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Gain of Function of a p53 Hot Spot Mutation in a Mouse Model of Li-Fraumeni Syndrome

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Summary

Individuals with Li-Fraumeni syndrome carry inherited mutations in the *p53* tumor suppressor gene and are predisposed to tumor development. To examine the mechanistic nature of these p53 missense mutations, we generated mice harboring a G-to-A substitution at nucleotide 515 of *p53* (*p53*$^{+/515A}$) corresponding to the p53R175H hot spot mutation in human cancers. Although *p53*$^{+/515A}$ mice display a similar tumor spectrum and survival curve as *p53*$^{+/-}$ mice, tumors from *p53*$^{+/515A}$ mice metastasized with high frequency. Correspondingly, the embryonic fibroblasts from the *p53*$^{515A/515A}$ mutant mice displayed enhanced cell proliferation, DNA synthesis, and transformation potential. The disruption of *p63* and *p73* in *p53*$^{-/-}$ cells increased transformation capacity and reinitiated DNA synthesis to levels observed in *p53*$^{515A/515A}$ cells. Additionally, p63 and p73 were functionally inactivated in *p53*^{515A} cells. These results provide in vivo validation for the gain-of-function properties of certain p53 missense mutations and suggest a mechanistic basis for these phenotypes.

Introduction

The *p53* tumor suppressor encodes a transcriptional regulator that controls cell cycle progression and apoptosis. In response to DNA damage or cellular stress, p53 activity increases, initiating a cascade of events that block cell division. Thus, the *p53* gene itself or the p53 pathway is derailed in the development of most if not all cancers. Somatic inactivation of p53 occurs in over 50% of human tumors, the majority of which are missense mutations in the DNA binding domain (Bartek et al., 1991; Greenblatt et al., 1994; Levine, 1993).

Inherited mutations in the *p53* tumor suppressor occur in individuals with Li-Fraumeni syndrome (LFS), a syndrome characterized by a predisposition to tumor development (Malkin et al., 1990; Srivastava et al., 1990). Individuals with p53 mutations are prone to early onset tumor development and develop multiple tumors (Li and Fraumeni, 1969a; Li and Fraumeni, 1969b; Li et al., 1988). In contrast to other cancer syndromes, a wide variety of tumor types are observed in LFS families; the most common are sarcomas, breast cancers, leukemias, brain tumors, and adrenocortical carcinomas (Evans and Lozano, 1997; Hwang et al., 2003). Most of the *p53* mutations in LFS are also missense mutations in the conserved regions of the p53 DNA binding domain, while a few are nonsense mutations that result in no p53 or a truncated p53 protein.

Functional consequences of missense mutations versus deletions of *p53* have been extensively characterized in vitro (Sigal and Rotter, 2000). All p53 missense mutations are loss-of-function mutations that mimic absence of p53 because of their inability to inhibit cell cycling or induce apoptosis. However, dominant-negative p53 mutants can inhibit the function of the normal p53 protein, usually through protein-protein interactions (Milner et al., 1991). The dominant-negative hypothesis is strongly supported by the observations that many mutant p53 proteins have an increased half-life (Finlay et al., 1988; Hinds et al., 1990; Slingerland et al., 1993) and that they oligomerize with wild-type p53, inhibiting its function (Farmer et al., 1992; Jeffrey et al., 1995; Milner et al., 1991; Sturzbecher et al., 1992). The formation of mixed wild-type and mutant p53 molecules converts wild-type p53 into the mutant conformation in vitro (Milner et al., 1991). Gain-of-function mutations, on the other hand, are those missense mutations in which mutant p53 has additional functions not seen in wild-type p53. For example, the p53R175H mutant, when overexpressed in a nontransformed cell line lacking *p53*, yields tumors in nude mice, while the parental cell line does not (Dittmer et al., 1993). Transgenic mice overexpressing the human p53R175H mutation in epithelial cells exhibit an increased susceptibility to chemical carcinogenesis with shorter latency of tumor development as compared to mice lacking *p53* (Li et al., 1998; Wang et al., 1998a, 1998b).

While these experiments suggest both dominant-negative and gain-of-function phenotypes of p53 mutants, they do not mimic the occurrence of p53 mutations in human tumors, since strong promoters drive expression of the mutant *p53* transgene to very high levels. The best experiments that assay these functions of p53 are ones in which mutant p53 is not vastly overproduced. Some of these experiments have been performed in tissue culture using retroviral vectors to express wild-type and/or mutant p53 in cells lacking endogenous *p53* (Chen et al., 1990). The introduction of another missense mutation with gain-of-function properties, p53R273H, yielded cells that are smaller, have a higher saturation density, and form colonies in soft agar as compared to cells infected with a single copy of the wild-type p53 retrovirus. However, and perhaps more importantly, a cell line containing one copy of each of the wild-type and mutant *p53* genes was indistinguishable from the

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cell line that expresses wild-type p53 alone. These data indicate that, in the heterozygous state, wild-type p53 is dominant over mutant p53. These data contrast with studies in murine ES cells in which the equivalent mutation was introduced into the *p53* locus. Upon exposure to γ radiation, heterozygous ES cells show a dominant-negative phenotype (de Vries et al., 2002). These opposing results suggest that, under different conditions, the same p53 mutation can or cannot function as a dominant negative. Importantly, these in vitro experiments cannot determine the effect of mutant p53 on survival and tumor spectrum in vivo. A mouse that contains a missense mutation in one of the endogenous *p53* genes would be invaluable to understanding p53 missense mutations in vivo.

Several mechanisms have been identified to contribute to the p53 gain-of-function phenotype (Frazier et al., 1998; Pugacheva et al., 2002; Sigal and Rotter, 2000). Most attention has focused on the recently identified p53 family members p63 and p73 (Benard et al., 2003; Soussi, 2003). Several p63 and p73 isoforms lacking the transactivation domain exist, called the ΔTA isoforms, that bind and inhibit the functions of full-length p63 and p73, respectively (Fillippovich et al., 2001; Ishimoto et al., 2002; Stiewe et al., 2002; Yang et al., 1998). These isoforms also inhibit the function of p53. The ΔTAp73 isoforms are upregulated in some tumors with wild-type p53, suggesting that they contribute to tumorigenesis through inactivation of p53 (Concin et al., 2004; Zaika et al., 2002). Additionally, data using mice with deletions of *p63* and *p73* indicate a cooperative nature of all p53 family members. p53-dependent apoptosis was nonexistent in cells lacking *p63* and *p73* (Flores et al., 2002). Thus, the interdependency of p63, p73, and p53 suggests that disruption of any of these components cripples p53 tumor suppression function. The ability of mutant p53 to bind and inhibit the function of p63 and p73 suggests that mutant p53 would be more detrimental than absence of p53.

To more accurately simulate the Li-Fraumeni syndrome and to examine the mechanistic nature of missense mutations on tumorigenesis in vivo, we generated and characterized a mouse model with an arg-to-his alteration (a G-to-A substitution at nucleotide 515) at p53 amino acid 172, corresponding to the p53R175H hot spot mutation in human cancers, by homologous recombination at the endogenous locus. $p53^{+/515A}$ mice developed tumors similarly to $p53^{+/-}$ mice, with one major difference: osteosarcomas and carcinomas from $p53^{+/515A}$ mice metastasized to various organs with high frequency. Thus, in vivo, mutant p53 has additional properties that allow metastasis. Mouse embryonic fibroblasts (MEFs) from mice with one or two copies of the $p53^{515A}$ allele showed increased growth rates and higher saturation densities. Moreover, these cells continued to undergo DNA synthesis under conditions in which *p53* heterozygous and null cells did not. Mechanistically, p53R172H bound p63 and p73 in mouse tumor cell lines and downmodulation of p63 and p73 in *p53* null MEFs increased transformation potential. Notably, these data indicate in vivo differences between loss of *p53* or presence of a *p53* mutation in tumor development and have significant implications for therapy.

Results

Generation of $p53^{515A}$ Knockin Mice

To establish a LFS mouse model with a specific p53 missense mutation, we generated mice with a targeted mutation at the endogenous *p53* locus using the cre-*lox*P system (Figure 1A). We engineered an arg-to-his substitution at amino acid 172 that corresponds to the hot spot mutation at arg 175 in humans. Southern blot analysis was used to verify proper targeting of the endogenous *p53* allele (Figure 1B). The *neomycin* gene was removed from the targeted allele by crossing with transgenic mice expressing Cre recombinase (CMV-*cre*). Sequence analysis of the entire coding region in the *p53* transcript from homozygous mutant mouse embryos indicated no other changes (data not shown). This allele has been designated the $p53^{515A}$ to indicate the G-to-A alteration at nucleotide 515.

MEFs were generated from mice heterozygous and homozygous for the $p53^{515A}$ mutation. The p53R172H mutant protein was present at high levels in $p53^{515A/515A}$ MEFs (Figure 1C). The levels of mutant p53 protein were not high in $p53^{+/515A}$ MEFs and could be due to the presence of the wild-type allele and its ability to activate the *mdm2* gene, which encodes a protein that degrades both wild-type and mutant p53 (Asher et al., 2003; Iwakuma and Lozano, 2003).

In Vivo Tumorigenesis

A cohort of *p53* mutant mice in the C57BL/6 background was generated to monitor tumor development and survival. The survival curves comparing $p53^{-/-}$, $p53^{515A/515A}$, and $p53^{-/515A}$ mice were identical, perhaps owing to the rapid nature of tumor development (Figure 2A). The survival curves of $p53^{+/515A}$ and $p53^{+/-}$ mice also overlapped and showed no statistical difference (Figure 2B). No differences between survival of male and female mice were observed (data not shown). Our survival data for $p53^{+/-}$ and $p53^{-/-}$ mice were consistent with previously published data (Harvey et al., 1993a; Jacks et al., 1994). The most common tumor types seen in mice with any of the above genotypes were lymphomas and sarcomas (Table 1). The majority of $p53^{-/-}$ and $p53^{515A/515A}$ mice developed lymphomas (56% and 70%, respectively), with a smaller percentage developing sarcomas (40% and 29%, respectively). $p53^{+/-}$ and $p53^{+/515A}$ showed an increased frequency of sarcomas (56% and 53%, respectively) with a concomitant decrease in lymphoma development (32% and 31.5%, respectively). $p53^{+/-}$ and $p53^{+/515A}$ also developed carcinomas at low frequency (12% and 15.5%), which are rare in $p53^{-/-}$ and $p53^{515A/515A}$ mice. Thus, in a C57BL/6 background, the comparison of *p53* mutant ($p53^{515A}$) and null alleles showed no differences in the kinds of tumors that these mice developed. However, the tumor spectrum of heterozygous mice showed one marked difference. The osteosarcomas and carcinomas in $p53^{+/515A}$ mice metastasized (Table 1 and Figure 2C). Tumors and metastases from $p53^{+/515A}$ mice expressed high levels of p53 (Figure 2C). None of the $p53^{+/-}$ heterozygous mice, in this and other cohorts that we have studied, exhibited metastasis (Liu et al., 2000, 2004). Thus, while the survival of mice lacking *p53* or inheriting a missense mutation did not vary, $p53^{+/515A}$

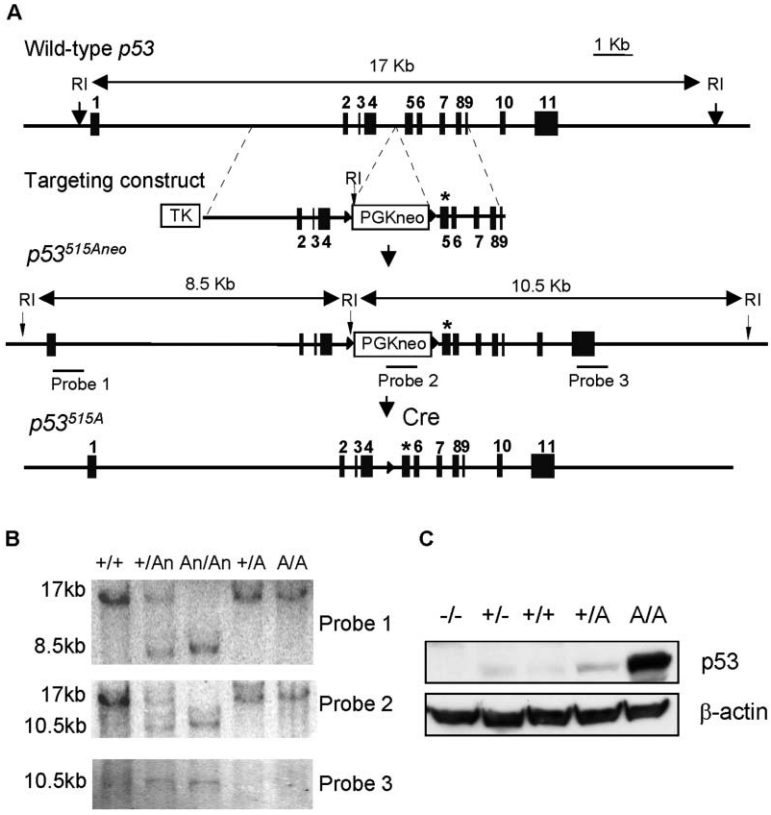

Figure 1. Knockin Strategy and Characterization of the $p53^{515A}$ Allele

(A) The cre-*lox*P-mediated strategy was used to generate the knockin allele. Mice with insertion of the *neomycin* (*neo*) gene flanked by *lox*P sites (triangles) and a missense mutation in exon 5 (asterisk) were generated and mated with CMV-*cre*-expressing mice to delete *neo*. The final product is a *p53* gene with a single *lox*P site in intron 4 and a single substitution at nucleotide 515.

(B) Southern blot analysis was performed on mouse tail DNAs of the various genotypes after digestion with EcoRI (RI). The banding patterns for three different probes before and after Cre recombination are shown.

(C) Western blot analysis of p53 protein levels in MEFs of various genotypes. +/+, $p53^{+/+}$; +/−, $p53^{+/-}$; −/−, $p53^{-/-}$; +/An, $p53^{+/515Aneo}$; An/An, $p53^{515Aneo/515Aneo}$; +/A, $p53^{+/515A}$; A/A, $p53^{515A/515A}$.

mice developed osteosarcomas and carcinomas that metastasized at high frequency.

Since the p53R172H mutant was not very stable in $p53^{+/515A}$ MEFs (see Figure 1C) but the equivalent mutant is stable in human tumors, we performed immunohistochemical analysis on some of the $p53^{+/515A}$ mouse tumors. The results consistently showed stable p53 in tumor cells but not adjacent normal tissue (Figure 2C), suggesting that other factors contribute to mutant p53 stability. One possible explanation for the increased levels of p53 in tumors is the loss of the wild-type *p53* allele. We therefore examined tumors for loss of heterozygosity (LOH) by SSCP (single-stranded conformation polymorphism) analysis using DNA isolated from tumors and normal tissues in $p53^{+/515A}$ mice by in situ laser capture as we have previously described (Figure 2D) (Liu et al., 2000). Only three of 13 tumors analyzed showed LOH, suggesting that loss of the wild-type *p53* allele is not a prerequisite for increased mutant p53 levels.

Analysis of p53R172H Function in the Presence of Wild-Type p53

Other experiments performed to determine the role of mutant p53 in inhibition of wild-type p53 function include crosses with the *mdm2* null allele and functional assays in $p53^{+/515A}$ mice. The *mdm2* null mouse dies early in embryogenesis, but this phenotype is completely rescued by deletion of p53 (Montes de Oca Luna et al., 1995; Jones et al., 1995). This mouse thus provides an in vivo assay to measure the functional significance of p53 mutations (Iwakuma et al., 2004). We hypothesized that, if p53R172H functioned as a true dominant negative, then mice null for *mdm2* and $p53^{+/515A}$ should be born. Crosses between $mdm2^{+/-}$ and $mdm2^{-/-}p53^{515A/515A}$ mice were performed. If the missense mutant has a dominant-negative activity in this context, then we expected 50% of the newborn mice to be $mdm2^{-/-}p53^{+/515A}$. This population of mice was not observed in ten offspring born (data not shown), suggesting that the wild-type *p53* allele in $p53^{+/515A}$ mice remained functional during embryogenesis. To examine p53-dependent apoptosis in response to DNA damage, embryos at 13.5 days gestation were irradiated and assayed for apoptosis in the region of the developing hypothalamus (Figure 2E). In this assay, *p53* null mice showed no evidence of apoptosis, while $p53^{+/-}$ mice showed a high number of apoptotic cells, although not quite as high as $p53^{+/+}$ mice. Under the same conditions, $p53^{+/515A}$ mice showed almost a complete absence of apoptosis, suggesting that, in response to DNA damage, the p53R172H mutant exerts a dominant-negative effect. These data and published in vitro data suggest that, in response to DNA damage, the mutant p53 can inhibit the function of wild-type p53 but not under all circumstances.

Proliferation Potential of MEFs

Previous studies of MEFs derived from p53 heterozygous crosses showed that $p53^{-/-}$ cells divided more rapidly than $p53^{+/-}$ and $p53^{+/+}$ cells and that $p53^{-/-}$ cells reached a higher saturation density than the other genotypes (Harvey et al., 1993b). We therefore compared growth rates and saturation densities of cells with the $p53^{515A}$ allele to determine if the presence of the p53 mutant protein bestowed a gain-of-function phenotype.

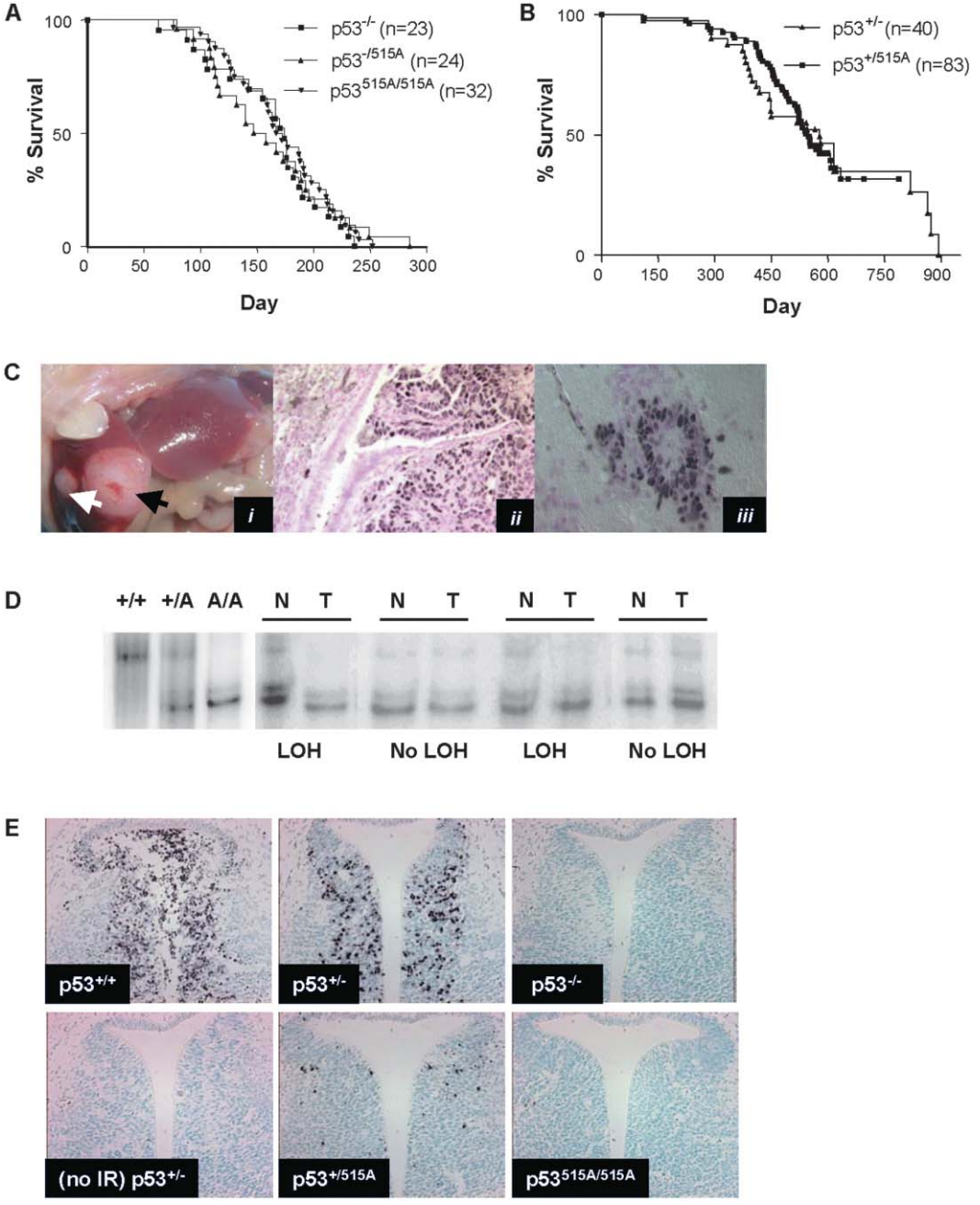

Figure 2. Survival Curves of $p53^{515A/515A}$, $p53^{-/515A}$, $p53^{-/-}$, $p53^{+/+}$, $p53^{+/-}$, and $p53^{+/515A}$ Mice

(A) Survival of $p53^{-/515A}$ mice (n = 24), $p53^{515A/515A}$ mice (n = 32), and $p53^{-/-}$ mice (n = 23) with respect to number of days is plotted.

(B) Survival of $p53^{+/515A}$ mice (n = 83) and $p53^{+/-}$ mice (n = 40) with respect to number of days is plotted.

(C) Metastatic phenotype of osteosarcoma and adenocarcinoma. (i) Osteosarcoma metastasis to the liver (arrows). (ii) p53 immunohistochemical staining of adenocarcinoma of the lung and (iii) its metastatic lesion in the brain.

(D) LOH study of tumors with DNA isolated from paraffin-embedded tumors (T) and normal tissues (N) in $p53^{+/515A}$ mice using laser-capture microscopy. A representative result of SSCP with four different normal and tumor tissues is shown with control DNA (+/+, $p53^{+/+}$; +/A, $p53^{+/515A}$; A/A, $p53^{515A/515A}$).

(E) TUNEL analysis of sections through 13.5 day mouse embryos. Apoptosis is analyzed in the region of the developing hypothalamus of the brain after IR.

The $p53^{515A/515A}$ and $p53^{+/515A}$ MEFs initially did not show any significant differences in growth rate compared to the $p53^{-/-}$ cell lines, but differences became apparent at day 4 (Figure 3A). The $p53^{515A/515A}$ and $p53^{+/515A}$ MEFs grew more rapidly than $p53^{+/-}$ and $p53^{+/+}$ cells, as expected, and grew even faster than $p53^{-/-}$ cells. By day 4, all cell lines reached saturation, but the $p53^{515A/515A}$ and $p53^{+/515A}$ MEFs reached a much higher saturation density compared to any of other genotypes. These data suggest that the $p53^{515A}$ mutation gives MEFs a growth ad-

Table 1. Tumor Spectrum and Metastatic Potential in *p53* Mutant Mice

Tumor Types	$p53^{-/-}$ (n = 19)	$p53^{515A/515A}$ (n = 33)	$p53^{+/-}$ (n = 21)	$p53^{+/515A}$ (n = 65)
Lymphoma	56%	70%	32%	31.5%
Unclassified	12 (48%)	27 (61%)	8 (32%)	23 (29%)
Diffuse large cell	2 (8%)	4 (9%)	0%	2 (2.5%)
Sarcoma	40%	29%	56%	53%
Osteosarcoma	0%	0%	6 (24%)	22 (28%)[a]
Unclassified	6 (24%)	9 (20%)	7 (28%)	12 (15%)
Angiosarcoma	3 (12%)	4 (9%)	1 (4%)	8 (10%)
Rhabdomyosarcoma	1(4%)	0%	0%	0%
Carcinoma	4%	0%	12%	15.5%
Adenocarcinoma	1 (4%)	0%	1 (4%)	3 (4%)[b]
Squamous	0%	0%	2 (8%)	7 (9%)[c]
Poorly differentiated	0%	0%	0%	2 (2.5%)
Tumor totals	25	44	25	79

[a] 23% metastasis (five out of 22).
[b] 67% metastasis (two out of three).
[c] 29% metastasis (two out of seven).

vantage allowing cells to bypass contact inhibition to a greater extent than $p53^{-/-}$ MEFs. This supports a gain of function for the p53 mutant allele.

Since MEFs containing mutant p53 exhibited a growth advantage, we decided to measure DNA synthesis upon contact inhibition. DNA synthesis was measured by [^{3}H] thymidine incorporation in MEFs of different genotypes at different time points after initial plating. $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ cell lines showed identical DNA synthesis profiles. DNA synthesis gradually declined over time, the cells reached a quiescent state at day 6, and the cells did not reenter the cell cycle (Figure 3B). On the other hand, DNA synthesis was always higher for cells containing mutant p53. The $p53^{515A/515A}$ and $p53^{+/515A}$ cell lines showed significantly different DNA synthesis profiles in this assay. While $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ cell lines never synthesized DNA again, cells with mutant p53 continued to synthesize DNA between days 6 and 10 (Figure 3B). Thus, again, the $p53^{+/515A}$ and $p53^{515A/515A}$ mutant MEFs showed obvious differences from cells lacking *p53*, providing additional evidence for a gain of function.

The p53R172H Mutant Increases Oncogenic *ras*-Induced Transformation of MEFs

p53 missense mutations cooperate with activated *ras* in transformation of normal rat embryo fibroblasts (Hinds et al., 1989, 1990). To examine whether cells with an endogenous p53 mutation exhibited an increased transformation potential, we infected $p53^{-/-}$ and $p53^{515A/515A}$ cells with a retroviral vector containing a mutant *Ha-ras*^{V12} cDNA and analyzed the focus-forming potential. $p53^{515A/515A}$ MEFs formed twice as many foci as $p53^{-/-}$ MEFs following *ras* transformation, while the vector controls formed no foci in both genotypes (Figures 3C and 3D). The size of the foci in the $p53^{515A/515A}$ mutant background also appeared larger. These findings support a role for the $p53^{515A}$ allele in cooperation with activated *ras* in transformation of cells, further supporting a gain-of-function hypothesis for the p53R172H mutant.

Gain of Function via p63 and p73

Several mechanisms have been proposed to account for the gain-of-function effect observed for p53 mutants. One mechanism involves the inhibition of activity of p53 family members p63 and p73 by interactions with mutant p53 (Di Como et al., 1999; Gaiddon et al., 2001; Strano et al., 2002). Several p63 and p73 isoforms bind the p53R175H mutant protein, and the interaction inhibits the transcriptional activity of p63 and p73 (Di Como et al., 1999; Gaiddon et al., 2001). To examine the mechanism of gain of function, we first asked whether endogenous murine p63 and p73 bound p53R172H. Tumor cell lines from a lung adenocarcinoma (93-1), from an osteosarcoma (318-1), and from its metastasis (318-M) were generated to examine the interaction of p63 and p73 with mutant p53. These tumors arose in $p53^{+/515A}$ mice, and the cell lines derived from these tumors had lost the wild-type *p53* allele (data not shown). In 93-1 and 318-M cells, endogenous p63 clearly interacted with mutant p53 by coimmunoprecipitation and Western analysis (Figure 4A). The interactions of p53 and p73 were also clearly visible in all three cell lines. In the reverse experiment, a p53-specific antibody coimmunoprecipitated endogenous p63 and p73 (Figure 4A).

These data suggest that the p53R172H mutant binds p63 and p73 in cell lines derived from tumors from $p53^{+/515A}$ mice. To determine if this interaction functionally inactivated p63 and p73, we used siRNA to inhibit expression of mutant p53 and assayed for transactivation of *p21*. p73 and p63 have previously been shown to transactivate the *p21* promoter through the p53-responsive element (Di Como et al., 1999; Gaiddon et al., 2001; Kaghad et al., 1997; Strano et al., 2002; Yang et al., 1998). 318-1 cells transfected with *p53* siRNAs showed a dramatic decrease in mutant p53 protein levels (Figure 4B). To measure the activity of p63 and p73, 318-1 cells that have lost the wild-type *p53* allele (data not shown) were transfected with vector, murine *p63*α, or HA-tagged *p73*α expression plasmids, with a *p21* promoter luciferase plasmid, and with control or *p53* specific siRNAs. In all cases, samples with *p53* siRNA showed increased *p21* promoter activity as compared to samples with control siRNA (Figure 4C). Both p63 and p73 were able to activate the *p21* promoter to a greater extent in the absence of mutant p53. Experiments using both *p63*α and *p73*α (at half the DNA concentration each) also showed increased *p21* promoter activity upon inhi-

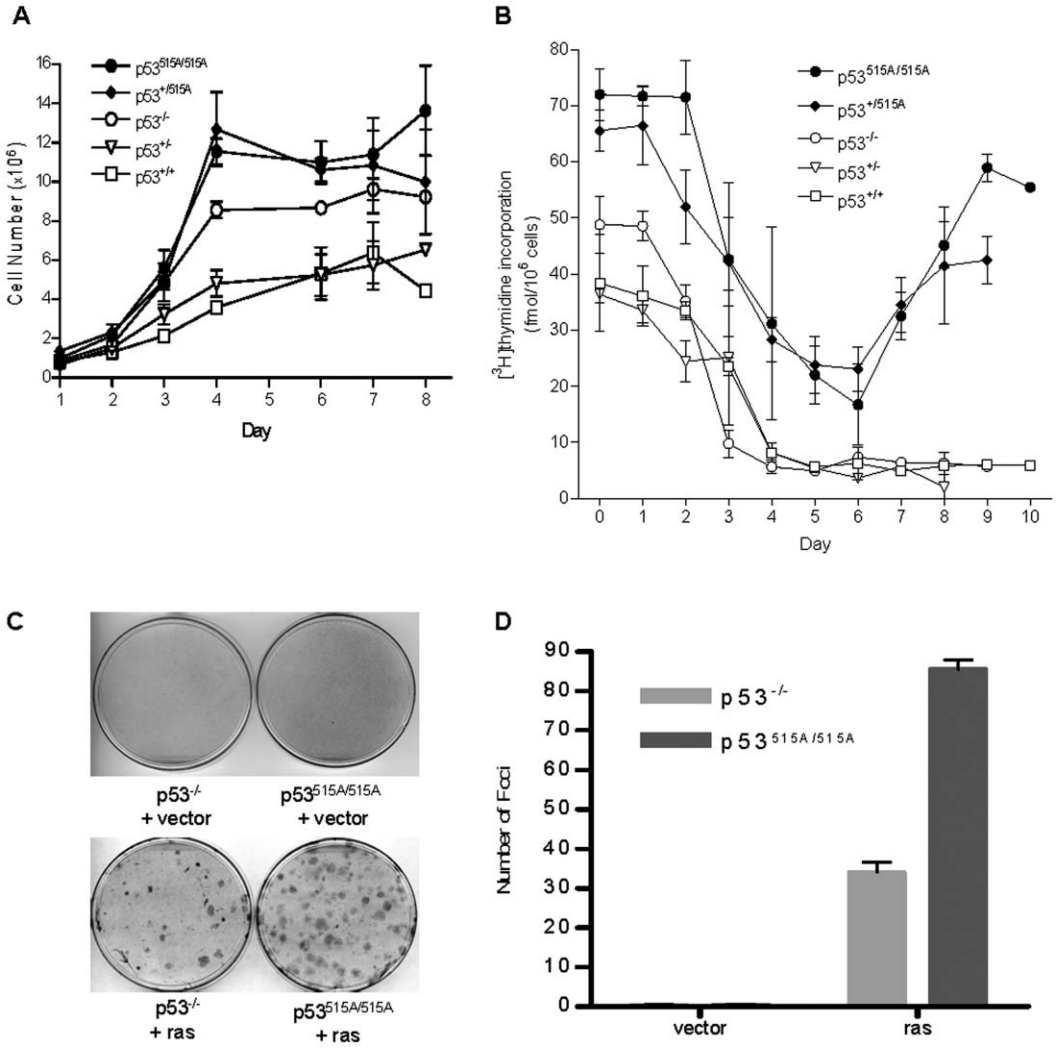

Figure 3. Proliferation and Transformation in MEFs

(A) To measure cell growth rates, MEFs of different genotypes were plated at day 0 and counted at indicated time points.

(B) Time course of DNA synthesis upon contact inhibition in MEFs as measured by incorporation of [^{3}H] thymidine.

(C and D) The transformation potential of *p53*$^{-/-}$ and *p53*$^{515A/515A}$ MEFs in cooperation with activated *ras* was measured using a focus-forming assay. Low-passage MEFs were infected with a retroviral vector containing an activated *Ha-ras*^{V12} cDNA or vector control and diluted with nontransfected cells before plating. Foci were counted after 14 days of growth.

bition of mutant p53. Even the addition of *p53* siRNA alone showed a slight increase in *p21* luciferase activity (Figure 4C). We also measured the ability of endogenous p63 and p73 to activate the endogenous *p21* promoter. In transfection experiments using 318-1 cells, the addition of *p53* siRNA inhibited the levels of endogenous *p53* and increased *p21* mRNAs, as measured by real-time RT-PCR (Figure 4D). These data indicate functional inactivation of p63 and p73 by the p53R172H mutant protein.

To determine the role of p63 and p73 in transformation, we downmodulated expression of *p63* and *p73* in focus-forming assays with MEFs of different genotypes. First, to check the specificity of siRNAs, FLAG-tagged *p63*α and myc- tagged *p73*α expression constructs were cotransfected with control siRNAs and siRNAs specific for *p63* or *p73* into H1299 cells. The *p63* and *p73* siRNAs showed almost complete inhibition of p63 and p73 protein levels by immunohistochemisty (Figure 5A) and Western blot analyses (Figure 5B), while the levels of p63 and p73 remained high with control siRNAs.

To examine if the siRNAs specific for *p63* and *p73* could inhibit *p63* and *p73* expression in MEFs, we performed Western blot (Figure 5C) and real-time RT-PCR analyses (Figure 5D). The data clearly show reduction of p63 and p73 protein levels. At the mRNA level, *p63* and *p73* mRNAs were barely detectable when the respective siRNAs were used. Thus, the *p63* and *p73* siRNA dramatically reduced the levels of endogenous mRNAs and proteins in MEFs.

Next, focus-forming assays were performed utilizing these *p63* and *p73* siRNAs. MEFs were infected with activated *ras*, followed by transfection with siRNAs for *p63* and *p73*, or control siRNA. Control transfection experiments with or without siRNA showed no significant effect on the number of foci that formed in *p53*$^{-/-}$ and

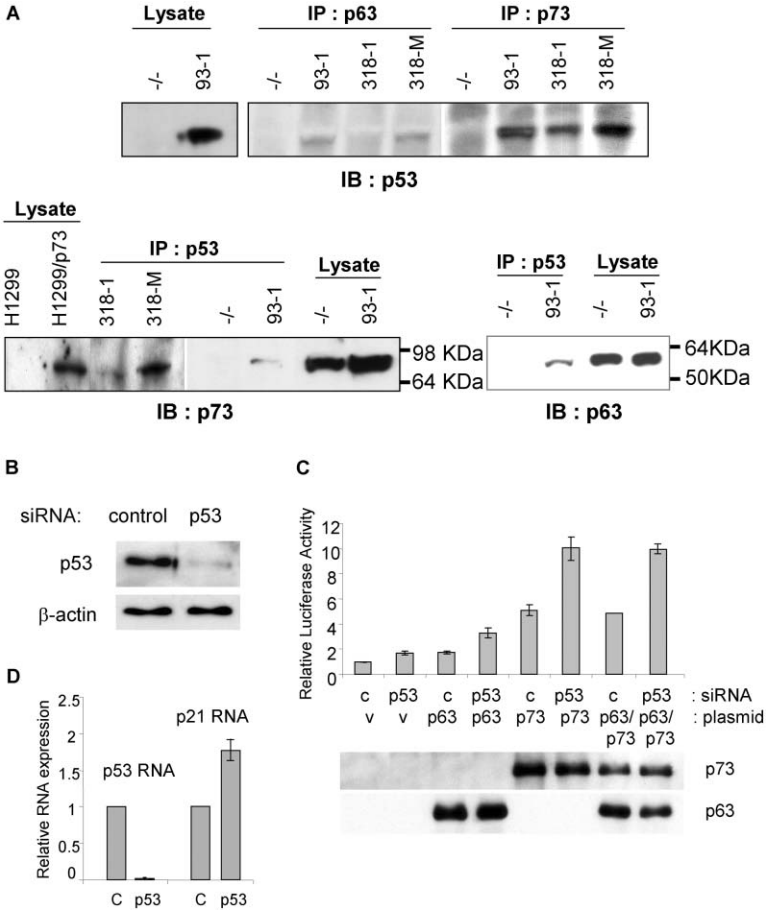

Figure 4. p53R172H Coimmunoprecipitated and Functionally Inactivated the TA Isoforms of p63 and p73

(A) Coimmunoprecipitation experiments were performed on a *p53*$^{-/-}$ (−/−) murine rhabdomyosarcoma cell line, a *p53*$^{+/515A}$ lung adenocarcinoma cell line (93-1), a *p53*$^{+/515A}$ osteosarcoma cell line (318-1), and a *p53*$^{+/515A}$ osteosarcoma cell line from a metastasis (318-M). All three tumor cell lines from *p53*$^{+/515A}$ mice had lost the wild-type *p53* allele. H1299 cells transfected with a *p73*α plasmid (H1299/p73) were used as a control for detection of p73.

(B) Control or *p53* siRNAs were transfected into 318-1 osteosarcoma cells derived from a *p53*$^{+/515A}$ mouse. After transfection, Western blotting was performed using p53 and β-actin-specific antibodies.

(C) Luciferase assays were performed in 318-1 cells to measure *p21* promoter activity. A *p21* reporter plasmid was cotransfected with either a vector plasmid or *p63*α and/or HA-tagged *p73*α-encoding plasmids in the presence of either control or *p53*-specific siRNAs. Relative luciferase activities were shown with standard deviations after normalization with *pRL-TK* values. Western blot analysis of p63 and p73 protein levels after transfection was performed using p63 and HA antibodies, respectively.

(D) Real-time RT-PCR was performed to measure the endogenous levels of *p53* and *p21* mRNAs after transfection with siRNA for *p53* in 318-1 cells.

p53$^{515A/515A}$ MEFs (Figures 5E and 5F). As in previous experiments (Figures 3C and 3D), *p53*$^{515A/515A}$ MEFs showed a 2-fold increase in the number of foci as compared to *p53*$^{-/-}$ MEFs. In contrast, *p53*$^{-/-}$ MEFs transfected with siRNAs specific for *p63* and *p73* showed a significant increase in the number of foci that formed. The foci number in *p53*$^{-/-}$ cells increased by approximately 2-fold, similar to the number seen in *p53*$^{515A/515A}$ MEFs and in *p53*$^{515A/515A}$ MEFs transfected with control siRNAs. In addition, *p53*$^{515A/515A}$ MEFs showed no noticeable difference in foci number when transfected with both *p63* and *p73* siRNAs, suggesting the increased number of foci is caused by the inhibition of p63 and/or p73 in *p53*$^{515A/515A}$ MEFs.

Inhibition of p63 and p73 in *p53*$^{-/-}$ MEFs Reinitiates DNA Synthesis

To examine the importance of p63 and p73 in the gain-of-function phenotype, we exploited another difference between mutant and wild-type cells. Cells with one or two copies of the *p53* null allele cease to replicate DNA after short-term culture (Figure 3B), while cells with one or two copies of the *p53*^{515A} allele reinitiate DNA synthesis. We therefore transfected siRNAs for *p63* and *p73*, or control siRNA into low passage MEFs, and monitored DNA synthesis with time. The control siRNA-transfected cells yielded similar results as previous experiments without transfection of siRNAs (compare Figures 3B and 6A). *p53*$^{-/-}$ and *p53*$^{+/+}$ MEFs did not incorporate [^{3}H] thymidine beyond day 6, while *p53*$^{515A/515A}$ MEFs continued to synthesize DNA (Figure 6A). *p63* and *p73* siRNA transfection experiments in *p53*$^{515A/515A}$ MEFs resulted in no change in the DNA synthesis profile, supporting the possibility that p63 and p73 are already inhibited in this cell line (Figure 6B). However, in *p53*$^{-/-}$ MEFs, *p63* and *p73* siRNA had a significant effect. Inhibition of p63 and p73 resulted in continued DNA synthesis beyond day 6, similar to *p53*$^{515A/515A}$ MEFs (Figures 6C and 6D). These findings provided further evidence that the p63 and/or p73 pathways are inhibited in *p53*$^{515A/515A}$ MEFs.

Discussion

In this study, we have generated a knockin allele of a common p53 mutation in a more faithful reproduction of the LFS. Several important observations arose from these studies. First, the osteosarcomas and carcinomas from *p53*$^{+/515A}$ mice metastasize, as in humans, to the lymph nodes, lung, liver, and brain, while tumors from *p53*$^{+/-}$ mice do not (Donehower et al., 1992; Jacks et al., 1994). These data are supported by a metastatic phenotype seen in tumors from mice with a hypomorphic allele of the same mutation (Liu et al., 2000). Since all mice in this study are in the same genetic background, C57BL/6, the data clearly indicate a gain-of-function phenotype for this p53 mutation in vivo. In the

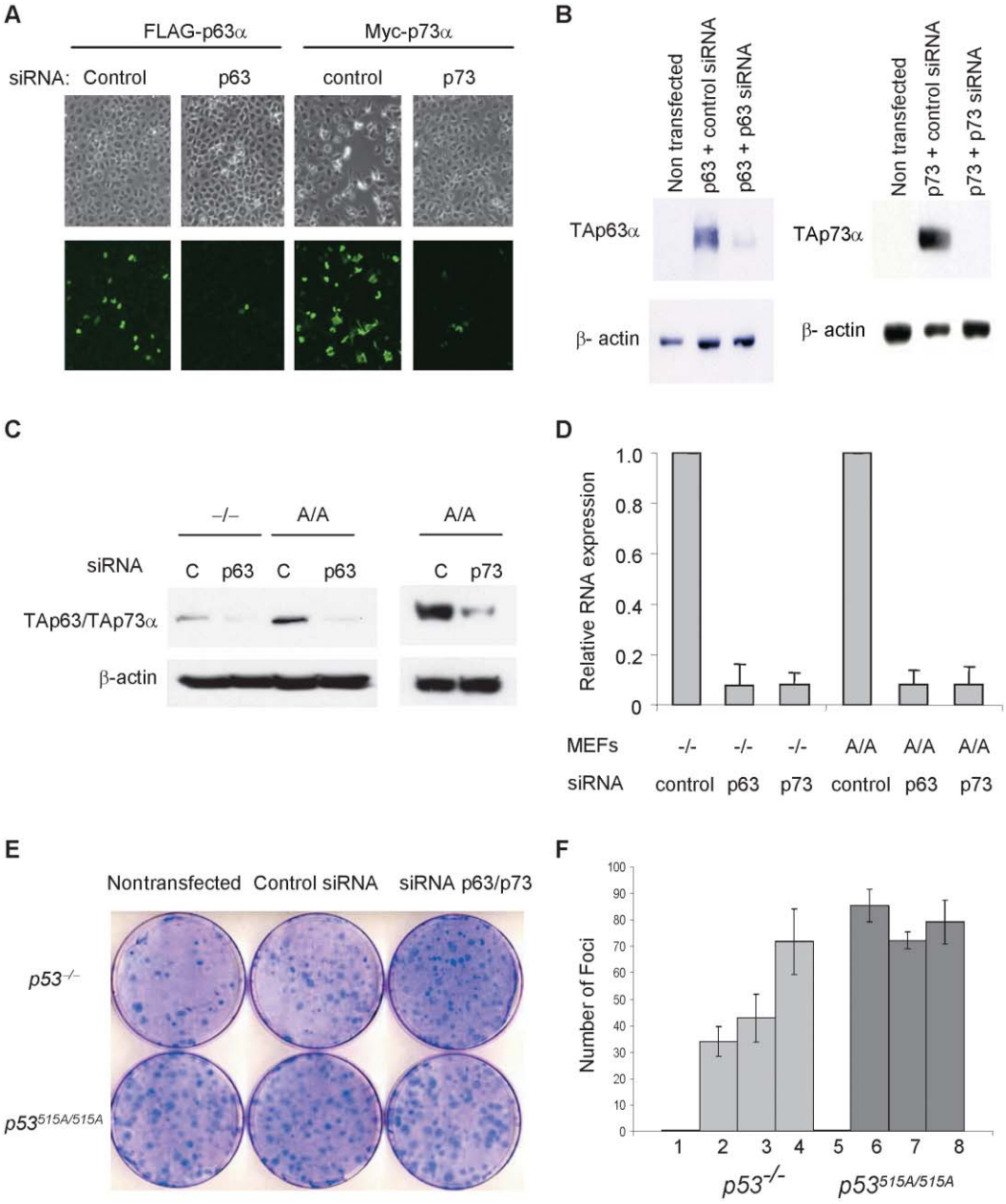

Figure 5. siRNAs Specific for Murine *p63* and *p73* Enhance Transformation Potential of $p53^{-/-}$ but Not $p53^{515A/515A}$ MEFs

(A) FLAG-tagged *p63*α or myc-tagged *p73*α expression plasmids were transfected with control siRNAs or specific siRNAs into H1299 cells, and cells were stained with anti-FLAG or anti-myc antibody to detect p63 or p73, respectively.

(B) Western blotting was performed using anti-p63 or anti-myc antibodies after FLAG-tagged *p63*α or myc-tagged *p73*α cotransfection with siRNAs.

(C) Western blot analysis of the levels of p63 and p73 expressed after transfection with *p63* and *p73* siRNA. C, control; –/–, $p53^{-/-}$; A/A, $p53^{515A/515A}$.

(D) Real-time RT-PCR was used to measure the levels of *p63* and *p73* mRNA after transfection with *p63* and *p73* siRNA, respectively.

(E) The transformation potential of $p53^{-/-}$ and $p53^{515A/515A}$ MEFs in cooperation with $Ha\text{-}ras^{V12}$ was measured following transfection of control and *p63*/*p73* siRNAs.

(F) Foci were counted after 14 days of growth and graphed. $p53^{-/-}$ and $p53^{515A/515A}$ MEFs were infected with vector (lanes 1 and 5), $Ha\text{-}ras^{V12}$ (lanes 2 and 6), $Ha\text{-}ras^{V12}$ and control siRNA (lanes 3 and 7), and $Ha\text{-}ras^{V12}$and *p63*/*p73* siRNA (lanes 4 and 8). Multiple plates were counted from duplicate experiments.

accompanying paper, Olive et al. (2004 [this issue of *Cell*]) also find a gain-of-function phenotype in mice inheriting the same mutation. However, in the $129S_4$/SvJae strain, they also see a different tumor spectrum in mutant heterozygous mice, as compared to $p53^{+/-}$ mice (Harvey et al., 1993c; Donehower et al., 1995). Thus, not only do *p53* mutant alleles elicit a gain of function but they also manifest different tumor incidences in different strains of mice.

To mechanistically examine the gain-of-function phe-

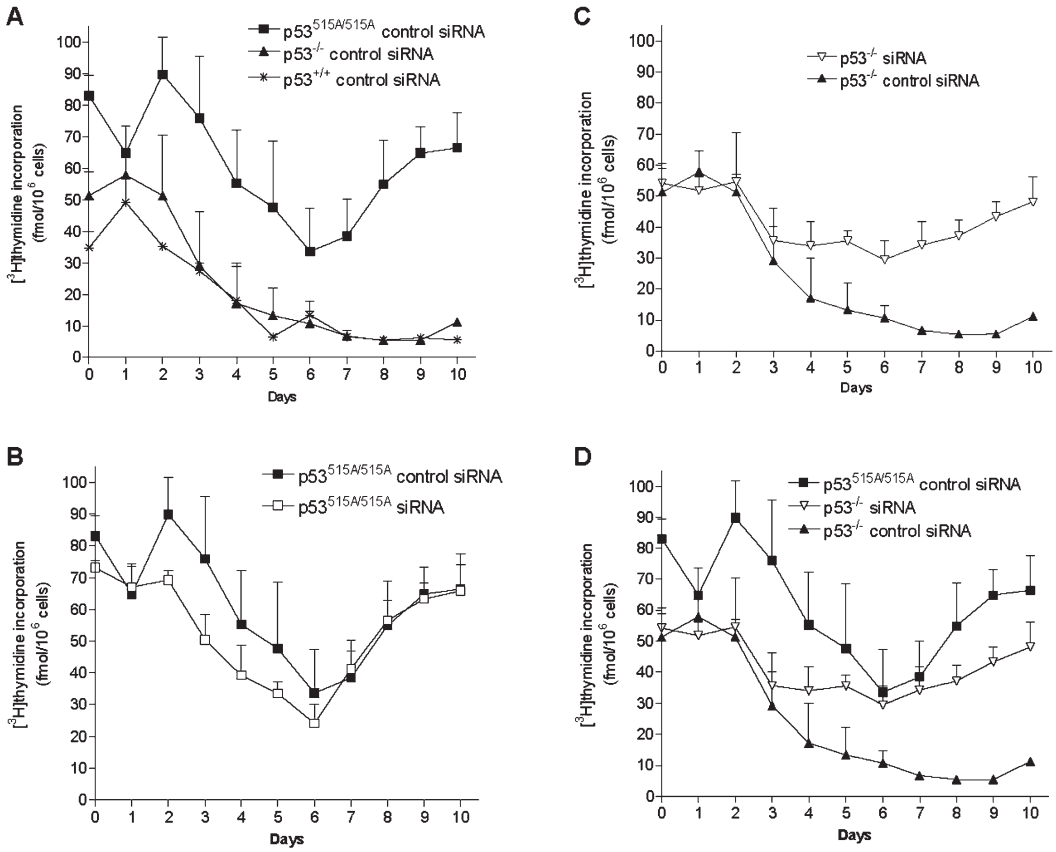

Figure 6. siRNAs Specific for Murine *p63* and *p73* Allowed $p53^{-/-}$ MEFs to Reinitiate DNA Synthesis

(A) [^{3}H] thymidine incorporation was measured at different times after plating in $p53^{+/+}$, $p53^{-/-}$, and $p53^{515A/515A}$ MEFs transfected with control siRNA.

(B) Comparison of DNA synthesis profiles for $p53^{515A/515A}$ MEFs with control and *p63*/*p73*-specific siRNAs.

(C) Comparison of DNA synthesis profiles for $p53^{-/-}$ MEFs with control and *p63*/*p73*-specific siRNA.

(D) $p53^{-/-}$ MEFs transfected with *p63*/*p73* siRNAs resulted in DNA synthesis trends that were very similar to $p53^{515A/515A}$ MEFs.

notype, we developed tissue culture-based assays using MEFs of various genotypes. $p53^{515A/515A}$ MEFs exhibit twice the transforming potential as compared to $p53^{-/-}$ MEFs. These differences are overcome by eliminating p63 and p73 in $p53^{-/-}$ cells. Experiments designed to measure DNA synthesis show the same phenotype. Whereas early passage *p53* null MEFs cease DNA synthesis, reinitiation of DNA synthesis occurs upon downmodulation of p63 and p73, similar to $p53^{515A/515A}$ MEFs. These data indicate that downmodulation of p63 and p73 in cells in culture increased transformation potential but do not exclude the possibility that other factors might also contribute. To correlate the tissue culture data with tumorigenesis in vivo, we showed the interaction of endogenous p63 and p73 proteins with mutant p53 in three independent tumor cell lines derived from $p53^{+/515A}$ mice that had lost the wild-type *p53* allele. Moreover, functional inactivation of p63 and p73 was observed in the same tumor cell lines. Our data indicate that mutant tumor cell lines producing p53R172H from a single copy of the gene lack functional p63 and p73. However, while we have observed the interaction of p63 and p73 with mutant p53 and have identified a role of p63 and p73 in a gain-of-function phenotype in MEFs and tumor cell lines, we have no direct evidence that p63 and p73 play a role in metastasis in vivo. Flores and Jacks (submitted) have generated $p53^{+/-}$ $p63^{+/-}$ and $p53^{+/-}$ $p73^{+/-}$ mice to analyze the role of p63 and p73 in tumorigenesis. Notably, these mice develop tumors that metastasize. Thus, the combined in vitro and in vivo data indicate that mutant p53 binds and inhibits the function of p63 and p73 and that haploinsufficiency for either *p63* or *p73* reproduces the metastatic phenotype in $p53^{+/-}$ mice.

The generation of $p53^{+/515A}$ mice and cells has allowed a detailed characterization of the effects of heterozygosity on wild-type and mutant p53 function. Importantly, the p53R172H protein in $p53^{+/515A}$ mice is not stable but becomes stable in tumors. In the developing embryo, the absence of *mdm2* resulted in a lethal phenotype in $p53^{+/515A}$ mice, suggesting that wild-type p53 is functional. Since the *mdm2* null phenotype is rescued by loss of *p53*, the lack of dominant-negative activity of p53R172H in embryogenesis is clear. The inability of the mutant p53 to function in the presence of a wild-type allele suggests that p53R172H cannot inactivate p63 and p73 in embryogenesis and is supported by the absence of lethal phenotypes, as seen with loss of *p63* and *p73* (Yang et al., 1999, 2000). Thus, in a heterozygous mouse in an in vivo situation, mutant p53 does not drive wild-type p53 into a mutant conformation as it does in vitro (Milner et al., 1991). This predicts that other

alterations must occur to stabilize mutant p53 in tumors. This may explain the lack of difference in survival between *p53*$^{+/-}$ and *p53*$^{+/515A}$ mice, since stabilization of p53 requires other changes for tumorigenesis. This change could be as simple as loss of wild-type p53, as seen in tumor cell lines from *p53*$^{+/515A}$ mice. However, the majority of the tumors (ten out of 13) retain the wild-type *p53* allele. Additionally, posttranslational modifications may be required to stabilize p53. In response to DNA damage, p53 is modified by phosphorylation and acetylation, although the importance of these modifications on p53 stability and function is controversial (Appella and Anderson, 2001).

The possibility that posttranslational modifications are needed to stabilize p53R172H is supported by DNA damage experiments. In response to DNA damage, p53-dependent apoptosis is vastly reduced in the embryonic brain of *p53*$^{+/515A}$ mice, suggesting that, in this scenario, the p53R172H mutant inactivates wild-type p53. p53R172H may also be binding and inactivating p63 and p73, a possibility that is supported by the observation that *p63* or *p73* null embryos also lack a p53-dependent apoptotic response (Flores et al., 2002). These data suggest that posttranslational modifications may be required for mutant p53 to inhibit the function of wild-type p53 and members of its family. Thus, further understanding of the regulation of mutant p53 stability is imperative.

This study showed in vivo differences between *p53*$^{+/-}$ and *p53*$^{+/515A}$ in that tumors from *p53*$^{+/515A}$ mice metastasized. Another mouse heterozygous for an arg-to-pro mutation at p53 amino acid 172, a less common mutation identified in human tumors, shows delayed tumor onset and no metastasis (Liu et al., 2004). Since these mice are otherwise genetically identical, the data indicate in vivo differences between mutant *p53* alleles in tumorigenesis. Thus, identification of the type of p53 mutation in human patients may be important to determine the course of therapy. Many reports suggest that the presence of p53 mutations in tumors correlated with resistance to chemotherapy and worse prognosis as compared to tumors lacking p53 mutations (Iacopetta, 2003; Lai et al., 2000; Powell et al., 2000; Skaug et al., 2000). Other reports show the opposite (Bataille et al., 2003; Lane and Hupp, 2003; Zhivotovsky et al., 1999). It is enticing to speculate that the kind of *p53* mutation that occurs and the posttranslational events that modify the mutant may be important in response to chemotherapy. Clearly, typing of *p53* mutations should be performed in human tumors to determine the relevance of our mouse models to human disease.

Experimental Procedures

Generation of *p53*^{515A} Knockin Mice

The targeting vector was generated by cloning a *lox*P-flanked PGK*neo* cassette into the second AccI site of intron 4 of a *p53* genomic fragment. An arginine-to-histidine substitution (CGC to CAC) at codon 172 was performed by site-directed mutagenesis. The resulting construct was then cloned into a vector containing a *thymidine kinase* (TK) cassette. The entire targeting construct was sequence verified (data not shown) and linearized with NotI. Targeting vector (25 μg) was electroporated into AB-1 embryonic stem (ES) cells. DNA from G418-resistant FIAU-sensitive ES colonies was subjected to Southern blot analysis. Two targeted ES cell clones were injected into C57BL/6 blastocysts and transferred into pseudopregnant CD1 female recipients. The resulting chimeras were mated with C57BL/6 females. Mice with the targeted allele were crossed with CMV-*cre* transgenic mice (also in the C57BL/6 background) to remove the *lox*P-flanked *neomycin* cassette. Mice carrying the *p53*^{515A} allele were crossed with C57BL/6 mice for more than four generations until the genetic background was greater than 90% C57BL/6 to establish the cohort for a tumor study. Genotyping was performed by polymerase chain reaction (PCR) analysis using primers previously described surrounding the *lox*P sites (Liu et al., 2004).

Cell Culture

Cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals) and penicillin (100 IU/ml)/streptomycin (100 μg/ml). MEFs were generated by crossing mice of the appropriate genotypes and collecting embryos at 13.5 dpc. For cell growth rates and saturation densities, 5×10^5 MEFs were plated on 100 mm tissue culture dishes and counted as needed. To monitor DNA synthesis, 1–3 $\times$ 10^6 cells were plated on 60 mm culture dishes in 4 ml DMEM with 20% FBS and left unperturbed. At the indicated days, MEFs were incubated with 1 mCi [^{3}H] thymidine for 2 hr before measuring the radioactivity incorporated into cells using liquid scintillation counting.

Focus-Forming Assays

Low-passage MEFs were infected with a retroviral vector containing an activated *Ha-ras*^{V12} cDNA and a puromycin resistance gene. Two days after infection, puromycin was added to select a pool of cells expressing *Ha-ras*^{V12}. Following selection, 2000 puromycin-resistant cells were mixed with 998,000 noninfected cells of the same genotype and plated. On day 14, the plates were fixed and stained with crystal violet in 70% ethanol, and foci were counted. Transfection with siRNAs was performed after viral infection and selection.

Tumor Samples

A cohort of mice was monitored for signs of illness or obvious tumor burden. Moribund mice were sacrificed, and the major organs and tumors were fixed with 4% formalin in PBS. Tissues were paraffin embedded and sectioned at 10 μm. All sections were stained with hematoxylin and eosin prior to pathological analysis. Immunohistochemical analysis was performed as previously described (Evans et al., 2001). For LOH study, DNA was isolated from paraffin-embedded tumors and normal tissues from *p53*$^{+/515A}$ mice by a pathologist using laser-capture microscopy and PCR amplified for SSCP analysis as previously described (Liu et al., 2000).

Design of siRNAs Specific for Murine *p53*, *p63*, and *p73*

siRNAs specific for murine *p53*, *p63*, and *p73* were designed and confirmed for uniqueness using the BLASTN program. The target sequence for *p53* is 5′-AAGTCTGTTATGTGCACGTAC-3′ (spanning amino acids 117–123), for *p63* is 5′-AAGAGACCGGAAGGCAGA TGA-3′ (spanning amino acids 134–141), and for *p73* is 5′-AAGGCAG AGTGTGGTTGTGCC-3′ (spanning amino acids 224−231).

Transfections and Luciferase Assay

For transfection experiments, lipofectamine reagent was used according to the manufacturer's protocol (Invitrogen). DNA and siRNAs were mixed in a 5:1 (μg) ratio. For Western blotting and immunostaining, cells were harvested 24–42 hr after transfection. For DNA synthesis and focus-forming assays, cells were used 48 hr after transfection. For luciferase assays, cells were seeded at 70% confluency 1 day before transfection. The *p21* reporter plasmid (*pGLp21Luc*) (0.7 μg) was used with an internal control, 0.15 μg of *pRL-TK* (Promega). Either *p63*α or *p73*α expressing plasmids (0.3 μg) were cotransfected with these luciferase plasmids. When both *p63* and *p73* were cotransfected, 0.15 μg of each plasmid was used. In addition, 2 μl of 20 μM control siRNA (Scramble II, Dharmacon) or *p53*-specific siRNA was also added to the transfection. The dual reporter luciferase assay system (Promega) was used to measure *p21* promoter activity according to manufacturer's instruction.

Real-Time RT-PCR

mRNAs were harvested using RNeasy kit (Qiagen). Reverse transcriptase reactions were performed using the First-Strand cDNA Synthesis Kit (Amersham Bioscience). Real-time PCR was performed to manufacturer's specifications (Applied Biosystems). The following primers were used: *p21*, CCTGACAGATTTCTATCACTCCA and CAGGCAGCGTATATCAGGAG; *p53*, ACATGACGGAGGTCGT GAGA and TTTCCTTCCACCCGGATAAG; *p63*, AAAGAACGGCGAT GGTACGA and GGTACAGCAGCTCATCATCTGG; *p73*, AGAGCATG TGACCGACATTGTT and TTCTACACGGATGAGGTGGCT; *GAPDH*, TCACCACCATGGAGAAGGC and GCTAAGCAGTTGGTGGTGCA. Primer sequences were obtained using the Primer Express program and checked for gene specificity by BLASTN. Relative mRNA expression was normalized to the value of *GAPDH* for each reaction.

Western Blot and Coimmunoprecipitation Analyses

Protein extracts (100 μg) from MEFs were run on polyacrylamide gels, transferred to Hybond P membranes (Amersham Pharmacia), and probed with antibodies for p53 (CM5, Novacastra; FL393, Santa Cruz) and β-actin (Sigma). The secondary antibody used was horseradish peroxidase conjugated (Amersham). Signal detection was carried out with an electrochemiluminescence kit (Pierce). For coimmunoprecipitation experiments, cells were lysed on ice in a buffer of 100 mM Tris-Cl (pH 8.0), 100 mM NaCl, 1% NP40, and proteinase inhibitors. Lysate (1.5 mg) was precleared with protein A agarose beads. The following antibodies were added according to the manufacturer's recommendations: p53, CM5 and FL-393; p63, Ab-1 (Neomarkers); and p73, Ab-2 (Oncogene Research).

Analysis of Apoptosis in 13.5 dpc Embryonic Brains

The in vivo TUNEL assay was performed as previously described (Liu et al., 2004).

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