigenic potential (Dittmer et al., 1993; Hsiao et al., 1994; Shaulsky et al., 1991). Many studies have ascribed novel biochemical properties to mutant p53 that diverge from the function of wild-type p53, including the ability to regulate distinct sets of target genes and the ability to participate in novel protein-protein interactions (reviewed in Cadwell and Zambetti [2001]). Little is known about the relevance of these observations in vivo.

Substantial efforts have been made to model LFS in mice. We and others engineered mice lacking expression of the p53 protein. *p53* knockout mice are highly cancer prone; however, in contrast to LFS patients, the tumor spectrum of *p53* knockout mice is fairly restricted; both heterozygous and homozygous *p53* knockout mice develop primarily sarcomas and lymphomas. Carcinomas in these mice are rare, particularly in *p53*-null mice (Donehower et al., 1992; Jacks et al., 1994; Purdie et al., 1994). A number of groups have produced transgenic strains that overexpress mutant *p53* from exogenous promoters (Harvey et al., 1995; Lavigueur et al., 1989; Li et al., 1998; Wang et al., 1998). In each case, mutant *p53* transgene expression resulted in accelerated spontaneous or carcinogen-induced tumor development, supporting a dominant effect for mutant *p53*. For example, a $p53^{135V}$ transgene conferred accelerated tumorigenesis and an altered tumor spectrum on $p53^{+/+}$ and $p53^{+/-}$ mice (Harvey et al., 1995). Similarly, Liu et al. (2000) found an increased incidence of carcinomas and

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metastatic osteosarcomas, compared to $p53^{+/-}$ mice, in mice heterozygous for an endogenous mutant allele of *p53* ($p53^{R172H\Delta g}$); however, analysis of these mice was confounded by the presence of an extraneous splice acceptor mutation.

In order to accurately model LFS in mice and to investigate the potential for dominant-negative or gain-of-function effects by mutant *p53*, we constructed and characterized two mouse strains with endogenous point mutations of *p53*. These strains harbor contact or structural mutations that together comprise two of the three most commonly mutated *p53* codons in human cancer. These mice closely mimic humans with LFS both genotypically and phenotypically. Moreover, data from these mice provide compelling evidence for gain-of-function effects by mutant *p53*.

Results

Generation of Germline Point Mutant *p53* Mice

Two conditional point mutant *p53* alleles were engineered into the endogenous murine *p53* locus. They encode the contact mutant $p53^{R270H}$ and the structural mutant $p53^{R172H}$, both of which are commonly found in spontaneous human tumors and in the germline of LFS kindreds. (Murine *p53* codons 270 and 172 correspond to human *p53* codons 273 and 175.) In order to enable tissue- or stage-specific expression of mutant *p53* under physiological conditions, conditional mutant alleles of *p53* were generated by introducing the LoxP-flanked transcriptional STOP cassette into intron 1 of the *p53* gene. This Lox-STOP-Lox (LSL) cassette was used by our group previously to create a conditional oncogenic allele of *K-ras*; it efficiently blocks expression of targeted loci prior to removal of the STOP element by Cre recombinase (Tuveson et al., 2004). Site-directed mutagenesis was used to introduce an arg→his mutation at either codon 270 or codon 172, resulting in the conditional mutant alleles $p53^{LSL\bullet R270H}$ and $p53^{LSL\bullet R172H}$ (Figure 1A). These alleles were verified by sequencing the entire *p53* open reading frame and all intron/exon boundries in the targeting vectors (data not shown). Both alleles were targeted into J1 embryonic stem cells, and germline transmission was achieved in multiple clones. Presence of the STOP cassette in intron 1 of *p53* in these mice was determined by PCR (Figure 1B), and the presence of each mutation was reconfirmed by sequencing (data not shown).

In order to study the effects of endogenous expression of the mutant *p53* alleles in all tissues of the mouse, $p53^{LSL\bullet R270H/+}$ and $p53^{LSL\bullet R172H/+}$ mice were crossed to protamine-Cre transgenic mice, which express Cre recombinase in haploid sperm (O'Gorman et al., 1997). Resulting $p53^{LSL\cdot R270H/+}$; *PrmCre*/+ and $p53^{LSL\cdot R172H/+}$; *PrmCre*/+ male mice were then crossed to wild-type $129S_4$/SvJae females to generate $p53^{R270H/+}$ and $p53^{R172H/+}$ mice (collectively referred to as "$p53^{M/+}$" mice). Tail DNA samples from offspring were analyzed by PCR (Figure 1C).

Successful generation of conditional and germline point mutant *p53* mice was verified by several means. *p53* cDNAs were derived from $p53^{M/+}$ and $p53^{LSL\cdot M/+}$ mouse brains and sequenced. As expected, both mutant and wild-type alleles were found to be expressed in $p53^{R270H/+}$ and $p53^{R172H/+}$ samples, while only the wild-type allele was expressed in $p53^{LSL\cdot R270H/+}$ and $p53^{LSL\cdot R172H/+}$ samples (Figure 1D and data not shown). In addition, cDNAs derived from $p53^{R270H/-}$ and $p53^{R172H/-}$ primary mouse embryo fibroblasts (MEFs; see below) were sequenced, confirming the presence of only the desired mutations (data not shown). Quantitative real-time PCR analysis for *p53* confirmed that the point mutant alleles are expressed at levels comparable to wild-type *p53* in MEFs (Figure 1E).

Mutant p53 protein often accumulates to high levels in human tumors, and some mutant proteins adopt an altered conformation. In particular, structural mutants assume a partially denatured conformation that results in the exposure of novel epitopes recognized by mutant-specific α-p53 antibodies. In contrast, contact mutants have been described either as "flexible" (reactive to both wild-type- and mutant-specific antibodies) or as fully wild-type in conformation (Webley et al., 2000; Yewdell et al., 1986). In order to examine the biochemical properties of mutant p53 proteins, mice that express only point mutant p53 were generated by crossing $p53^{M/+}$ mice to $p53^{+/-}$ mice (Jacks et al., 1994) to generate $p53^{R270H/-}$ and $p53^{R172H/-}$ mice (collectively referred to as $p53^{M/-}$ mice). These mice appeared grossly similar to $p53^{-/-}$ mice at birth and throughout development (see below). They were born in expected Mendelian ratios except for a slight decrease in the number of $p53^{M/-}$ females, a phenotype that is also observed in $p53^{-/-}$ animals due to female-specific exencephaly (Sah et al., 1995). Figure 1F shows a Western blot for p53 performed on MEF whole-cell lysates confirming that mutant p53 accumulated to high levels in these cells. In addition, these experiments also revealed that the contact mutant $p53^{R270H}$ accumulated to higher levels in $p53^{M/-}$ MEFs than the structural mutant $p53^{R172H}$. This effect was reproduced using three different antibodies against p53 (data not shown). As shown in Figure 1G, the structural mutant $p53^{R172H}$ was preferentially immunoprecipitated by the mutant-specific antibody PAb 240 while the contact mutant $p53^{R270H}$ was preferentially immunoprecipitated by the wild-type-specific antibody PAb 1620.

$p53^{M/+}$ Mice Develop Distinct Tumor Spectra

In order to evaluate whether the $p53^{M/+}$ mouse strains are improved models of LFS and to compare the effects of *p53* point mutation to *p53*-null mutation, a cohort of 37 $p53^{R270H/+}$, 41 $p53^{R172H/+}$, and 37 $p53^{+/-}$ mice were aged and monitored for the onset of malignancy. As shown in Figure 2A, the life spans of $p53^{R270H/+}$, $p53^{R172H/+}$, and $p53^{+/-}$ mice were similar with mean survival times of 15.8, 15.2, and 15.4 months, respectively (Kaplan-Meyer log-rank test). Each mouse was subjected to a complete necropsy, and histopathological analysis of all tissues regardless of whether tumors were apparent. The pathology of each mouse in this study is available in the Supplemental Data at http://www.cell.com/cgi/content/full/119/6/847/DC1/.

The $p53^{M/+}$ mice demonstrated significant differences in their tumor spectra, both compared to the $p53^{+/-}$ mice as well as to each other (Figure 2B). The $p53^{R270H/+}$ mice had an increased incidence of carcinomas compared to $p53^{+/-}$ mice (χ^2, $p = 0.017$). In particular, 7/36

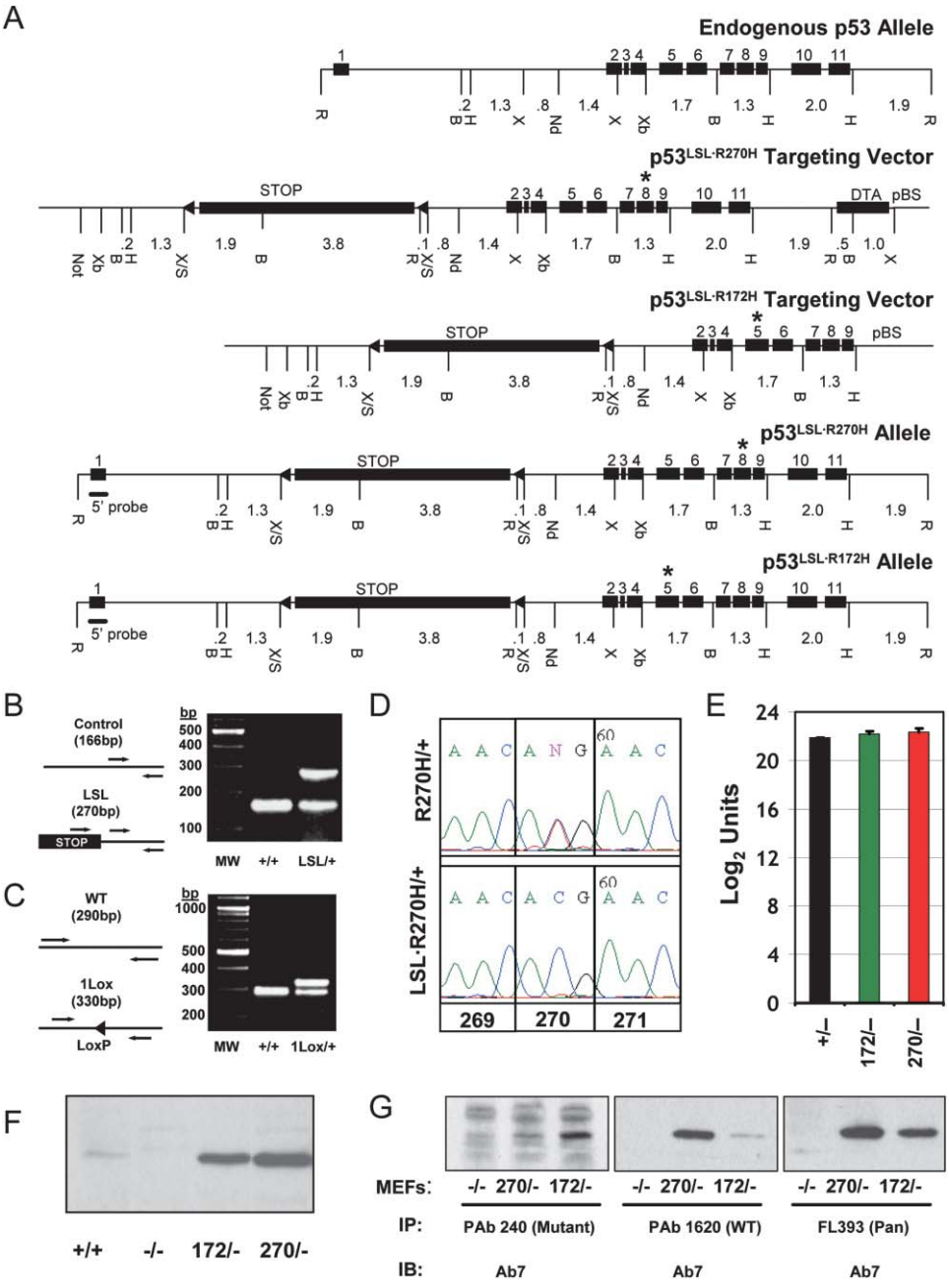

Figure 1. Generation, Targeting, and Verification of Conditional Point Mutant *p53* Alleles

(A) The endogenous *p53* locus is depicted at top, followed by the $p53^{LSL\text{-}R270H}$ and $p53^{LSL\text{-}R172H}$ targeting vectors. Asterisks indicate the sights of point mutations. Targeted mutant *p53* alleles are shown at bottom. Restriction sites are indicated: R, EcoRI; B, BamHI; H, HindIII; X, Xho; Nd; NdeI, Xb, Xba; S, Sal.

(B) PCR amplification of conditional mutant alleles results in a 270 bp mutant product and a 166 bp control product.

(C) PCR primers flanking the LSL cassette yield a 290 bp wild-type band and a 330 bp mutant p53 band.

(D) Sequence traces from $p53^{LSL\text{-}R270H/+}$ (bottom) and $p53^{R270H/+}$ (top) mouse brain cDNAs. N indicates expression of both the wild-type allele (C) and the mutant allele (T).

(E) qRT-PCR analysis of *p53* in WT, $p53^{R172H/-}$ and $p53^{R270H/-}$ MEFs. Histograms depict $\log_2$ induction relative to background in $p53^{-/-}$ MEFs and are the average of three different MEF lines, each assayed in triplicate. Error bars indicate standard error.

(F) Western blot analysis of MEF whole-cell lysates shows accumulation of mutant p53 proteins.

(G) Conformation of mutant p53 proteins was determined by immunoprecipitation of p53 proteins from 1000 μg of MEF whole-cell lysates with 10 μl of either *p53* Ab5 (ORP), *p53* Ab3 (ORP), or FL-393 (Santa Cruz), followed by Western blotting with p53 Ab7 (ORP).

$p53^{R270H/+}$ mice developed lung adenocarcinomas, including several with malignant features commonly seen in human lung adenocarcinoma such as nuclear atypia (5/7), desmoplasia (4/7), and metastasis (2/7). Other carcinomas found in $p53^{R270H/+}$ mice included five squamous cell carcinomas, two hepatocellular carcinomas, a tran-

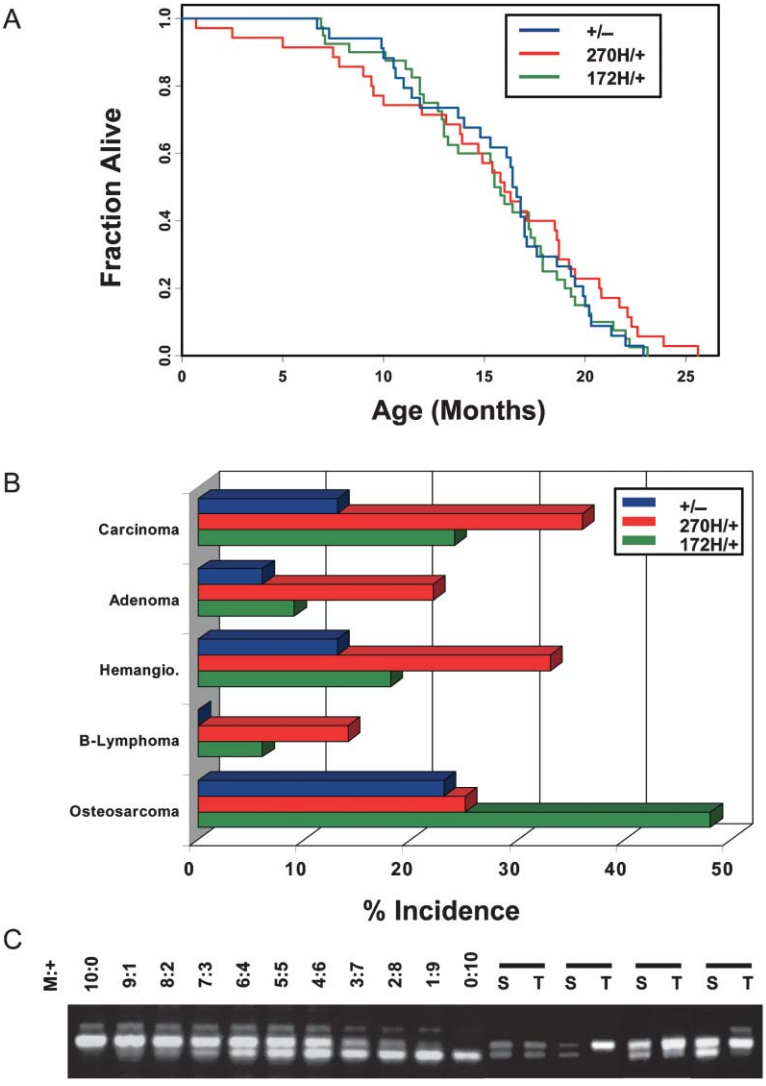

Figure 2. Mice Heterozygous for Mutant p53 Develop Novel Tumors

(A) Kaplan-Meyer plot demonstrates no difference in survival time between $p53^{+/-}$, $p53^{R270H/+}$, and $p53^{R172H/+}$ mice.

(B) Distinct tumor spectra in $p53^{M/+}$ mice. Histograms show the fraction of $p53^{+/-}$ (blue), $p53^{R270H/+}$ (red), and $p53^{R172H/+}$ (green) mice that developed the indicated tumors. See Supplemental Figure S1 on the *Cell* website for additional data.

(C) PCR analysis was performed on matched tumor (T) and somatic (S) DNA samples to look for LOH. At left is a dilution series of $p53^{M/M}$ and $p53^{+/+}$ DNA for reference.

sitional cell carcinoma of the kidney, and an intestinal carcinoma (Supplemental Figures S2A–S2C on the *Cell* website). Many of these tumors were invasive or showed evidence of distal metastases (Supplemental Figures S2D and S2E on the *Cell* website). Immunohistochemistry (IHC) was performed on some tumors to further support their epithelial origin (Supplemental Table S1 and Supplemental Figure S3A on the *Cell* website). In contrast to the $p53^{R270H/+}$ mice, only four low-grade carcinomas were found in 37 $p53^{+/-}$ mice, similar to previous reports (Donehower et al., 1992; Jacks et al., 1994). The single lung carcinoma observed in a $p53^{+/-}$ mouse was well differentiated and lacked severely dysplastic nuclei or stromal deposition. Premalignant epithelial lesions such as adenomas and papillomas were also more common in the $p53^{R270H/+}$ mice than in the $p53^{+/-}$ mice (Supplemental Figure S1 on the *Cell* website).

In addition to carcinomas, $p53^{R270H/+}$ mice also had an increased incidence of B cell lymphomas. 5/36 $p53^{R270H/+}$ mice developed B cell lineage tumors (χ^2, $p = 0.031$; Supplemental Figures S3B and S3C on the *Cell* website). They generally arose in the spleen or mesenteric lymph nodes and spread through the abdominal cavity, sometimes invading adjacent organs such as the kidneys and intestines (Supplemental Figure S3B on the *Cell* website). Overall, $p53^{R270H/+}$ mice had an increased tumor burden compared to $p53^{+/-}$ mice; 44% of $p53^{R270H/+}$ mice had developed multiple tumors at the time of necropsy compared to 19% of $p53^{+/-}$ mice (χ^2, $p = 0.03$).

In contrast to the frequent carcinomas in $p53^{R270H/+}$ mice, the most frequent tumors in the $p53^{R172H/+}$ mice were osteosarcomas. Roughly twice as many $p53^{R172H/+}$ mice developed osteosarcomas as did $p53^{+/-}$ and $p53^{R270H/+}$ mice (χ^2, $p = 0.043$ for $p53^{+/-}$ mice, $p = 0.039$ for $p53^{R270H/+}$ mice). These tumors developed primarily in trabecular regions of vertebrae or longbones. Diffuse osteopetrosis was commonly observed concurrent with the osteosarcomas. In addition, the $p53^{R172H/+}$ osteosarcomas metastasized more frequently than those in the $p53^{+/-}$ or $p53^{R270H/+}$ mice, generally to the liver, lung, or spleen (Supplemental Figures S2E and S2F on the *Cell* website). Only four osteosarcomas in $p53^{R172H/+}$ mice did not metastasize, including two early lesions found in mice that died from other causes. The $p53^{R172H/+}$ mice

also developed carcinomas slightly more frequently than $p53^{+/-}$ mice (χ^2, p = 0.38); however, they did not display the high frequency of lung adenocarcinomas noted in $p53^{R270H/+}$ mice.

Tumors that develop in humans with hereditary *p53* mutations frequently lose the remaining wild-type *p53* allele. It was therefore of interest to determine the status of the wild-type *p53* allele in tumors from $p53^{M/+}$ mice. The mutant *p53* reaction described earlier (Figure 1C) was modified to be quantitative by terminating the reaction after 29 cycles rather than 35. The sensitivity of this assay was confirmed by running a dilution series of $p53^{+/+}$ and $p53^{M/M}$ samples (Figure 2C). PCR analysis of DNA derived from $p53^{M/+}$ tumors revealed evidence of loss of heterozygosity (LOH) in 10/19 tumors analyzed (4/10 $p53^{R270H/+}$ and 6/9 $p53^{R172H/+}$), further validating these mice as models for LFS.

Dominant Effects of Mutant p53 in Primary Cells

There are several potential explanations for the differences in tumor spectra between the $p53^{M/+}$ mice and $p53^{+/-}$ mice. Mutant *p53* may act in a dominant-negative manner to interfere with the function of the wild-type *p53* allele. Alternatively, the mutant *p53* alleles may contribute novel protumorigenic functions. These two mechanisms are not mutually exclusive. In order to assess the dominant effects of endogenous mutant *p53*, several well-established assays for *p53*-dependent functions were performed on $p53^{M/+}$ MEFs and thymocytes. For example, *p53* can act to restrain cell proliferation, as $p53^{-/-}$ MEFs cycle more quickly than wild-type or $p53^{+/-}$ MEFs. Therefore, we measured cell proliferation in $p53^{M/+}$ MEFs by analyzing the fraction of cells in S phase via FACS analysis of bromodeoxyuridine (BrdU) and propidium iodide (PI) incorporation. As expected, a large fraction of $p53^{-/-}$ MEFs were in S phase during exponential growth compared to wild-type or $p53^{+/-}$ MEFs. Interestingly, both the $p53^{R270H/+}$ and $p53^{R172H/+}$ MEFs had a larger S phase fraction than did $p53^{+/-}$ MEFs, with the $p53^{R172H/+}$ MEFs appearing comparable to $p53^{-/-}$ MEFs in this assay (Figure 3A, solid bars and Supplemental Figure S4A on the *Cell* website). Evidence of an elevated proliferation rate in $p53^{R172H/+}$ MEFs was also apparent when they were submitted to the classical 3T3 passaging protocol (Todaro and Green, 1963). $p53^{R172H/+}$ MEFs grew as rapidly as $p53^{-/-}$ MEFs for four passages before entering replicative senescence. Immortalized clones quickly escaped senescence and PCR analysis demonstrated loss of the wild-type *p53* allele in these clones (Supplemental Figure S4B on the *Cell* website and data not shown). *p53* is also required for G_1 arrest in MEFs after DNA damage (Kastan et al., 1992). Surprisingly, the DNA damage-induced G_1 arrest response was intact in $p53^{M/+}$ MEFs: after treatment with the DNA damaging agent doxorubicin, $p53^{M/+}$ MEFs arrested in the G_1 phase to a similar extent as wild-type cells (Figure 3A). Thus, physiological expression of point mutant *p53* in MEFs appears to dominantly interfere with cellular functions related to basal proliferation but does not affect DNA damage-induced G_1 arrest.

As DNA damage-induced G_1 arrest in MEFs is primarily dependent on the ability of p53 to transactivate the cell cycle inhibitor *p21*, this pathway was investigated in

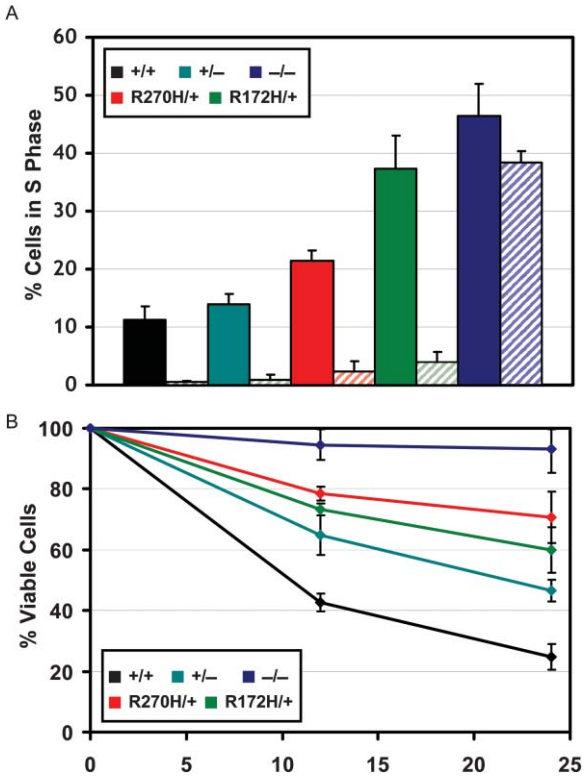

Figure 3. Dominant Effects by Mutant p53 in Primary Cells

(A) S phase fraction in $p53^{M/+}$ MEFs. Cell cycle analysis was performed on exponentially growing MEFs. Histograms show the percent of untreated cells (solid bars) or doxorubicin-treated cells (hashed bars—0.2 μg/ml, 12 hr) in S phase as measured by FACS analysis of bromo-deoxyuridine and propidium iodide incorporation. Histograms show the average values of four independently derived MEF lines for each genotype. Error bars show standard deviation. See Supplemental Figure S3A on the *Cell* website for additional data.

(B) Apoptosis in thymocytes from $p53^{M/+}$ mice. Thymocytes were isolated from 6- to 9-week-old mice and treated with 5Gy of γ radiation. Apoptosis was measured by FACS analysis of Annexin-V expression and propidium iodide exclusion. Graph depicts the percentage of viable cells (Annexin-V-negative, propidium iodide-negative) at 12 and 24 hr post treatment, normalized to untreated controls. The average values from at least three mice per genotype are shown.

$p53^{M/+}$ MEFs. As shown in Figure 4A, the amount of total p53 in untreated $p53^{M/+}$ MEFs was elevated compared to $p53^{+/+}$ or $p53^{+/-}$ MEFs and increased further upon treatment with doxorubicin. p21 levels, however, were comparable to those found in $p53^{+/-}$ MEFs, suggesting that mixed mutant and wild-type p53 tetramers are competent to transactivate at least some target genes. An IP-Western for MDM2 supports this notion and also suggests that the accumulation of high levels of mutant p53 in these cells is not due to a loss of MDM2 transactivation by mutant p53.

Finally, in order to analyze the ability of $p53^{M/+}$ cells to undergo *p53*-dependent apoptosis, primary thymocytes were isolated from 6- to 8-week-old mice. The apoptotic response of $p53^{M/+}$ thymocytes to 5Gy of γ radiation was assayed by FACS analysis for Annexin-V expression and propidium iodide exclusion. The relative fraction of

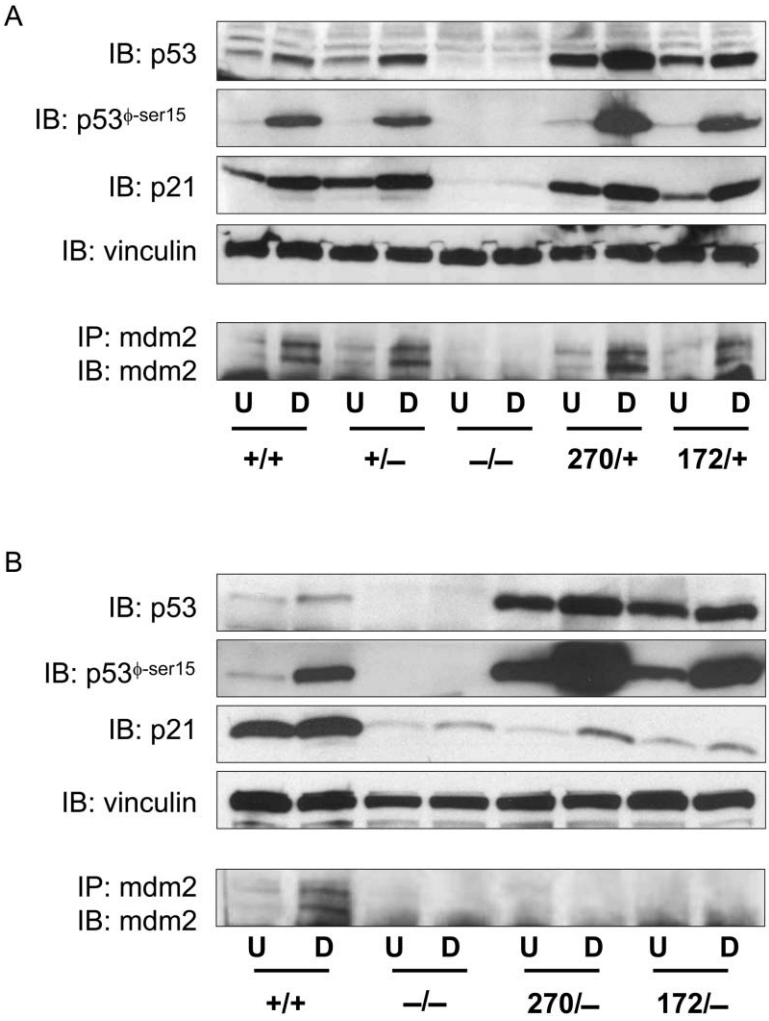

Figure 4. *p53* Target Gene Expression in Mutant *p53* Cells

(A) Immunoblots for p53, ϕ-ser^{15}-p53, and p21 on MEF whole-cell lysates are shown. MDM2 immunoprecipitates from the same extracts are also shown.

(B) Immunoblots for p53, ϕ-ser^{15}-p53, and p21 on MEF whole-cell lysates are shown. MDM2 immunoprecipitates from the same extracts are also shown.

viable cells after doxorubicin treatment is shown in Figure 3B. As expected, $p53^{-/-}$ thymocytes were completely resistant to radiation-induced apoptosis through 24 hr, whereas only 20% of wild-type thymocytes survived to this time point. Both $p53^{R270H/+}$ and $p53^{R172H/+}$ thymocytes were partially resistant to γ-induced apoptosis. The $p53^{M/+}$ thymocytes were more resistant to apoptosis than $p53^{+/-}$ cells, consistent with a dominant effect by point mutant proteins on apoptosis. These results are similar to previous experiments on point mutant *p53* thymocytes generated by $Rag2^{-/-}$ blastocyst complementation (de Vries et al., 2002).

p53 Gain of Function: Mutant *p53* Predisposes Mice to Epithelial and Endothelial Tumors

To directly evaluate the *p53* gain-of-function hypothesis in vivo, spontaneous tumor development in mice expressing only mutant p53 was compared to that in mice lacking p53. If the point mutant *p53* alleles possess inherent oncogenic functions, $p53^{M/-}$ mice could have a more pronounced tumor phenotype than *p53*-null mice. Forty-one $p53^{-/-}$, forty $p53^{R270H/-}$, and forty-five $p53^{R172H/-}$ mice were generated as described above. All of the mice required euthanasia or died by 8.5 months of age, with a mean survival time of 4.4, 4.5, and 4.6 months for the $p53^{-/-}$, $p53^{R270H/-}$ and $p53^{R172H/-}$ mice, respectively. There was no significant difference in the survival of *p53* mutant mice compared to *p53*-null mice (Kaplan-Meyer log rank test, Figure 5A).

In support of the mutant *p53* gain-of-function hypothesis, two significant differences were observed in the tumor spectra of the $p53^{M/-}$ mice compared to $p53^{-/-}$ mice (Figure 5B). In previous studies, $p53^{-/-}$ mice primarily developed lymphomas and sarcomas and rarely developed carcinomas. Likewise, none of the $p53^{-/-}$ mice in this study developed carcinomas. In contrast, carcinomas were observed in 18% of the $p53^{R270H/-}$ mice and 16% of $p53^{R172H/-}$ mice (χ^2, $p = 0.008$ for $p53^{R270H/-}$ and $p = 0.015$ for $p53^{R172H/-}$). Carcinomas found in the point mutant *p53* mice arose in a variety of different tissues including lung, small intestine, colon, breast, skin, liver, and pancreas. These tumors developed in mice ranging from 1.5 to 6.9 months of age and, in one case, two different primary carcinomas were observed in the same mouse. Most of the carcinomas showed evidence of invasion, metastasis, or other features commonly seen in advanced human carcinomas such as desmoplasia and stromal invasion (Figures 6A–6E). In

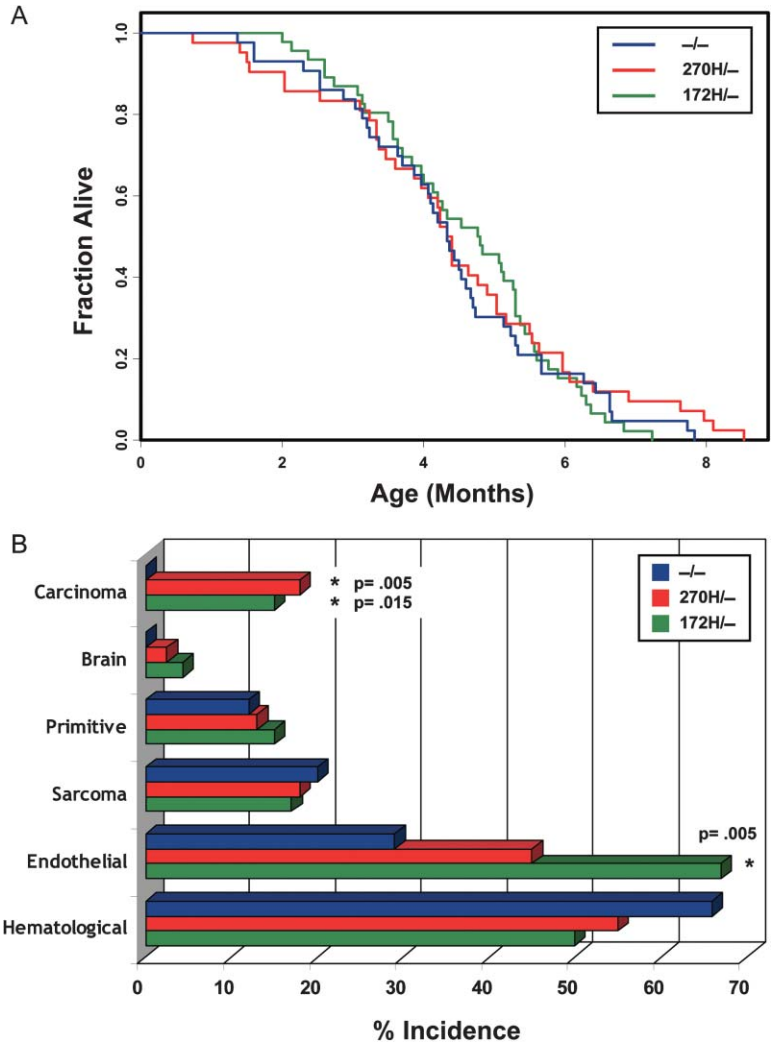

Figure 5. Point Mutant *p53* Mice Develop Novel Tumors Compared to *p53* Knockout Mice

(A) Kaplan-Meyer plot demonstrates no difference in survival time of $p53^{-/-}$, $p53^{R270H/-}$, and $p53^{R172H/-}$ mice.

(B) Histograms show the fraction of $p53^{-/-}$ (blue), $p53^{R270H/-}$ (red), and $p53^{R172H/-}$ (green) mice that developed the indicated tumors.

most instances, the epithelial origin of these tumors was readily apparent through histopathology; however IHC was also used to support these diagnoses (Supplemental Figures S3E–S3F and Supplemental Table S1 on the *Cell* website).

In addition to epithelial tumors, the point mutant *p53* mice also demonstrated an increased incidence of hemangiosarcomas (Figures 5B and 6F). The $p53^{R172H/-}$ mice, in particular, showed a significant increase to 62% incidence (27/45), compared to 32% (13/41) in the $p53^{-/-}$ mice (χ^2, $p = 0.005$). These tumors were highly aggressive, demonstrated marked nuclear atypia, and stained positively for the endothelial tumor marker Fli-1 (data not shown).

Similar to previous studies, 66% of the $p53^{-/-}$ mice in this study developed hematological malignancies, primarily T cell lymphomas. A slight decrease in the incidence of hematological malignancies was observed in $p53^{M/-}$ mice (55% of $p53^{R270H/-}$ mice and 50% of $p53^{R172H/-}$ mice), although there was no shift in the survival time of $p53^{M/-}$ mice with lymphomas. The other tumors observed in $p53^{M/-}$ mice were found at frequencies similar to that of $p53^{-/-}$ mice. Overall, 32% of $p53^{-/-}$ mice developed multiple tumors, compared to 43% of $p53^{R270H/-}$ mice (χ^2, $p = 0.31$) and 57% of $p53^{R172H/-}$ mice (χ^2, $p = 0.02$).

Mutant p53 Accumulation in Tumors

Tumors cells from LFS patients often show nuclear accumulation of mutant p53 protein. In order to determine whether tumors from $p53^{M/+}$ mice were similar in this respect, IHC for p53 was performed on tumors from the aging studies. (Supplemental Figures S6A–S6C and Supplemental Table S2 on the *Cell* website). Interestingly, 12/16 carcinomas from $p53^{M/+}$ mice exhibited substantial nuclear accumulation of p53 protein. Three of the four tumors that did not accumulate p53 were low-grade lung adenocarcinomas. More advanced lung adenocarcinomas stained positively for p53 accumulation in patches that correlated with more malignant histology (Supplemental Figure S6C on the *Cell* website). In addition, 11/13 osteosarcomas in $p53^{M/+}$ mice were found to accumulate p53 to high levels in at least a subset of cells.

Several carcinomas that stained positively for p53 also exhibited stromal desmoplasia. In each case, the stro-

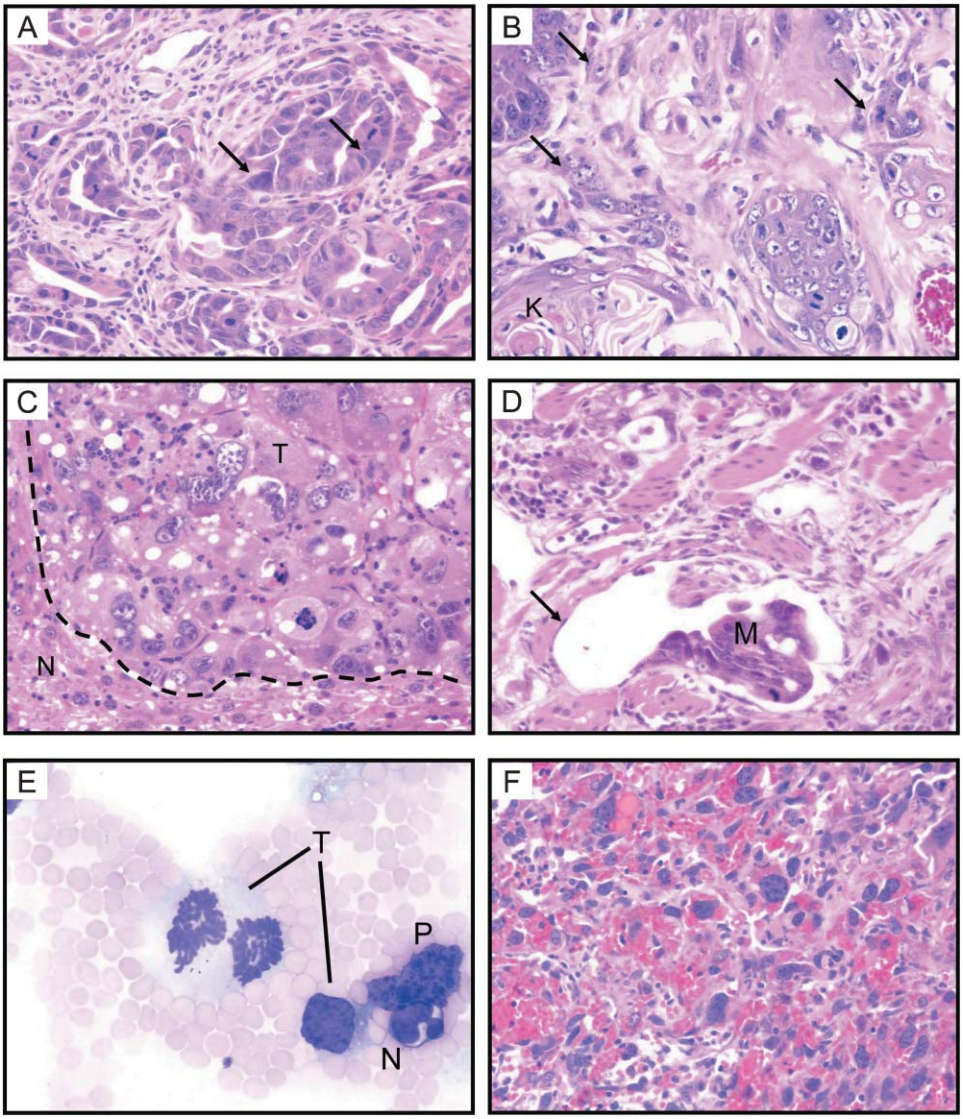

Figure 6. Histopathology of Point Mutant *p53* Tumors Stained with Hematoxylin and Eosin

(A) A moderately differentiated $p53^{R172H/-}$ colon adenocarcinoma with a high mitotic index, severe nuclear dysplasia (arrows), desmoplasia, and stromal invasion (400X).

(B) A $p53^{R172H/-}$ squamous cell carcinoma with well-differentiated cells forming a keratin pearl (K) and poorly differentiated cells alone or in small clusters (arrows) surrounded by tumor stroma (400X).

(C) A highly pleomorphic hepatocellular carcinoma (T) from a $p53^{R270H/-}$ mouse. Note the bizarre, enlarged nuclei found within the tumor compared to the nuclei of adjacent normal hepatocytes (N) (400X).

(D) Intralymphatic metastasis (M) from a $p53^{R172H/-}$ colon adenocarcinoma. Arrow indicates the lymphendothelium (400X).

(E) Wright-Giemsa-stained peripheral blood smear from a $p53^{R172H/-}$ mouse with a colon adenocarcinoma. Circulating tumor cells are indicated (T), including an actively dividing polyploid cell with a roughly 8N complement of chromosomes. Nearby are a normal neutrophil (N) and a cluster of platelets (P) (1000X).

(F) A $p53^{R172H/-}$ hemangiosarcoma (400X).

mal cells did not express high levels of p53 (Supplemental Figures S6A and S6B). Likewise, normal tissues from $p53^{M/+}$ mice did not show evidence of p53 accumulation. These data might indicate that residual *p53* function in $p53^{M/+}$ mice is sufficient to restrain p53 accumulation in normal but not neoplastic cells. Alternatively, the tumor cells that accumulated p53 might have lost their wild-type *p53* allele. Indeed, in determining whether LOH had occurred in $p53^{M/+}$ tumors (see above), it was observed that LOH in a tumor generally correlated with the accumulation of mutant p53 protein. However, there were several exceptions: tumors were found that had undergone LOH but did not accumulate mutant p53 and, conversely, that accumulated mutant p53 but retained heterozygosity. Furthermore, if mutant p53 protein accumulation is dependent on a loss of wild-type p53 function, it would follow that mutant p53 would accumulate to high levels in the normal tissues of $p53^{M/-}$ mice,

which lack any wild-type *p53* function. To test this directly, IHC for p53 was performed on tumors and normal tissues from $p53^{M/-}$ mice. As shown in Supplemental Table S2 on the *Cell* website, p53 was readily detected in the majority of, but not all, $p53^{M/-}$ tumors. However, in no case was p53 accumulation observed in normal tissues from these mice. Furthermore, as shown in Supplemental Figures S6D–S6F on the *Cell* website, mutant p53 does accumulate in normal tissues of $p53^{M/-}$ mice after γ irradiation, but only in the same cells and tissues observed to accumulate p53 in wild-type animals. Thus, tumor-associated increases in p53 staining cannot be ascribed solely to the *p53* genotype of the cell, but rather would appear to reflect a secondary event that causes mutant p53 stabilization. Interestingly, thymic lymphomas, which occur frequently in $p53^{-/-}$, as well as $p53^{M/-}$ mice, consistently showed a lack of p53 accumulation, perhaps indicating that the initiation of this tumor requires only the loss of *p53* function.

Loss of Wild-Type Function in $p53^{M/-}$ Primary Cells

Point mutant *p53* alleles have alternatively been reported to be completely or partially defective in transactivation (Forrester et al., 1995). In order to determine whether the $p53^{R270H}$ and $p53^{R172H}$ mutants had lost wild-type *p53* activity, the cellular assays described above were carried out on $p53^{M/-}$ and $p53^{-/-}$ MEFs. No difference was seen between the point mutant MEFs and $p53^{-/-}$ MEFs in basal S phase fraction or in doxorubicin-induced cell cycle arrest (Supplemental Figure S5A on the *Cell* website). $p53^{M/-}$ MEFs were also found to be immortal by 3T3 assay, but they were unable to form foci at high density or to form colonies in soft agar, similar to $p53^{-/-}$ MEFs (Supplemental Figure S5Bon the *Cell* website and data not shown). Likewise, there was no difference between $p53^{-/-}$, $p53^{R270H/-}$, and $p53^{R172H/-}$ MEFs in their abilities to induce colony formation at low density or their abilities to proliferate in low serum (data not shown). Finally, mutant *p53* alleles were defective in inducing apoptosis in primary thymocytes after γ irradiation, similar to $p53^{-/-}$ thymocytes (data not shown).

In order to assess the ability of mutant p53 to transactivate target genes, Western blotting for p53, $p53^{\phi\text{-ser15}}$, p21, and MDM2 was performed on protein extracts from $p53^{M/-}$ MEFs. Although mutant p53 accumulated and was phosphorylated in response to doxorubicin treatment, p21 and MDM2 were not induced in mutant *p53* MEFs (Figure 4B).

Mutant p53 Gain of Function in Tumor-Derived Cells

Previous work has demonstrated that overexpression of mutant p53 protein in *p53*-null cells results in aggravation of the tumorigenic phenotype of those cells. The reverse experiment, however, has not been performed on contact or structural mutant p53-bearing cells. We therefore utilized a conditional lentiviral shRNA expression construct, pSico-p53 (Ventura et al., 2004), to knock down mutant p53 expression in an endogenous setting. To allow selection of cells bearing the conditional shRNA, a puromycin resistance cassette was substituted for the GFP cassette of pSico-p53, to give $pSico^{P}$-p53.

In an effort to find a cellular system sensitive to the expression of mutant *p53*, a cell line, Os1, was derived from an osteosarcoma metastasis that arose in a $p53^{R172H/+}$ mouse. Analysis of DNA from Os1 cells indicated that they had lost their wild-type *p53* allele (data not shown). Os1 cells were infected with $pSico^{P}$-p53, selected in puromycin, and then superinfected with either a Cre-expressing adenovirus (AdCre) or a control adenovirus (AdEmpty). The percentage of cells infected by AdCre was greater than 90%, as assessed by the renewed susceptibility of the cells to puromycin (the puromycin resistance cassette is lost upon activation of shRNA expression by Cre). To control for potential side effects of expressing Cre recombinase, parental Os1 cells were also treated with AdCre (Figure 7A). The infected cells were expanded, and protein was collected from cells in the presence or absence of the DNA damaging agent (cisplatin). As seen in Figure 7B, *p53* shRNA expression resulted in substantial knockdown of mutant p53, both before and after treatment with cisplatin.

In the course of expanding the cells, it was noticed that the *p53* shRNA-expressing cells grew more slowly than the control cells. To quantify this effect, a proliferation assay was performed on *p53* shRNA-expressing and control Os1 cells. As seen in Figure 7C, Os1 cells proliferate more slowly after knockdown of mutant p53. As this experiment took place several passages subsequent to the initial adenoviral infections, the experiments were repeated just 48 hr after infection with AdCre or AdEmpty. As seen in Figure 7D, the inhibition of proliferation was even more dramatic immediately after infection. This effect was visualized by diluting the cells 1:500 with wild-type MEFs and allowing them to grow to high density (Figure 7E).

It was previously reported that mutant p53 can bind to and inhibit the function of family-member proteins *p63* and *p73*. In order to determine whether this occurs in Os1 cells, p73 immunoprecipitates were probed with an antibody against p53. As shown in Figure 7F, mutant p53 was coimmunoprecipitated with p73, but not by a mouse antibody specific for an unrelated protein. Similar results were seen with an antibody against p63 and a second antibody against p73 (data not shown). Furthermore, qPCR experiments demonstrated an increase in p63 and p73 target gene expression following knockdown of mutant p53 (data not shown). These results support the inhibition of p63 and p73 by protein-protein interaction as a potential mechanism for the observed gain of function by mutant p53.

Discussion

p53 was described 25 years ago as a cellular protein bound by the SV40 large T antigen and as a tumor antigen expressed in carcinogen-induced mouse tumors (DeLeo et al., 1979; Lane and Crawford, 1979; Linzer and Levine, 1979). *p53* was originally considered to be an oncogene due to its ability to cooperate in the transformation of cells (Eliyahu et al., 1984; Parada et al., 1984) and its overexpression in tumors and transformed cells. However, a series of observations changed the perception of *p53* function in tumorigenesis, suggesting that it acted instead as a tumor suppressor gene. First, *p53* cDNAs that had been used in oncogene cooperation

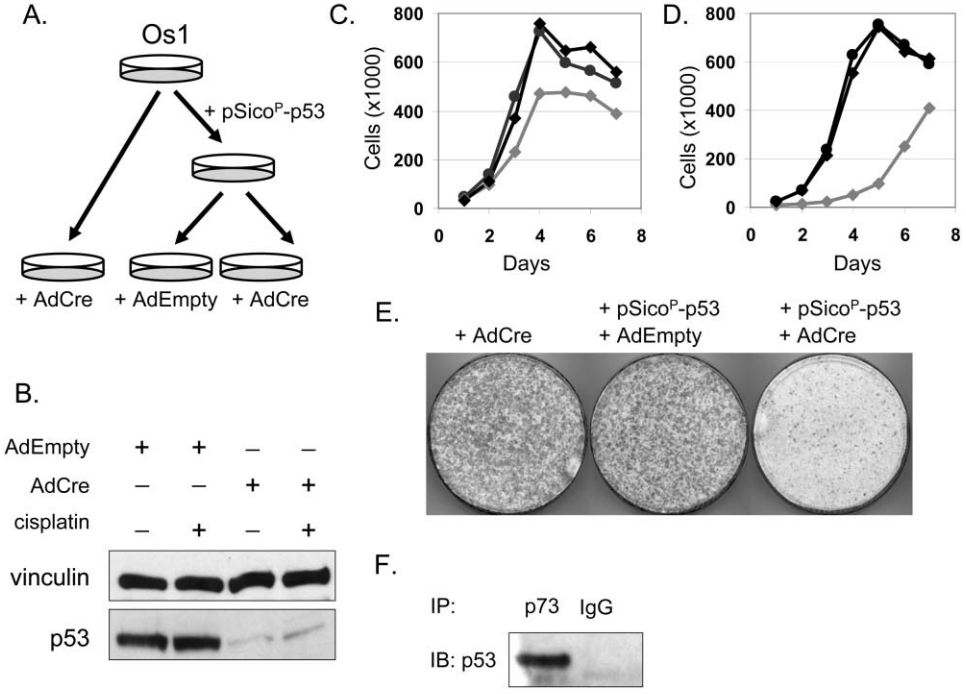

Figure 7. Mutant p53 Gain of Function in Tumor-Derived Cells

(A) Os1 cells were infected with pSico^P-p53 and AdCre to knock down endogenous mutant p53. Control cells were infected with AdCre alone or pSico^P-p53 + AdEmpty.

(B) Western blot for p53 in Os1 + pSico^P-p53 after infection with AdCre or AdEmpty demonstrates a strong shRNA-mediated knockdown of mutant p53. The membrane was also probed with an antibody against vinculin as a loading control.

(C and D) Os1 cells proliferate more slowly after mutant p53 knockdown. 3×10^4 cells/well were plated in 12 well dishes, and cells from two wells were counted daily. Experiments were initiated either three passages after adenoviral infection (C) or immediately following infection (D).

(E) Os1 cells proliferate to higher density prior to mutant p53 knockdown. Immediately following adenoviral infection, 2×10^4 Os1 cells were diluted into 1×10^6 wild-type MEFs and plated in 10 cm dishes. After 1 week, cells plates were stained with Wright-Giemsa. Darker staining in plates without mutant p53 KD reflect the ability of Os1 cells to rapidly grow to high density rather than their ability to form classic foci.

(F) Mutant p53 is coimmunoprecipitated by p73 Ab2 (ORP), but not by a control antibody. Immunoblot was performed using p53 Ab7.

assays were found to be mutant, and wild-type p53 cDNAs were shown to inhibit cellular transformation (Finlay et al., 1989; Hinds et al., 1989). Second, *p53* was determined to be mutated frequently and at numerous codons in a wide range of human tumors and was seen to undergo loss of heterozygosity with high frequency (Baker et al., 1990; Hollstein et al., 1991). Finally, loss-of-function mutations in *p53* in the mouse led to profound cancer predispositions. In this light, the tumor-associated point mutations in p53 were generally viewed to be acting in a dominant-negative manner to inhibit wild-type p53.

While the tumor-suppressive functions of wild-type *p53* are not in question, there have been persistent reports that point mutant p53 proteins can affect transformation independent of wild-type p53. These studies have led to the suggestion that point mutant *p53* has gain-of-function oncogenic properties. However, because these experiments have involved ectopic overexpression of the mutant p53 allele, they have been interpreted with caution. Here we describe a definitive test for the existence of oncogenic functions of point mutant p53.

Several important conclusions can be drawn from the analysis of mice with knockin alleles of tumor-derived mutations in p53. First, $p53^{M/+}$ mice are models of Li-Fraumeni syndrome that better recapitulate the human familial syndrome than do $p53^{+/-}$ mice. Moreover, $p53^{R270H/+}$ mice develop distinct tumor spectra from $p53^{R172H/+}$ mice, indicating that different *p53* point mutations confer subtly different functions. Specific functional assays on primary cells derived from $p53^{M/+}$ mice also support this conclusion. $p53^{M/-}$ mice develop tumor spectra distinct from *p53*-null mice, and tumor cells derived from mutant *p53* mice are sensitive to the shRNA-mediated knockdown of mutant *p53*, strongly supporting a gain-of-function effect by mutant *p53*.

$p53^{M/+}$ Mice Are Models of Li-Fraumeni Syndrome

Of the 20 or so tumor suppressor genes associated with human familial cancer syndromes, *p53* is unusual in that it is commonly altered through missense mutation rather than deletion and because mutant p53 proteins are upregulated in tumors. A few LFS kindreds have alterations akin to null alleles of p53. An analysis of seven such families concluded that these patients developed fewer cancers overall and at an older age than LFS patients with point mutations in the p53 DBD (Birch et al., 1998). However, a separate study found no difference

between point mutant and truncation mutant LFS kindreds (Hwang et al., 2003). The small number of these patients combined with their diverse genetic backgrounds complicates this analysis. In contrast, our work shows a clear difference in tumor spectra in $p53^{M/+}$ mice compared to $p53^{+/-}$ mice. Although there was no change in survival time of mice with different genotypes, the $p53^{M/+}$ mice developed more tumors and a different spectrum of tumors compared to $p53^{+/-}$ mice. Moreover, $p53^{M/+}$ mice more accurately recapitulate the phenotype of LFS patients in that they develop a broad spectrum of tumors, including a variety of carcinomas, soft tissue and bone sarcomas, leukemias, and even a glioblastoma multiforme (the most common brain tumor observed in LFS patients). The most obvious omission from this tumor spectrum was breast carcinomas, the most common tumor in LFS patients. This omission could be due to the action of a background modifier not present in the $129S_4$/SvJae genetic background. Indeed, when crossed to the BALB/c background, $p53^{+/-}$ mice develop mammary carcinomas at high frequency (Kuperwasser et al., 2000). This observation raises the prospect that crossing the point mutant *p53* mice onto BALB/c or other backgrounds will further refine the accuracy of these models.

Differences between Contact and Structural Mutant p53

While numerous studies have reported biochemical differences between contact and structural mutant p53 proteins (Halevy et al., 1990; Hinds et al., 1990), our work represents the first clear evidence of a differential effect on tumorigenesis in vivo. $p53^{R270H/+}$ mice develop an increased incidence of carcinomas and B cell lymphomas compared to $p53^{+/-}$ mice. $p53^{R172H/+}$ mice develop an increased incidence of metastatic osteosarcomas (and a trend toward more carcinomas). Among LFS patients, few connections between particular mutations and phenotypes have been described.

The molecular basis for the difference in tumor spectra between $p53^{R270H/+}$ and $p53^{R172H/+}$ mice is unclear, but data derived from primary cells isolated from these mice uncovered some functional differences between the contact and structural mutant proteins in the presence of wild-type p53. For example, $p53^{R270H/+}$ MEFs proliferated only modestly faster than $p53^{+/-}$ MEFs while $p53^{R172H/+}$ MEFs doubled nearly as fast as $p53^{-/-}$ cells. In contrast, neither mutant interfered with the induction of G_1 arrest after DNA damage in MEFs.

Dominant Effects of Mutant p53

In addition to effects on the basal proliferation rate in MEFs, mutant p53 exhibited dominant effects on radiation-induced apoptosis in thymocytes; $p53^{M/+}$ thymocytes had levels of apoptosis intermediate to $p53^{+/-}$ and $p53^{-/-}$ thymocytes. The simplest explanation for this effect is that both $p53^{R270H}$ and $p53^{R172H}$ interfere with the ability of wild-type p53 to transactivate apoptotic target genes through a dominant-negative mechanism. However, these experiments do not rule out dominant gain-of-function effects by mutant p53. That is, the decrease in radiation-induced apoptosis in $p53^{M/+}$ thymocytes may be due to interference with the function of wild-type p53 or interference with other factors involved in radiation-induced apoptosis.

Similarly, the novel tumor phenotypes of $p53^{M/+}$ mice could be due to either dominant-negative effects, dominant gain-of-function effects or a combination of both. For example, one interpretation of the development of lung carcinomas in $p53^{R270H/+}$ mice is that this allele confers a dominant gain-of-function effect because lung carcinomas are rarely observed in $p53^{-/-}$ mice. However, most $p53^{-/-}$ mice die before six months of age. Were null mice to avoid other tumors for 18 months, perhaps they too would be predisposed to lung adenocarcinoma. In order to assess this properly, *p53* function would need to be examined specifically in the lung without other background tumors. The conditional point mutant *p53* mice presented in this work will be useful for studying the effects of mutant *p53* in particular tissues or at specific developmental stages.

Mutant p53 Gain of Function

This work presents the first clear in vivo evidence for gain-of-function effects by mutant *p53*. Since the initial descriptions of *p53* knockout mice, there have been persistent questions as to why they do not develop more carcinomas when p53 is so often mutated in this class of tumors. As discussed above, one explanation is that background modifiers that promote the development of carcinomas in humans may be absent from certain inbred laboratory mouse strains. However, our data support a model in which epithelial carcinogenesis is enhanced by the production of point mutant p53, rather than the loss of p53. $p53^{M/-}$ mice spontaneously develop a variety of carcinomas in addition to the normal complement of tumors previously associated with *p53*-null mutations in mice. Furthermore, mutant p53 accumulation occurred commonly in carcinomas from $p53^{M/-}$ mice but not in lymphoblastic lymphomas. It is also notable that two of the tumors commonly found in *p53*-null mice, lymphoblastic lymphomas and teratocarcinomas, rarely harbor *p53* mutations in humans. Rather, both tumors are associated with functional inactivation of *p53*, such as through HDM2 overexpression (Eid et al., 1999; Zhou et al., 1995). Likewise, HDM2 overexpression is found in over one-third of human sarcomas, the third major tumor type in *p53*-null mice (Cordon-Cardo et al., 1994; Oliner et al., 1992). These correlations further support the idea that p53 inactivation (combined with alterations in other genes) is sufficient for tumorigenic progression in a subset of tissues.

In concurrent work, Guillermina Lozano and colleagues have also generated a $p53^{R172H}$ mouse through similar techniques (Lang et. al, 2004 [this issue of *Cell*]). Although they observed a similar spontaneous tumor phenotype in heterozygous mutant p53 mice, they did not observe an increase in carcinomas or other tumors in $p53^{R172H/R172H}$ mice. This may be due to differences in strain background: Lozano's mice were enriched for C57Bl/6 while our mice were enriched for $129S_4$/SvJae.

The data presented here provide strong support for the protumorigenic effects of point mutant *p53*. At the molecular level, several mechanisms have been proposed to explain mutant p53 gain-of-function effects (reviewed in Cadwell and Zambetti [2001]). For example,

mutant p53 may be able to transactivate novel target genes such as *mdr*, *BAG-1*, or *c-myc*. Alternatively, mutant *p53* may participate in novel protein-protein interactions. Perhaps the most well-characterized interaction is with the *p53* family members, p63 and p73. These two genes share a high degree of homology with p53 and are capable of transactivating several p53 target genes in response to DNA damage. *p63* and *p73* are also activated by DNA damage, similar to *p53* (Irwin et al., 2000; Jost et al., 1997; Yang et al., 1998). Several reports have shown that mutant p53 can bind to and inactivate p63 and p73 (Di Como et al., 1999; Gaiddon et al., 2001; Marin et al., 2000; Strano et al., 2000). We also found mutant p53 capable of binding to its family members in tumor cells derived from a $p53^{R172H}$ osteosarcoma metastasis. The multiple splice variants of *p63* and *p73* complicate our understanding of their role in tumorigenesis. Although deletions of *p63* or *p73* are rare in human tumors, overexpression of ΔNp63 is common in human squamous cell carcinomas (Hibi et al., 2000), and overexpression of ΔNp73 correlates with poor outcomes in human neuroblastomas (Casciano et al., 2002). Nonetheless, the classification of p63 and p73 as tumor suppressor genes has yet to be commonly accepted. It is therefore striking that compound mutation of p53 with either p63 or p73 results in mice bearing tumor spectra reminiscent of those seen in the mutant p53 mice presented in this study (E. Flores et. al, submitted). Taken together, these data strongly implicate the inhibition of p63 or p73 as a potential mechanism for point mutant p53 gain of function.

Experimental Procedures

Details of the construction of the $p53^{R270H/+}$ and $p53^{R172H/+}$ mice are provided in Supplemental Data on the *Cell* website, along with information on immunohistochemistry, quantitative real-time PCR, and lentiviral shRNA experiments.

Aging Study

Aging cohorts were produced by mating $p53^{R270H/-}$ or $p53^{R172H/-}$ mice to $p53^{+/-}$ mice. (Nineteen $p53^{+/-}$ mice and thirty $p53^{-/-}$ mice were derived from the $p53^{R270H}$ cross, while eighteen $p53^{+/-}$ mice and fourteen$^{-/-}$ mice were from the $p53^{R172H}$ cross.) Aging mice were between 93% and 97% enriched for the $129S_4$/SvJae genetic background. Routine testing of sentry mice indicated the colony was pathogen free. Ill or distressed mice were euthanized by CO_2 asphyxiation and all tissues except skin were fixed in 10% formalin/PBS for 24 hr (soft tissues) or Bouin's fixative for 3 weeks (bones). Tissues were embedded in paraffin, sectioned at 5 microns, dewaxed, and stained with hematoxylin and eosin. Tumors were identified with the assistance of a veterinary pathologist (R.B.).

Immunoblotting

Standard techniques were employed for immunoprecipitations and Western blotting. One milligram samples were immunoprecipitated with 10μl of FL-393 (Santa Cruz), *p53* Ab3 (Oncogene Research Products [ORP]), *p53* Ab5 (ORP), p73 Ab2 (ORP), or MDM2 Ab2 (ORP). Western blots were probed with the following antibodies: *p53* Ab7 (1:2500, ORP JA1308), SC-1616 (1:2000, Santa Cruz), SC-6426 (1:1000, Santa Cruz), MDM2 Ab-2 (1:1000, ORP).

Cellular Assays

Thymocyte apoptosis was performed as described previously (de Vries et al., 2002). For cell cycle analysis, 1×10^6 MEFs were plated in 10 cm^2 plates in DME/HEPES + 10% FBS + 5 mM L glutamine + Pen/Strep. After 24 hr, if indicated, cells were treated with 0.2 μg/ml doxorubicin. After 12 hr BrdU was added to 10 μM for 4 hr. Cells were collected in trypsin, quenched with medium, and washed in PBS; FACS analysis was performed as described (Kastan et al., 1992). Tumor-derived cell lines were grown on plastic in DME/HEPES + 10% FBS + Pen/Strep.

Acknowledgments

We apologize to our colleagues for not citing many primary references due to space constraints. We would like to thank S. O'Gorman and L. Attardi for the PrmCre mice. We also thank A. deVries for initiating work on mutant p53, M. McLaughlin for pathology expertise, E. Flores for technical advice, A. Ventura for advice on shRNAs and current and former members of the Jacks Lab for advice and critical review of the manuscript. K.P.O. is a David Koch fellow. D.A.T. acknowledges support from HHMI (Physician Postdoctoral Research Fellow) and the AACR-PANCAN career development award. T.J. is an Investigator of HHMI and this work was supported in part by the NCI Mouse Models of Human Cancer Consortium.

Received: May 7, 2004
Revised: August 24, 2004
Accepted: October 18, 2004
Published: December 16, 2004

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