Cell

EXTENDED EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and Transfection

Mouse embryonic fibroblasts (MEFs) were isolated from 13.5 day postcoital embryos obtained by heterozygous intercross according to the standard procedures. H1299 cells and MEFs were cultured in DMEM medium supplemented with 10% FBS or heat-inactivated FBS (MEF only) and 1% non-essential amino acids (MEF only) plus 200 unit/ml penicillin/streptomycin. Cells were maintained at 37°C in a 5% CO2 incubator. For cell growth rate analysis, 3×10^4 MEFs of different genotypes were seeded in 6-well plates at day 0 and counted daily with trypan blue dye exclusion. The K-to-R mutation of mouse p53 at K117, K161 and K162 was performed using QuickChange Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's instructions. Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Chromatin Immunoprecipitation Assay (ChIP)

MEFs or transfected H1299 cells were incubated in culture media and crosslinked with 1% formaldehyde with gentle shaking for 10 min at room temperature, and neutralized with a final concentration of 0.125 M glycine. After two washes with cold PBS, cells were harvested in ice cold lysis buffer (10 mM Tris-Cl [pH 8.0], 85 mM KCl, 0.5% NP-40, 5 mM EDTA, and fresh proteinase inhibitor cocktail) and incubated on ice for 10 min. Nuclei were harvested, suspended in cold RIPA buffer (10 mM Tris-Cl [pH 8.0], 150 mM NaCl, 0.1% SDS, 0.1% DOC, 1% Triton X-100, 5 m MEDTA, and fresh proteinase inhibitor cocktail), and sonicated to about 500 bp. Cell extracts were then blocked with protein A/G beads (Millipore), and the supernatants were used for immunoprecipitation by various antibodies. After eight washes by RIPA buffer, the proteins were eluted from the beads by 0.5 ml elution buffer (0.1 M NaHCO3 and 1% SDS). After reverse crosslinking, the DNA samples were recovered by phenol extraction and ethanol precipitation. The purified DNA was then analyzed by PCR within linear amplification range followed by 2% agarose gel electrophoresis.

Cell and Tissue Staining

Immunofluorescence cell staining was performed using standard protocols. For cell cycle analysis, MEFs were seeded and grow on coverslips in 6-well plates overnight and then irradiated with 5 or 10 Gy of γ irradiation and cultured for 23 hr, the cells were pulse-labeled with 10 μM BrdU (Sigma-Aldrich) for 45 min and then fixed with 4% paraformaldehyde (Sigma-Aldrich) for 20 min at room temperature. Then the percentage of BrdU-positive cells was determined by immunofluorescence using anti-BrdU antibody (BD Biosciences). Images were taken by Nikon eclipse 80i fluorescence microscopy using Photometrics CoolSNAP HQ^2 camera. For immunohistochemical staining, tissues from mice either untreated or exposed to different dose of γ irradiation were collected four hours later and fixed with 10% formalin overnight, then processed, paraffin-embedded, sectioned and stained with anti-mouse p53 (CM5; Leica Microsystems) and cleaved caspase 3 (Asp175; Cell Signaling Technologies) antibodies according to the standard procedures.

Senescence-Associated-β-Galactosidase (SA-β-gal) Staining

The SA-β-gal staining was performed as described previously (Dimri et al., 1995). Cells were fixed for 5 min at room temperature in 3% formaldehyde, washed twice with PBS, and then incubated at 37°C for 16 hr with fresh SA-β-gal stain solution (1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactosidase (X-Gal), 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$).

RNA Isolation and qRT-PCR

Total RNA was isolated from MEF cells or mouse tissues using TRIzol (Invitrogen) and treated with DNase I (Ambion). 1 μg total RNA was reverse-transcribed using SuperScript III First-Strand Synthesis SuperMix (Invitrogen) and random primers following manufacturer's protocol. PCR was performed in triplicate using SYBR green mix (Applied Biosystems), and a 7500 Fast Real-Time PCR System (Applied Biosystems) under the following conditions: 15 min at 95°C followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. qRT-PCR assay and data analysis were performed as described before (Bookout and Mangelsdorf, 2003) and Primers used for RT-PCR and qRT-PCR were shown in Primers For ChIP and RT-PCR.

Flow Cytometry

To analyze apoptosis in vivo, mice were either untreated or exposed to 5 Gy of whole-body γ irradiation. Four hours later, mice were sacrificed and thymocytes in single-cell suspension were prepared and stained with annexin-V-FITC (BD Biosciences) for analysis. Cells were sorted using a Becton Dickinson FACScalibur machine and data were analyzed using CellQuest.

Western Blotting

Cell or tissue lysates were prepared in RIPA buffer (50 mM Tris-HCl [pH 8], 150 mM NaCl, 0.1% SDS, and 0.5% Na deoxycholate, 1% NP40 and fresh proteinase inhibitor cocktail). Protein extracts were analyzed by western blotting according to standard protocols using primary antibodies specific for p53 (CM5, Leica Microsystems), P-S15p53 (9284, Cell Signaling Technologies), Mdm2 (Ab5, Millipore), p21 (SX118, Santa Cruz), PUMA (p4743, Sigma-Aldrich), cleaved caspase 3 (Asp175, Cell Signaling Technologies), Arf (5-C3-1, Santa Cruz), Gls2 (C-term E-513, Abgent), Tigar (E-2, Santa Cruz) and β-actin (A3853, Sigma-Aldrich). HRP-conjugated

anti-rabbit,-mouse and -rat secondary antibodies (GE Healthcare) were used and signal was detected using an ECL western blotting detection system (GE Healthcare).

Primers for ChIP and RT-PCR

Primers used for the p53 ChIP assay in human cells in Figures 3D and 6E were shown as follows: human *p21* forward 5′-TGATTGGCTTTCTGGCCGTC-3′, human *p21* reverse 5′-CCAGCCTCTTCTATGCCAGAGC-3′, human *MDM2* forward 5′-GTCAAGTTCAGACACGTTC-3′, human *MDM2* reverse 5′-CCTCCAATCGCCACTGA ACAC-3′, human *PUMA* forward 5′-CGTA CATCGGTCGGTCTGTGTACG-3′, human *PUMA* reverse 5′-CCAGACACCGGGACAGTCG-3′, human *GLS2* forward 5′-AGCCAA ATAAGCCCTCCAACCC-3′, human *GLS2* reverse 5′-TGTGGTTTCGCCATATCGGT C A-3′, human *TIGAR* forward 5′-CGGCAGGTCTTAGATAGCTT-3′, human *TIGAR* reverse 5′-GGCAGCCGGCATCAAAAACA-3′. RT-PCR analysis of mouse *Glut3* or β*-Actin* as a control was carried out using the following primer pairs: *Glut3* forward 5′-ATG GGGACAACGAAGGTGACCC CATCTCTG-3′, *Glut3* reverse 5′-TGAGCTACCAGAAT CCCAACAACG-3′, β*-Actin* forward 5′-ATGGATGACGATATCGCTGCGC-3′, and β*-Actin* reverse 5′-GCAGCACAGGGTGCTCCTCA-3′.

qRT-PCR Primers

For the quantitative real-time PCR (qRT-PCR) analysis of mouse transcripts the following primers were used: *Mdm2* forward 5′-GGACTCGGAAGATTACAGCCTGA-3′, *Mdm2* reverse 5′-TGTCTGATAGACTGTGACCCG-3′, *p21* forward 5′-AGATCCACAG CGATATCCAGAC-3′, *p21* reverse 5′-ACCGAAGAGACAACGGCACACT-3′, *Puma* forward 5′-ACGACCTCAACGCGCAGTACG-3′, *Puma* reverse 5′-GAGGAGTCCCATG AAGAGATTG-3′, *Bax* forward 5′-CAGGATGCGTCCACCAAGAA-3′, *Bax* reverse 5′-AG TCCGTGTCCACGTCAGCA-3′, *Killer/DR5* forward 5′-CGGGCAGATCACTACACCC-3′, *Killer/DR5* reverse 5′-TGTTACT GGAA CAAAGACAGCC-3′, *Noxa* forward 5′-TCGCAAA AGAGCAGGATGAG-3′, *Noxa* reverse 5′-CAC TTTGTCTCCAATCCTCCG-3′, *14-3-3*σ forward 5′- GTGTGTGCGACACCGTACT-3′, *14-3-3*σ reverse 5′-CTCGGCTAGGTAGC GGTAG-3′, *Gadd45*α forward 5′-CCGAAAGGATGGACACGGTG-3′, *Gadd45*α reverse 5′-TTATCGGGGTCTACGTTGAGC-3′,*Ccng1* forward 5′-CGTGTCC TCAGTTCTTTGGC TTTGACACG-3′, *Ccng1* reverse 5′-GATGCTTCGCCTGTACCTTCATT-3′, *Tigar* forward 5′-AAAGACATGG CGGTGAAGTACG-3′, *Tigar* reverse 5′-CGGATTAGCGGCTTGCCT TCCG-3′, *Sco2* forward 5′-AGGCCAGTGGGTGCTGATGTA-3′, *Sco2* reverse 5′-CGGA CTCCGCCTCTAGCTTCCG-3′, *Gls2* forward 5′-GAACAAGATGGCTGGGAACGA-3′, *Gls2* reverse 5′-CGGAGCCGATGGCGTAATTCCG-3′, *Aldh4*α forward 5′-CGCCTGGCT GGAGAGTGTGGCG-3′, *Aldh4*α reverse 5′-GGCC GCCGTACTCGAATG-3′, *Dram1* forward 5′-GGGCATCGTAGCCAACTTCC-3′, *Dram1* reverse 5′-CGGTGAAAGCCAGA AGCG CACCG-3′, *Gamt* forward 5′-CGTCCGGTTGCAGTAGGTGAGGACG-3′, *Gamt* reverse 5′-GACACGTACCCGCTGTCTGAAG-3′, *Gpx1* forward 5′-CGGGGTGGTGCT CGGTTTCCCG-3′, *Gpx1* Reverse 5′-CCAGGTCGGACGTACTTGAGG-3′, *Gss* forward 5′-CGGAAAAGTTGCTGTGGTGTACTTCCG-3′, *Gss* reverse 5′-CTCTAGCATCAGGC GTGCTTCC-3′, *Sesn1* forward 5′-GCAGTTA CAGGAATGCCGAGAG-3′, *Sesn1* reverse 5′-CGGACTGGTGTAACTTCATCATCGTCCG-3′, *Sesn2* forward 5′-CACCTTCGCCTC CCAGTGA-3′, *Sesn2* reverse 5′-CGCACTGAAGCTGCCTCATGCG-3′, *Pai-1* forward 5′-TTGTCCAGCGGGACCTAGAG-3′, *Pai-1* reverse 5′-AAGTCCACCTGTTTCACCATA GTCT-3′, *Pml* forward 5′-CCAGCGTCCTGCCACAGT-3′, *Pml* reverse 5′-GGTGCGATA TGCATTCAGTAACTC-3′, and β*-Actin* forward 5′-GGCTGTATTCCCCTCCATCG-3′, β*-Actin* reverse 5′-CCAGTTGGTAACAATGC CATGT-3′.

SUPPLEMENTAL REFERENCES

Dimri, G.P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., Medrano, E.E., Linskens, M., Rubelj, I., Pereira-Smith, O., et al. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. Proc. Natl. Acad. Sci. USA *92*, 9363–9367.

Bookout, A.L., and Mangelsdorf, D.J. (2003). Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. Nucl. Recept. Signal. *1*, e012.

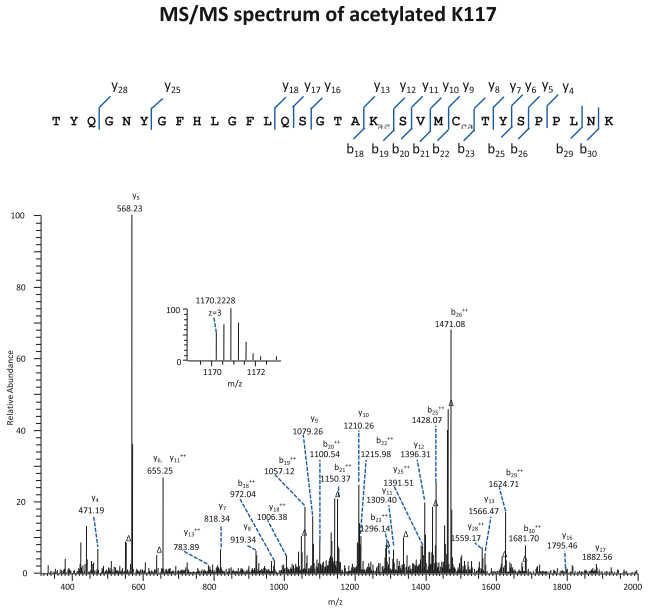
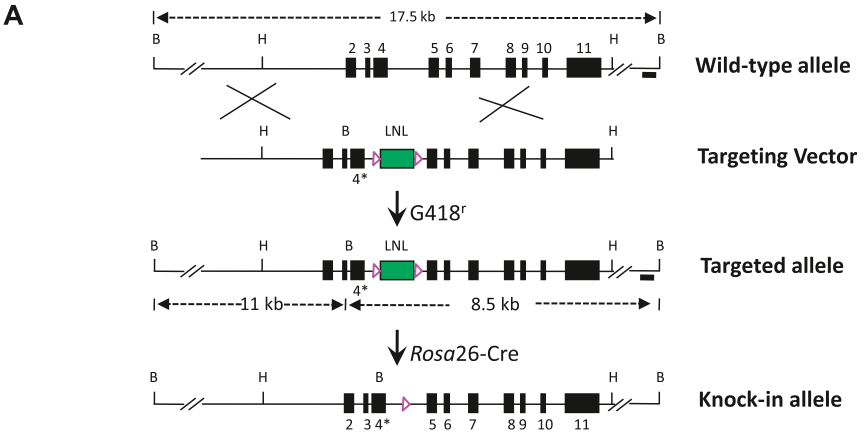
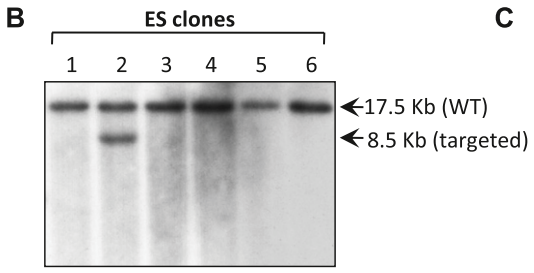
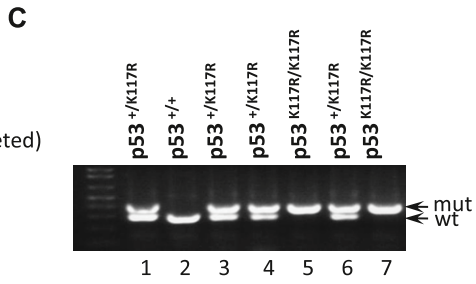


Figure S1. Mass Spectrometry Analysis of the Mouse p53-Derived Peptides Containing K117, Related to Figure 1

The fragmentation spectrum of ^{99}TYQGNYGFHLGFLQSGTAK$_{ac}$SVMC$_{ca}$TYS PPLNK^{129} showed the presence of peptides with acetylation at K117. The mouse p53 protein was prepared as described in the Experimental Procedures. "C$_{ca}$" designates carbamidomethyl cysteine induced by in vitro alkylation.







D Mendelian ratio

Heterozygous Intercross	Expected frequency	Observed frequency
$p53^{+/+}$	25% (34/136)	25% (34/136)
$p53^{+/K117R}$	50% (68/136)	51.5% (70/136)
$p53^{K117R/K117R}$	25% (34/136)	23.5% (32/136)

Figure S2. Generation and Genotype Analysis of $p53^{K117R/K117R}$ Mice, Related to Figure 1

(A) Targeting scheme for generation of $p53^{K117R}$ mice. The targeting construct contains a 5′ homology region with exons 1-4, a floxed neomycin resistant gene cassette (LNL) and a 3′ homology region with exons 5-11. 4* designates the mutated exons creating a BglII restriction site at K117. Targeted ES clones are selected by G418 and detected by Southern blot with the indicated probe and BglII digestion as containing an 8.5 kb band. Mice generated from correctly targeted ES clones were mated with *Rosa26-Cre* mice to excise the LNL cassette and generate mice with only the targeted $p53^{K117R}$ mutations. Dashed lines show the size of the fragments generated from the indicated allele after BglII digestion. B, BglII; H, HindIII.

(B) Southern blot analysis of targeted ES clones using the 3′ external probe indicated in (A). Genomic DNA of ES clones was digested with BglII and correctly targeted clone 6 shows a wild-type 17.5 kb BglII band and a recombinant 8.5 kb BglII band.

(C) PCR genotyping of the $p53^{K117R}$ mutant allele. The wild-type and mutant PCR products are 300 bp and 390 bp, respectively.

(D) Table showing the expected and observed Mendelian ratios from $p53^{K117R}$ heterozygous breedings.

(E) Sequencing analysis of p53 transcripts expressed from $p53^{+/+}$ and $p53^{K117R/K117R}$ MEF cells. The K-to-R mutation at K117 of p53 is highlighted in the mutated $p53^{K117R}$ allele.

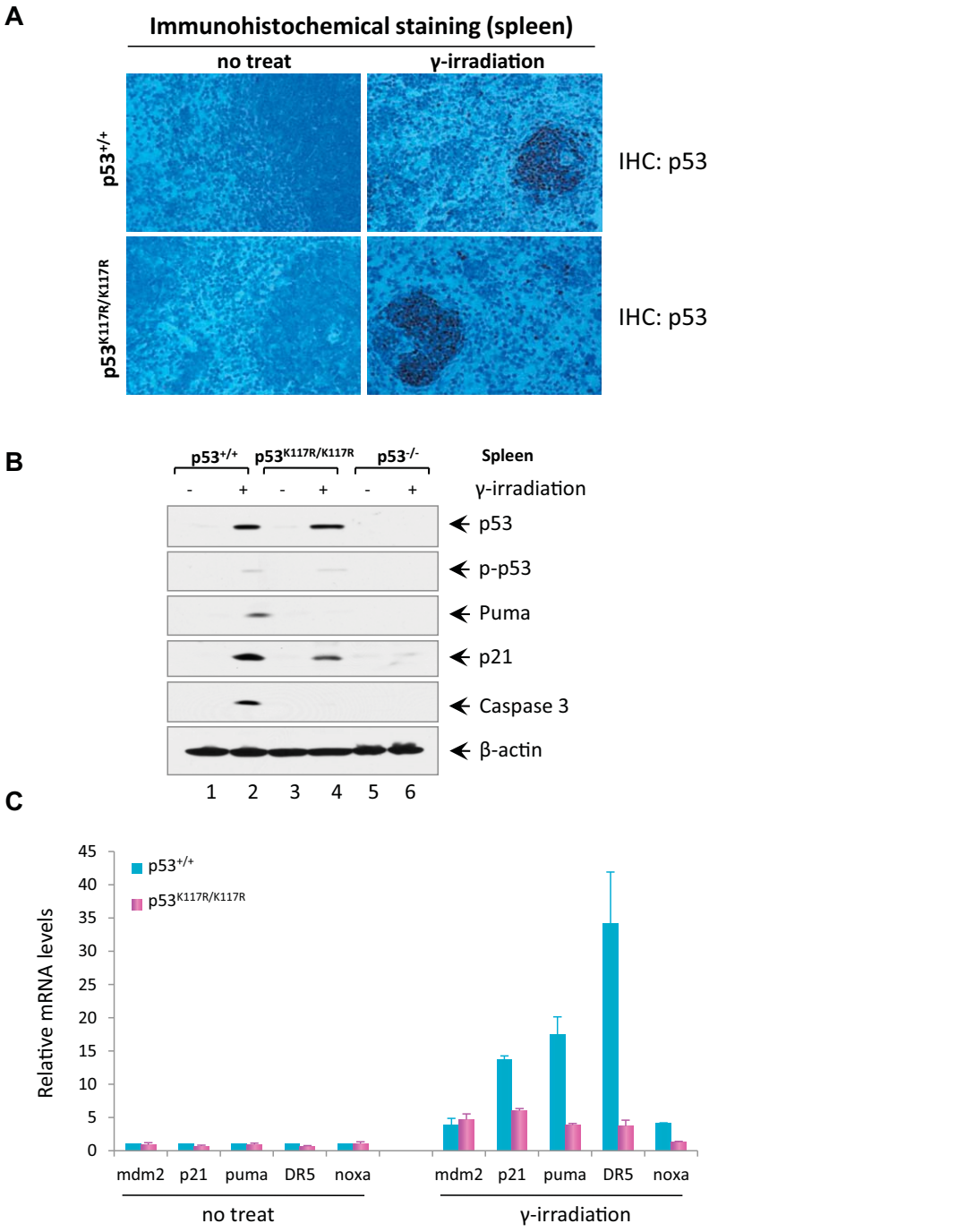


Figure S3. p53^{K117R} Retains Normal Transactivation Activity for p21 and Mdm2 but Impairs Activation of Proapoptotic Target Genes in Spleen, Related to Figure 1

(A) Representative immunohistochemical staining of p53 in spleens of p53$^{+/+}$ and p53$^{K117R/K117R}$ mice. Eight-week-old mice were either untreated or exposed to 12.5 Gy of γ irradiation. Four hours later, spleens from these mice were fixed overnight then processed, paraffin-embedded, sectioned and stained with anti-mouse p53 (CM5) antibodies according to standard protocol.

(B) Western blot analysis of the spleens of p53$^{+/+}$ and p53$^{K117R/K117R}$ mice. Mice were either untreated or exposed to 12.5 Gy of γ irradiation, four hours later, splenocytes were isolated and analyzed for the expression of indicated p53 target genes and cleaved caspase 3.

(C) qRT-PCR analysis of p53 target genes after DNA damage treatment. Mice were treated as described in (A), then spleens from p53$^{+/+}$ and p53$^{K117R/K117R}$ mice were removed for total RNA extraction. Expression of p21, Mdm2 and proapoptotic genes PUMA, Noxa and Killer/DR5 were analyzed by quantitative real-time RT-PCR. The relative amount of specific mRNA was first normalized to β-actin and then to untreated wild-type sample values. Error bars show ± SEM of three independent experiments.

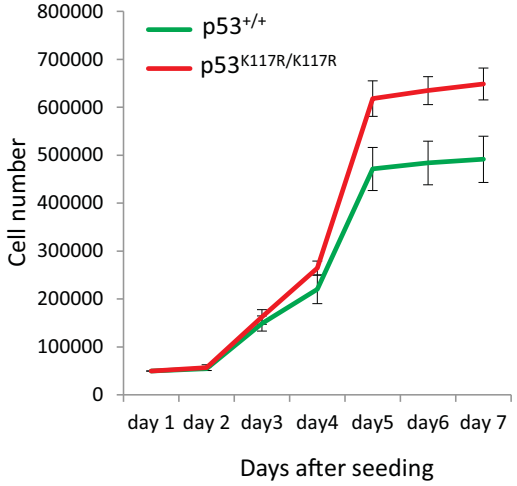


Figure S4. Proliferation Analysis of $p53^{+/+}$ and $p53^{K117R/K117R}$ MEFs, Related to Figure 2

To measure the growth rates, 3×10^4/well MEFs were seeded into 6-well plates at day 0 and counted at indicated time points. Error bars represent ± SD of three independent MEF lines.

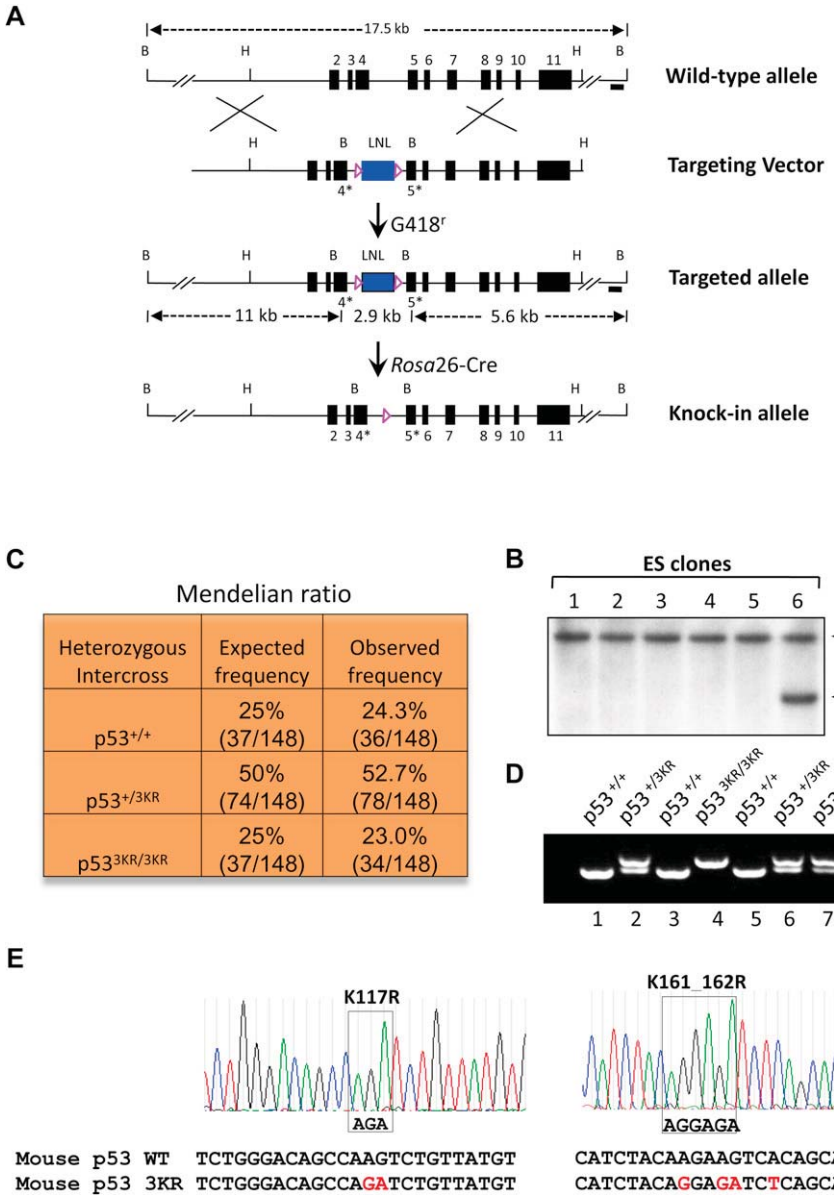


Figure S5. Generation of p53$^{3KR/3KR}$ Mice, Related to Figure 4

(A) Targeting strategy used to generate p53$^{3KR/3KR}$ mice. Targeted ES cells are selected by G418 and detected by Southern blot with the indicated probe and BglII digestion as containing a 5.6 kb band. Mice generated from correctly targeted ES clones were mated with *Rosa26-Cre* mice to remove the LNL cassette and generate mice with only the targeted p53^{3KR} mutations. 4* and 5* designate the mutated exon 4 and exon 5 creating a BglII restriction site at K117 and K162. B, BglII; H, HindIII.

(B) Southern blot analysis of targeted ES clones using the 3′ external probe indicated in (A). Genomic DNA of ES clones was digested with BglII and correctly targeted clone 6 shows a wild-type 17.5 kb BglII band and a targeted 5.6 kb BglII band.

(C) PCR genotyping of the p53^{3KR} mutant allele. The wild-type and mutant PCR products are 300 bp and 390 bp, respectively.

(D) Table showing the expected and observed Mendelian ratios from p53^{3KR} heterozygous breedings.

(E) Sequencing analysis of p53 transcripts expressed from p53$^{+/+}$ and p53$^{3KR/3KR}$ MEF cells. The K-to-R mutations at K117, K161 and K162 of p53 are highlighted in the mutated p53^{3KR} allele.

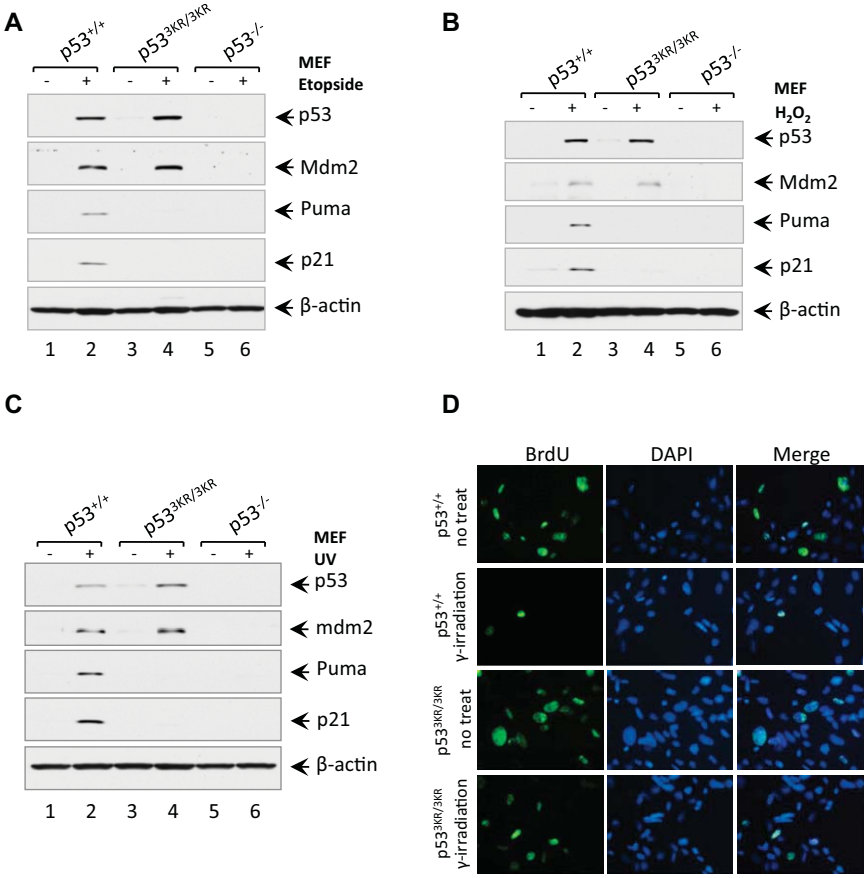
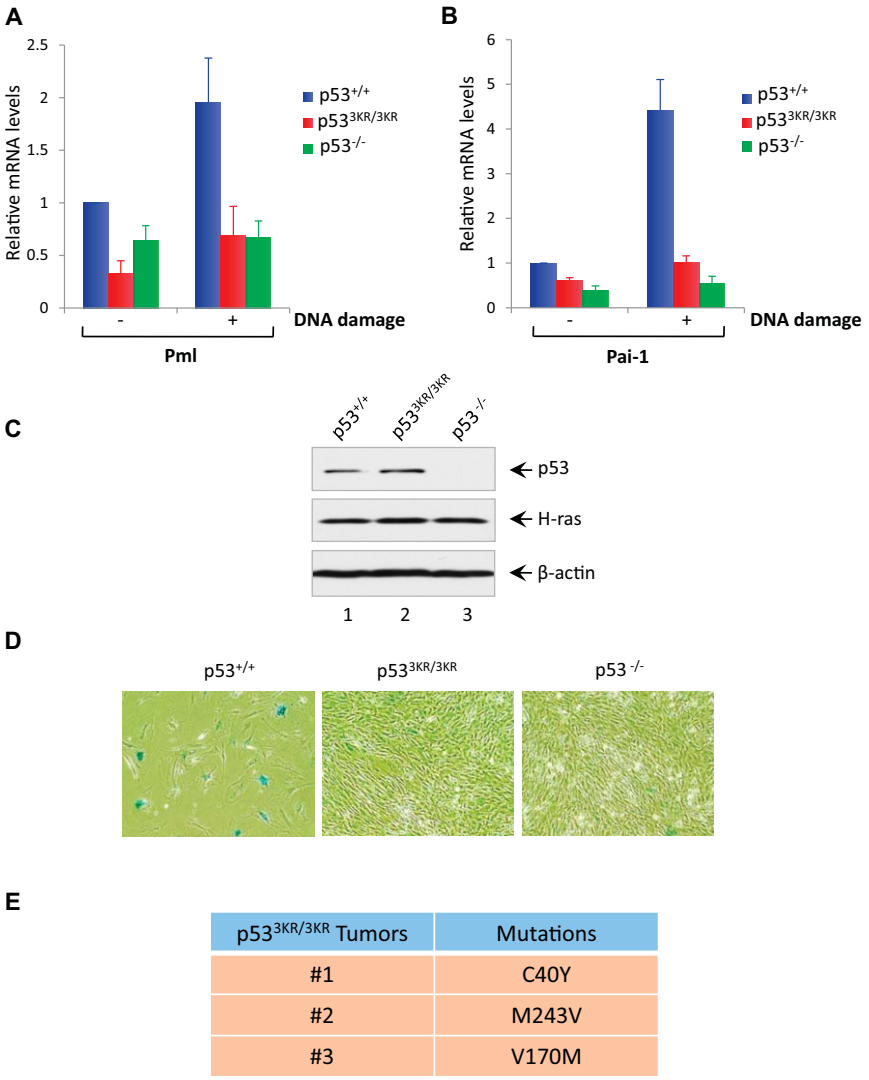


Figure S6. p53-Mediated Cell-Cycle Arrest and Apoptosis Were Abrogated in $p53^{3KR/3KR}$ MEFs in Response to Different Stresses, Related to Figure 4

(A–C) Western blot analysis of p53, Mdm2, PUMA and p21 in $p53^{+/+}$ and $p53^{3KR/3KR}$ and $p53^{-/-}$ MEFs either untreated or treated with 20 μM etopside for 16 hr (A), 400 μM H_2O_2 for 24 hr (B) or UV (30 J/m^2) for 6 hr (C). β-actin serves as a loading control.

(D) Cell-cycle arrest analysis of $p53^{+/+}$ and $p53^{3KR/3KR}$ MEFs either left untreated or exposed to 5 Gy of γ irradiation. 23 hr after irradiation, MEFs were pulsed with 10 μM BrdU for 45 min, and then cells were fixed and processed for immunofluorescence analysis for BrdU (green). The nuclei were stained with DAPI (blue).



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$p53^{3KR/3KR}$ Tumors	Mutations
#1	C40Y
#2	M243V
#3	V170M

Figure S7. $p53^{3KR/3KR}$ Primary MEFs Lose Activation of Senescence Markers after DNA Damage Treatment and Also Fail to Undergo Oncogene-Induced Senescence, Related to Figures 5 and 6

(A and B) qRT-PCR was performed to analyze the expression of the senescence-related target genes Pml and Pai-1 in $p53^{+/+}$ and $p53^{3KR/3KR}$ MEFs either untreated or treated with 0.2 μg/ml dox for 8 hr (A) and in $p53^{+/+}$ and $p53^{3KR/3KR}$ mice thymus either untreated or exposed to 5 Gy of γ irradiation (B). Data are shown as averages ± SEM from three different experiments.

(C) Western Blot analysis of p53 and H-ras in $p53^{+/+}$ and $p53^{3KR/3KR}$ and $p53^{-/-}$ MEFs (passage 2) infected with retrovirus expressing oncogenic H-ras. β-actin serves as a loading control.

(D) Images of SA-β-gal staining of $p53^{+/+}$ and $p53^{3KR/3KR}$ and $p53^{-/-}$ MEFs (passage 2) infected with oncogenic H-ras-expressing retrovirus. The infected MEFs were cultured for 7 days, then fixed and stained for β-galactosidase activity as described in the Extended Experimental Procedures.

(E) Sequencing analysis of p53 transcripts expressed from $p53^{3KR/3KR}$ tumor samples.