Phosphorylation of Ser312 contributes to tumor suppression by p53 in vivo

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The tumor suppressor p53 is a master sensor of stress, and post-translational modifications are key in controlling its stability and transcriptional activities. p53 can be phosphorylated on at least 23 Ser/Thr residues, the majority of which are phosphorylated by stress-related kinases. An exception is Ser315 in human p53 (Ser312 in mouse), which is predominantly phosphorylated by cell cycle-related kinases. To understand the biological importance of Ser312 phosphorylation in vivo, we generated p53Ser312Ala knock-in mice. We show here that, although Ser312 is not essential for mouse life span under normal physiological conditions, Ser312Ala mutation dampens p53’s activity during embryonic development. This is evident from its partial rescue of embryonic lethality caused by Mdm4 deletion. In agreement with the notion that Ser312 mutation weakens p53 function, Ser312Ala mice are also more susceptible to tumorigenesis following a sublethal ionizing radiation dose. Importantly, in the cohort studied, Ser312 mutation predisposes mice to develop thymic lymphomas and liver tumors, partly due to p53Ser312Ala’s inability to fully induce a set of p53 target genes including p21 and cyclin G1. Thus, we demonstrate that phosphorylation of Ser312 is required for p53 to function fully as a tumor suppressor in vivo.

Mdm4 | lymphoma | liver | knock-in | mouse

One of the best studied tumor suppressor genes is p53. The importance of its tumor suppressive role is illustrated in humans by the congenital Li-Fraumeni syndrome, where a mutation in the p53 gene results in the development of a range of malignancies, and the observation that the p53 gene undergoes inactivating mutations in around 50% of human tumors. p53 is normally present in the cell at low levels but increases in abundance in response to cellular stresses such as DNA damage, largely due to inhibition of its proteasomal degradation. Once elevated, it prevents potentially harmful damage being incurred following cell division, either by stalling the cell cycle until the damage is repaired or by preventing division entirely by inducing apoptosis or senescence. This is achieved largely by its ability to act as a transcription factor and by its regulation of target genes involved in various biological functions.

p53 is subject to many posttranslational modifications, such as acetylation and phosphorylation, which seem to both stabilize p53 and activate its transcriptional activity (1, 2). p53 is phosphorylated at 23 sites, mostly found within the N- and C-terminal domains that form the molecule’s regulatory regions. Most of these serine and threonine residues are phosphorylated by stress-related kinases such as ATM/ATR and Chk2. However, Ser315 of human p53 (Ser312 in mouse) is phosphorylated by kinases involved in cell cycle progression, such as Aurora kinase A and the cyclin-dependent kinases cdk2 and cdk9 (3–6). Ser315 phosphorylation is required for the E2F family to enhance p53-mediated transcription and apoptosis (7) and is one of the sites phosphorylated when it interacts with the peptidyl-prolyl isomerase Pin1 following DNA damage to alter its conformation and increase its stability and transactivation activity (8, 9). Although phosphorylation at Ser315 in these instances seems to elevate p53 stability and positively contribute to tumor suppression, its phosphorylation by Aurora kinase A (involved in chromosome segregation and function) results in its destabilization (5). Following endoplasmic reticulum stress, phosphorylation of Ser315 alongside Ser376 results in p53’s relocation to the cytoplasm, inhibiting p53-mediated apoptosis (10). This suggests that Ser315 phosphorylation may obstruct p53’s role in tumor suppression.

To establish a physiological role for the phosphorylation of p53 at Ser315, we generated mice possessing a Ser → Ala mutation at codon 312 (equivalent to human codon 315). p53Ser312Ala mice were born normally and displayed no overt phenotype. However, following a sublethal dose of ionizing radiation (IR), they were more susceptible to tumor development, particularly in the thymus and liver. We conclude that Ser312 phosphorylation contributes to tumor suppression by p53 in response to ionizing radiation in vivo.

Results

Phosphorylation of p53 at Ser312 Does Not Affect Mouse Life Span

Ser315 in murine p53 is equivalent to Ser312 in human p53 (Fig. 1A). To study the physiological effects of phosphorylating p53 at Ser312, knock-in mice were generated carrying a TCT → GCC (Ser → Ala) mutation in codon 312 of the mouse p53 gene (Fig. S1). The mutation was identified by PCR using primers specific for either the wild-type (WT) or mutant allele. p53 cDNA was isolated by RT-PCR from p53WT, heterozygous (p53312WA) and mutant (p53312AA) littermates, and the mutation was confirmed by sequencing (Fig. 1B). The entire p53 ORF was sequenced to confirm that no other mutations were present.

Mice homozygous for Ala at codon 312 (p53312AA mice) were viable, displayed no obvious developmental abnormalities, were born at the expected frequency (Fig. 1C), and survived to maturity. Cohorts of 11 p53WT, 25 p53312AA, and 18 p53312AA mice with a similar gender distribution for each genotype were followed over a period of 2 y to determine whether the mutation had a detrimental effect on life span. The p53312AA mice survived for a time period comparable to the p53WT and p53312AA mice and did not develop spontaneous tumors at a significantly higher rate (P > 0.05) (Fig. 1D).

To verify that phosphorylation was not occurring in the p53312AA mice, an antibody was raised against the phosphorylated Ser312 residue in WT p53. Using this antibody, Ser312 was phosphorylated in p53WT mouse embryonic fibroblasts (MEFs) but not in p53WT MEFs following immunoprecipitation with an antibody to total p53 (Fig. 1E, top left). As Ser312 has been reported to be phosphorylated in response to UV (11), UV-irradiated MEFs were analyzed with the anti-phosphoserine 312 antibody. Although Ser312...
phosphorylation is undetectable in nonirradiated MEFs, Ser312 is phosphorylated 8h after UV irradiation in WT, but not p53312A/A cells, confirming the antibody’s specificity (Fig. 1E, Top Right). Next, we determined whether Ser312 is phosphorylated following IR. Phosphorylation of p53 at Ser312 could not be detected using this antibody after IR (Fig. 1E, Middle). As MEFs are primary cells, we transformed them with adenovirus E1A and analyzed Ser312 phosphorylation after IR in cells undergoing oncogenic stress. Ser312 was able to be phosphorylated in p53WT E1A-transformed cells, although not in p53312A/A cells (Fig. 1E, Bottom). We measured the ability of p53WT versus p53312A/A to influence the ability of E1A to immortalize MEFs. The difference was very small and not statistically significant, which agrees with the subtle effect of the mutation (Fig. S2).

p53312A/A Partially Rescues Embryonic Lethality Caused by Loss of Mdm4. To investigate whether Ser312 could affect the activity of p53, we used a system in which p53 activity was elevated. In mice, the targeted deletion of Mdm4, an inhibitor of p53, results in increased p53 activity and embryonic lethality. This embryonic lethality can be entirely overcome by the simultaneous deletion of p53 (12–14). To investigate whether the Ser312Ala mutation could affect p53 activity in vivo, we isolated embryos from intercrosses between Mdm4−/−p53312A/A mice. The Mdm4 deletion was caused by a gene trap insertion in the Mdm4 gene, 194 bp upstream of exon 2 (12). In an Mdm4−/− background at embryonic day 10.5 (E10.5), only 50% of embryos were obtained with WT p53 or p53312A/+ compared with the expected Mendelian ratios (Fig. 2A and Table 1). The embryos isolated with genotypes of either Mdm4−/−p53WT or Mdm4−/−p53312A/A were grossly abnormal when compared with WT embryos (Fig. 2B). However, the number of Mdm4−/−p53312A/A embryos obtained at E10.5 was 100% of the predicted number based on Mendelian segregation, suggesting that Ser312Ala mutation may reduce p53’s activity.

This hypothesis was supported by the frequency of embryos recovered at later stages of development (up to E13.5). Sixty-nine embryos were examined at E12.5 and, as expected, none of them carried a Mdm4−/+p53312A/A genotype. We obtained a reduced number of Mdm4−/−p53312A/A embryos (69%) (Fig. 2A and Table 1), and 75% of the predicted number for Mdm4−/−p53312A/A. At E12.5, although some of the embryos possessed severe growth defects, others (4/6 for Mdm4−/−p53312A/A−/−) had developed further, as shown in Fig. 2B. A similar trend was also seen in embryos collected at E13.5 (Fig. 2A and Table 1). In a cohort of 106, we unexpectedly obtained 2 Mdm4+/−p53312A/A embryos.

Embryos isolated at E13.5 were used to make MEFs. Of the two Mdm4−/−p53WT embryos isolated at this stage, only one produced cells that grew, albeit slowly (Fig. 2C). Similarly, although six
Mdm4−/− p53Ser312Ala mice were able to proliferate in culture (Fig. 2C). Very few Mdm4-null embryos were obtained at E14.5 irrespective of p53 status (Table 1). Therefore, p53Ser312Ala mutation in one or both alleles can partially protect against embryonic lethality caused by Mdm4 loss, suggesting that Ser312 phosphorylation is necessary for p53 to be fully active.

### Tumor Predisposition in p53Ser312Ala Mice Exposed to Ionizing Irradiation

To discover whether p53Ser312Ala mice were more susceptible to tumor formation following exposure to a carcinogenic inducer, we exposed 3-wk-old mice to a single 4-Gy dose of IR. The group included 16 p53WT (8 females and 8 males), 21 p53Ser312Ala/+ (10 females and 11 males), and 22 p53Ser312Ala−/− (12 females and 10 males) mice. This cohort had a mixed 129SvEv/C57BL6/J male background and included 40% of the cohort had developed tumors, compared with 1/21 (4.8%) heterozygous and 1/16 (6.3%) p53WT mice. Among a group of age-matched mice not exposed to IR, none developed tumors during this time period. No tumors were observed in mice of any genotype between 52 and 70 wk. After 70 wk, the mice began to develop a variety of tumors. When overall tumor incidence was assessed after 90 wk, p53Ser312Ala−/− mice were significantly more susceptible to tumor development following IR exposure than their p53WT or p53Ser312Ala/+ counterparts (p53Ser312Ala−/− vs. p53Ser312Ala/+; P = 0.0094; p53Ser312Ala−/− vs. p53WT; P = 0.0066) (Fig. 3A).

Table 1. Partial rescue of Mdm4−/− embryonic lethality by p53Ser312Ala

<table>
<thead>
<tr>
<th>p53</th>
<th>E10.5</th>
<th>E12.5</th>
<th>E13.5</th>
<th>E14.5</th>
</tr>
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<tbody>
<tr>
<td>Mdm4WT</td>
<td>WT</td>
<td>4 (4)</td>
<td>7 (4)</td>
<td>9 (7)</td>
</tr>
<tr>
<td>312 A/+</td>
<td>12 (8)</td>
<td>21 (9)</td>
<td>16 (13)</td>
<td>8 (8)</td>
</tr>
<tr>
<td>312 A/A</td>
<td>6 (4)</td>
<td>13 (4)</td>
<td>4 (7)</td>
<td>5 (4)</td>
</tr>
<tr>
<td>Mdm4−/−</td>
<td>WT</td>
<td>5 (8)</td>
<td>9 (9)</td>
<td>16 (13)</td>
</tr>
<tr>
<td>312 A/+</td>
<td>19 (15)</td>
<td>8 (18)</td>
<td>37 (27)</td>
<td>21 (16)</td>
</tr>
<tr>
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<td>5 (8)</td>
<td>2 (9)</td>
<td>11 (13)</td>
<td>12 (8)</td>
</tr>
<tr>
<td>Mdm4−/−</td>
<td>WT</td>
<td>2 (4)</td>
<td>0 (4)</td>
<td>2 (7)</td>
</tr>
<tr>
<td>312 A/+</td>
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<td>6 (9)</td>
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<tr>
<td>312 A/A</td>
<td>4 (4)</td>
<td>3 (4)</td>
<td>5 (7)</td>
<td>0 (4)</td>
</tr>
<tr>
<td>Total</td>
<td>61</td>
<td>69</td>
<td>106</td>
<td>64</td>
</tr>
</tbody>
</table>

The number of embryos identified at increasing stages of development following a cross of Mdm4−/− p53Ser312Ala−/− mice. The numbers in parentheses are the predicted numbers based on Mendelian segregation. E, embryonic day.

The increased tumor susceptibility of p53Ser312Ala−/− mice in response to IR implies that phosphorylation of murine p53 at Ser312 contributes to p53’s tumor suppression in response to IR. We observed biphasic tumor onsets (25–52 wk and 70–90 wk) in this study, with an intervening 18-wk tumor-free period. Importantly, p53Ser312Ala−/− mice were significantly more prone to tumor development than p53WT or p53Ser312Ala+ mice in both phases.

### Mice Carrying the Ser312Ala Mutation Are Initially Susceptible to Lymphoma Development

The tumors that developed within the first phase of tumor onset (25–52 wk) were almost all lymphomas, the exception being a sarcoma found in the intestinal wall of a p53Ser312Ala−/− mouse. The lymphomas found between 26 and 40 wk of age were all thymic lymphomas (n = 5; 22.7% of all p53Ser312Ala−/− mice). In contrast, lymphomas that arose between 42 and 52 wk (n = 3; 13.6%) were generalized lymphomas that had disseminated to the liver and kidney (Fig. 4B). The affected tissues from the three mice with generalized lymphomas were stained for B- and T-cell markers to determine their origin. One stained positively for the T-cell marker CD3 (Fig. 4B), whereas the other mice did not show positive staining for either CD3 or the B-cell marker B220. Disregarding the sarcoma, p53Ser312Ala−/− mice were significantly more susceptible to lymphoma development (p53Ser312Ala−/− vs. p53Ser312Ala+/−; P = 0.0034; p53Ser312Ala−/− vs. p53WT; P = 0.0345) (Fig. 4A).

All of the p53Ser312Ala−/− mice that developed lymphomas (n = 8) had a white coat. Five of the eight mice were female, indicating that there was no gender bias in lymphoma development in this cohort. The majority of p53Ser312Ala−/− and p53WT mice with white coats were tumor-free at this stage of the study. These data demonstrate that p53Ser312Ala−/− mice are predisposed to lymphomas and that their genetic background may influence tumor development.

As a link has been made between the development of thymic lymphomas in p53-null mice and the resistance of their thymocytes to DNA damage-induced apoptosis (15–18), we investigated whether the Ser312Ala mutation could compromise p53’s induction of apoptosis in response to IR. Thymocytes from p53WT and p53Ser312Ala−/− mice were exposed to a variety of apoptotic stimuli, including IR, and apoptosis was assessed by Annexin V binding (Fig. S3). Little difference was detected in the levels of apoptosis induced by any of the stimuli. Therefore, apoptotic failure is not behind the increased incidence of thymic lymphomas seen in p53Ser312Ala−/− mice.

### Irradiated p53Ser312Ala Mice Are Susceptible to Liver Hyperplasia at a Later Stage

Following the occurrence of the lymphomas, no tumors were observed in mice of any genotype for a period of 18 wk. After 70 wk, however, the mice began to develop various tumors. Most notably liver tumors (predominantly in p53Ser312Ala−/− mice). Of the surviving 13 p53Ser312Ala−/− mice, 5 (38.5% of remaining p53Ser312Ala−/− mice, 22.7% of total p53Ser312Ala−/− cohort) developed liver tumors between 70 and 90 wk (Fig. 5A). In contrast, only 1 p53Ser312Ala+ mouse (1/20) (5.0% of remaining mice, 4.8% of total) developed liver tumors.

**Fig. 3.** p53 Ser312Ala mutation predisposes mice to tumor development following IR. (A) Survival curve showing overall tumor incidence over the course of the study. (B) The incidence of different tumor types by genotype. p53Ser312Ala−/− mice were significantly more prone to lymphomas and liver tumors. The incidence of lung tumors (the other main tumor group found during this study) was not significant.
was carried out using mRNA from the livers of two pairs of p53WT mice and p53312A/A mice. This was again found to be statistically significant (p53312A/A vs. p53312A/+; P = 0.0339; p53312A/A vs. p53WT; P = 0.0234) (Fig. 5A). The p53312A/A mice that developed liver tumors were mainly black (4/5 mice), and 4/5 of the liver tumors occurred in females, suggesting that there might be a gender and strain bias in this cohort for the development of liver tumors.

Histological analysis revealed that, although the hyperplasias in these mice were frequently benign, one liver displayed hepatocellular carcinoma (Fig. 5B, Lower Left and Lower Right) that had metastasized to the large intestine. The surrounding liver tissue in the afflicted mice was also frequently found to show circular, unstained areas consistent with the presence of lipid droplets. In addition, one p53312A/A mouse (female, black coat), killed at this point as it had developed a prolapse, was found to have a highly abnormal, fatty liver although it had not developed any hyperplasia. Therefore, a p53 Ser312Ala mutation can confer sensitivity to liver hyperplasia following IR.

**p53 Ser312Ala Mutation Dampens the Transcriptional Activity of p53.**

To better understand the mechanism by which Ser312 phosphorylation contributes to p53’s tumor suppression, we undertook a genome-wide gene array analysis using the Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay. Due to the increased incidence of liver tumors in p53312A/A mice, the analysis was carried out using mRNA from the livers of two pairs of p53WT and p53312A/A littermates that were either untreated or isolated 6 h after receiving a 4-Gy dose of IR. A p53 response has been previously detected in the liver at this time point (19) and is consistent with the phosphorylation of p53 at Ser312 seen in E1A-transformed MEFs (Fig. 1E). As expected, the expression levels of many genes, including some well-known p53 targets, were altered in p53312A/A mouse livers, with or without IR. However, the spectrum of genes affected differed considerably between the two samples (Fig. 6A). There were 124 genes whose expression was changed in p53WT but not in p53312A/A mice; however, these genes were not recognized p53 target genes.

The recognized p53-responsive genes whose expression differed to the greatest degree were p21 and cyclin G1 (Table 2). To validate our array data, quantitative RT-PCR was performed to measure the expression levels of p21 mRNA in p53WT versus p53312A/A mouse livers, with or without IR. Without IR, the levels of p21 and cyclin G1 mRNA in WT and mutant livers were comparable. However, after IR exposure, the increase in p21 and cyclin G1 mRNA was significantly less in p53312A/A mice than in WT mice (Fig. 6B and Table S2). In agreement, there was a small reduction in the level of p21 protein induced in p53312A/A livers in response to IR (Fig. 6C). This is further supported by the data obtained in p53WT versus p53312A/A MEFs (Fig. 6D), where p21 expression was lower with mutant p53, especially in the absence of Mdm4. These data suggest that phosphorylation at Ser312 enhances p53’s transcriptional activity.

By fine-tuning the actions of p53, Ser312 phosphorylation may have only a subtle effect on the expression of its target genes. However, when these effects are combined, they may be sufficient to mount a cellular response that is biologically significant following a genotoxic challenge such as IR. Our data demonstrate that despite possessing a mutation at only a single phosphorylation site, the Ser312Ala mutation confers increased susceptibility to tumorigenesis following IR exposure, especially in the thymus and, unusually, in the liver.

**Discussion**

In this study, we used knock-in mice carrying a Ser312Ala mutation in the p53 gene to investigate how phosphorylation at this site...
Table 2. Known p53 target genes found to be differentially expressed in both p53WT and p53312A/A samples upon irradiation

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Fold change (p53WT)</th>
<th>Fold change (p53312A/A)</th>
<th>Fold change (p53WT:p53312A/A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDKN1A</td>
<td>9.90</td>
<td>6.60</td>
<td>−3.30</td>
</tr>
<tr>
<td>CCNG1</td>
<td>3.25</td>
<td>2.15</td>
<td>−1.10</td>
</tr>
<tr>
<td>GADD45G</td>
<td>6.82</td>
<td>5.95</td>
<td>−0.87</td>
</tr>
<tr>
<td>PLK3</td>
<td>5.49</td>
<td>4.74</td>
<td>−0.75</td>
</tr>
<tr>
<td>PHLD3A</td>
<td>3.04</td>
<td>2.89</td>
<td>−0.15</td>
</tr>
<tr>
<td>BTG2</td>
<td>3.57</td>
<td>5.01</td>
<td>1.44</td>
</tr>
</tbody>
</table>

These genes are among the 117 overlapping genes in Fig. 6A. Data indicate the fold change in each gene after irradiation.

As with the untransformed MEFs in Fig. 1E, we were unable to detect phosphorylation of p53 Ser312 in WT liver or thymus following IR, despite numerous attempts. However, Ser312 was phosphorylated in irradiated E1A-transformed MEFs, suggesting that phosphorylation at this site occurs in cells already undergoing chronic (in this case, oncogenic) stress. If exposure to IR causes such a stress in vivo, the failure of p53312A/A mice to phosphorylate

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Ser312 in these conditions may prevent p53 from efficiently countering this stress, making the mice susceptible to tumor growth.

Existing studies show that IR-induced p53 in liver does not induce apoptosis, but instead induces p21-mediated cell cycle arrest (19, 31, 32). Restoration of p53 in vivo also causes liver tumor regression via p53-mediated senescence (33). The fact that p53312A/+ mice are prone to liver tumor development suggests that Ser312 mutation may hinder p53 by suppressing its ability to regulate cell cycle progression. This is supported by our observation that Ser312A mutation predisposes mice to lymphoma without affecting p53-mediated thymocyte apoptosis (Fig. 4 and Fig. S3). The reduced expression of p21 seen in the liver of p53312A/A mice, and MEFs derived from p53312A/A mice following DNA damage, correlates with previous reports that human p53 phosphorylated at Ser315 has an increased binding affinity for DNA (34). The resulting elevated expression of p21 is reversed by mutation of Ser315 to Ala or by inhibition of cdks by roscovitine (7, 11).

An increase in the expression of p21, but not proapoptotic genes such as Bax, has been reported in the livers of WT mice following IR exposure (35, 36). The elevated levels of p21 persisted for at least 10 wk, implying a central role for p21 in preventing proliferation following DNA damage in the liver (35). A reduction in p21 levels alongside altered p53 expression has also been observed in human hepatocellular carcinomas (36). Reduced p21 expression could also explain the partial rescue of the Mdm4−/− phenotype, as Mdm4−/− p21−/− embryos have a similar phenotype to that seen in the Mdm4−/−p53312A−/− mice (37). However, the number of genes whose expression varies between p53WT and p53312A/A mice (Fig. 6A) indicates that the difference in p21 expression is merely one element in a much more complex scenario. The fact that p21−/− mice do not develop liver tumors, either spontaneously or following IR exposure, would seem to confirm this (38).

This study has demonstrated that a single phosphorylation site is an important participant in tumor suppression by p53, depending upon which function is required and the site of suppression. Therefore, the many posttranslational modifications of p53 may act in a highly specific manner to subtly modify its behavior, and it is important that the contexts in which this occurs be determined.

Materials and Methods
Mice. The p53312A/A knock-in mice were generated by inGenious Targeting Laboratory. Mdm4−/− mice were a generous gift from J.-C. Marine (University of Leuven) (12). All animal procedures were approved by local ethical review and licensed by the UK Home Office.

Antibodies. The p53 phospho-Ser312 polyclonal antibody was made by immunizing rabbits with the peptide CT5ApsPPGKQKPK, followed by purification against the nonphosphorylated peptide to remove nonspecific antibodies. For more details, see SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Generation of Mice. A 19-kb genomic DNA fragment containing exons 6–10 of the TRP53 gene was cloned from a mouse 129Sv/Ev λ genomic library. The targeting vector was constructed using a 1.1-kb DNA fragment as the short arm, which was a PCR fragment from primers 53P20 and 53P19. Primer 53P20 is located 330 bp downstream of exon 10, with a sequence of 5'-CCAC-CACCACACATCACACC-3'. Primer 53P19 is located upstream of exon 10, with a sequence of 5'-CTCATGCTCT-GAGGCTGTGCC-3'. The short arm was inserted into the 5' end of the Neo gene cassette (flanked by LoxP sites) using a PspOMI site. The codon mutation (TCT → GCC) was introduced by PCR-directed mutagenesis. The PCR fragment was cloned into the 3' end of the neo cassette using a natural BamHI site, and a 6.5-kb BamHI genomic fragment was cloned into the BamHI site to serve as the long arm.

The targeting vector was linearized using NotI and transfected into 129Sv/Ev (ItIL1) embryonic stem (ES) cells. After G418 selection, surviving clones were screened by PCR to identify recombinants. A correctly targeted ES cell line was microinjected into C57BL/6J blastocysts to produce chimeras, which were mated to generate germline transmission of the mutation. The neo cassette was removed by mating the mice carrying the mutated codon with EIIaCre loxp deleter mice (FVB) (The Jackson Laboratory). The progeny were genotyped to verify Neo deletion and mated with C57BL/6 mice to identify non-mosaicNeo-deleted mice.

Genotyping. The TGT → GCC mutation was genotyped by PCR. A common PCR primer, 5'-CCCCITAAACCCCAATGCTCC-3', was used with either primer mp53S312 (5'-GCCACCTGCA-CAAGCGCCCT-3') to detect the WT allele or with primer mp53A312 (5'-GCCACCTGCAACAAGCGGCC-3') for the mutant allele. Bases in bold text indicate either the wild type (TCT) or mutant (GCC) codons.

Tumor Induction and Analysis. Mice were exposed to 4 Gy IR from an X-ray source at 3 wk of age and observed for signs of tumor growth or general ill health. Postsacrifice, all tissues were fixed for histological analysis as described previously (1). Statistical significance was determined using the log-rank test.

Quantitative Real-Time PCR. The primer sequences used for quantitative real-time PCR (qRT-PCR) were mGAPDH (forward: AAC TTT GGC ATT GTG GAA GG; reverse: ACA CAT TGG GGG TAG GAA CA), m-β-Actin (forward: TGT TAC CAA CTG GGA CCA CA; reverse: GGG GTG TTG AAG GTC TCA AA), mp21 (forward: TCT TGT GCT TTG ACA CGG; reverse: CTC TTC AGT CGT TTT CAC A), and m-Cyclin G1 (forward: AGG TCT GCG GCT TGA AAC TA; reverse: TCA GTC CAA CAC ACC CAA GA).

Gene Expression Profiling. The Affymetrix GeneChip Whole Transcript Sense Target Labeling Assay was used to amplify and reverse-transcribe total RNA and to biotinylate sense-strand DNA targets. Arrays were hybridized with the labeled-target hybridization mixture by rotation at 60 rpm in an Affymetrix GeneChip hybridization oven at 45 °C for 16 h, washed in an Affymetrix GeneChip Fluidics station FS 450, and scanned by an Affymetrix Gene Chip scanner 3000 7G system. Scanned image files (.CEL) were processed, normalized (RNA-Sketch Quantile), and log-transformed by Expression Console Software (Affymetrix). Normalized intensity files were statistically analyzed to calculate t-statistics and P values. The P values were further adjusted by the false discovery rate (<5%) approach and enriched for data displaying over twofold change.

Cell Culture. Mouse embryonic fibroblasts (MEFs) were prepared from embryonic day 13.5 embryos generated by intercrossing p53312A/+ mice and were cultured in DMEM + 10% FBS. The MEFs were transformed using the retroviral vector pBabe-puro E1A. Colony analysis was performed on Giemsa-stained dishes after 3 wk selection in puromycin. Growth analysis was performed by seeding the cells in 6-cm dishes at 3 × 10^5, counting the total number of cells after 3 d before reseeding the cells again at 3 × 10^5 cells/dish.

Assessment of Apoptosis. Thymocytes were removed from mice (7–12 wk) and, after exposure to apoptotic stimuli, incubated with Annexin V-FITC (Abcam) and analyzed by FACS.

Immunoblotting and Antibodies. Lysates were prepared in a solution of 8 M urea, 1 M thiourea, 0.5% CHAPS, 50 mM DTT, and 24 mM spermine, p53 was immunoprecipitated using anti-p53 mAb 242 in Nonidet P-40 lysis buffer. The p53 phospho-Ser312 polyclonal antibody was made by immunizing rabbits with the peptide CTSApSPQKKK, followed by purification against the nonphosphorylated peptide to remove nonspecific antibodies. Other antibodies used were: CM5 (anti-p53), anti-p21 SX118 (mAb), anti-actin (C-2) (Santa Cruz Biotechnology), and anti-CD3 rabbit polyclonal antibody (Abcam).

RNA Extraction. Total RNA was extracted from mouse liver by homogenization in TRIzol (Invitrogen). Genomic DNA was removed with DNaseI and RNA enriched with RNase columns (Qiagen). Quantity and integrity of extracted RNA was assessed using a NanoDrop Spectrophotometer and an Agilent 2100 Bioanalyzer, respectively.

Quantitative Real-Time PCR. For cDNA synthesis, 2 μg of total RNA was reverse-transcribed with random primers using a Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase kit (Invitrogen), qRT-PCR was carried out with 1 μl of cDNA, using SYBR Green master mix (Applied Biosystems), and analyzed using the StepOne Plus Real Time PCR Detection System (Applied Biosystems). The relative abundance of specific mRNA levels was calculated by normalizing to both GAPDH and β-actin expression by the 2−ΔΔCt method (2).

Gene Expression Profiling. Gene expression profiling was determined using Mouse Gene 1.0 ST (MoGene) arrays (Affymetrix).


**Fig. S1.** Generation of p53^{312AA} mutant mice. Schematic showing the targeted mutation of the murine p53 gene and illustrating the targeting vector ("mutant") containing the TCT → GCC mutation in exon 9.

**Fig. S2.** Impact of p53^{312AA} mutation on cell growth and senescence. (A) Nontransformed MEFs were counted and reseeded at 3-d intervals, and the fold growth in the intervening period was calculated. (B) Colony assay of E1A immortalized MEFs. MEFs were immortalized with retroviral E1A alongside an empty vector control, and colonies were allowed to grow under antibiotic selection before being stained with Giemsa and counted. Data are representative of three independent experiments. The p value was calculated using the Student’s t test.

**Fig. S3.** Induction of apoptosis in p53^{312AA} thymocytes. Apoptosis in thymocytes from p53^{WT} and p53^{312AA} mice detected by Annexin V binding. Apoptosis was assessed 6 h after exposure to 5 Gy ionizing radiation (IRR), anti-Fas (clone JO-1; 0.5 μg/mL) (Fas), etoposide (10 μM) (Etop), or thapsigargin (50 nM) (TG). Con indicates untreated cells.
Fig. S4. Susceptibility of mice to tumors by coat color. (A) Black p53^{312A/A} mice are significantly more liable to develop liver tumors ($P < 0.05$) (Left) than black wild type or p53^{312A/+} mice, although there is no difference in their overall tumor susceptibility (Right). No black wild-type mice developed liver tumors. (B) White p53^{312A/A} mice are significantly more susceptible to both lymphomas ($P < 0.05$) (Left) and tumors in general ($P < 0.05$) (Right) than either wild-type or p53^{312A/+} mice with white coats.

Table S1. Coat-color distribution versus genotype in the cohort of mice used in the tumor study

<table>
<thead>
<tr>
<th></th>
<th>p53^{WT}</th>
<th>p53^{312A/+}</th>
<th>p53^{312A/A}</th>
<th>Total</th>
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<tbody>
<tr>
<td>Agouti</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td>10</td>
</tr>
<tr>
<td>Black</td>
<td>7</td>
<td>7</td>
<td>7</td>
<td>21</td>
</tr>
<tr>
<td>White</td>
<td>6</td>
<td>9</td>
<td>13</td>
<td>28</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>21</td>
<td>22</td>
<td>59</td>
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Table S2. Summary of the array analysis data of two representative p53 target genes

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<th>Gene symbol</th>
<th>Ref Seq ID</th>
<th>Affymetrix ID</th>
<th>Fold change</th>
<th>p53^{WT} Treated vs. untreated</th>
<th>p53^{WT} Treated vs. untreated</th>
<th>p53^{312A/A} Treated vs. untreated</th>
<th>p53^{312A/A} Treated vs. untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCNG1 (Cyclin G1)</td>
<td>NM_009831</td>
<td>10385271</td>
<td>NC</td>
<td>3.25</td>
<td>0.001023</td>
<td>2.15</td>
<td>0.010745831</td>
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<td>CDKN1A (p21)</td>
<td>NM_007669</td>
<td>10443463</td>
<td>NC</td>
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<td>0.004587</td>
<td>6.6</td>
<td>5.72293E-05</td>
</tr>
</tbody>
</table>

NC, no change.