Supporting Information

Bedelbaeva et al. 10.1073/pnas.1000830107

SI Methods

Animals. MRL/MpJ (MRL), LG/J, SM/J, CDKN1A$^{-/-}$, and B6129F2 female mice were obtained from the Jackson Laboratory and C57BL/6 (B6) female mice were obtained from Taconic Laboratories. RI lines were described previously (1). Congenic mice were derived by breeding MRL female mice to B6 males, selected for healing with continuous breeding to B6 male mice (Fig. S1).

Cell Culture. Primary ear skin fibroblasts were established from MRL/MpJ, C57BL/6, LG/J, SM/J, RI, and congenic animals and grown in DMEM-10% FBS supplemented with 2 mM L-glutamine, 100 IU/mL penicillin streptomycin and maintained at 37 °C, 5% CO_2, and 21% O2. Cells were split 1:5 as needed to maintain exponential growth and avoid contact inhibition. Passage numbers were documented and cells from early passages (<P20) frozen in liquid nitrogen and used in the described experiments.

IHC. Primary ear skin fibroblasts were grown in DMEM with 10% FBS at 37 °C in a humidified 5% CO_2 incubator. For IHC staining, coverslips were rinsed with 1× PBS, the cells were fixed in cold methanol (−20 °C) for 10 min, rinsed with 1× PBS, treated with 0.1% Triton-X100, and then incubated with the appropriate primary and secondary antibodies.

Tissue from normal ears and from the small intestine were fixed with Prefer fixative (the active ingredient is glyoxal) (Anatech) overnight and then washed in H20. Tissue was embedded in paraffin and 5-μm thick sections cut. Before staining, slides were dewaxed in xylene and rehydrated. Antigen retrieval was performed by autoclaving for 20 min in 10 mM Sodium Citrate, pH 6.0. Tissue sections were then treated with 3% H_2O_2 and nonspecific binding was blocked with 4% BSA (A7906; Sigma) for 1 h.

The primary antibodies used for IHC were as follows: Anti-p53 mouse monoclonal antibody DO1 (sc-126; Santa Cruz Biotechnology, Inc.) was used at a dilution of 1:50 for both cultured cells and for paraffin sections. Anti-phospho-H2A.× (Ser139) rabbit polyclonal antibody (07-164; Upstate Biotechnology, Inc.) was used at a dilution of 1:1,000 for cultured cells and 1:200 for paraffin sections. Anti-TopBP1 polyclonal rabbit antibody (ab2402; Abcam) was used at a concentration of 1:2,000 for cultured cells. Anti-Caspase 3 polyclonal rabbit antibody specific for the active form of caspase 3 (AF835; R&D Systems, Inc.) was used at a dilution of 1:2,000 for paraffin sections. Anti-Rad51 (H-92) polyclonal rabbit antibody (sc-8349; Santa Cruz) was used at a dilution of 1:100 for cultured cells.

Secondary antibodies specific for mouse primary antibody include FITC-conjugated goat anti-mouse antibody used at a dilution of 1:200 (F2653; Sigma) and biotin-conjugated donkey anti-mouse antibody used at a dilution of 1:1,000 (715-065-151; Jackson ImmunoResearch Lab). Secondary antibodies specific for rabbit primary antibodies include FITC-conjugated goat anti-rabbit used at a dilution of 1:500 (A-11008; Molecular Probes), FITC-conjugated mouse monoclonal anti-rabbit antibody used at a dilution of 1:100 (F4890; Sigma), Alexa Fluor568 goat anti-rabbit antibody used at a dilution of 1:400 (A11036; Molecular Probes), and biotin-conjugated mouse monoclonal anti-rabbit antibody used at a dilution of 1:1,000 (B5283; Sigma).

Tissue sections were incubated with primary antibody overnight at 4 °C followed by blocking with 4% BSA for 1 h. After washing, sections were incubated with biotinylated antibody for 1 h at room temperature. For visualization, slides were treated with the Vectastain ABC Kit (PK-6100; Vector) at RT for 1 h. After multiple washes with 1× PBS, the sections were stained with the peroxidase substrate DAB (SK-4100; Vector).

Western Blot Analysis. Cells and tissue samples were homogenized and run on SDS PAGE gels, transferred, and stained with multiple antibodies. Cells and tissue samples were homogenized in lysis buffer (50mM Tris HCl, Triton X100-0.05%, SDS-0.1%) using glass-on-glass homogenizers (DUALL #20; Kontes Glass Co.) at 4 °C for 15 min, and then centrifuged. The OD was measured using an Ultraspec 3000 (Pharmacia Biotech) followed by mixing with 2× Laemmli loading buffer and heating at 95 °C for 5 min. Protein was loaded onto 10-15% SDS PAGE gels as described in online supplement.

After protein was transferred to Immobilon-P (Millipore), blots were incubated for blocking with 5% nonfat dry milk/TBST for 4 hrs, incubated with primary antibodies at 4 °C overnight, washed with TBST, then followed by an HRP-coupled secondary antibody. Finally, a chemiluminescent HRP substrate (WBKLS0100; Millipore) was used for development of bands using HyBlot CL autoradiography film.

For p53, normal and postinjury ear tissue was loaded onto a 10% gel. The primary antibody, mouse monoclonal antibody DO-1 (sc-126; Santa Cruz) was used at a dilution of 1:1,000. The secondary antibody #37 was used at a dilution of 1:10,000.

For γH2AX, fibroblasts and ear tissue were prepared and loaded onto a 15% gel.

The primary antibody, rabbit anti-phosphohistone H2A.X (ser139) antibody (2577; Cell Signaling) was used at 1:1,000. Secondary antibody was used at a dilution of 1:10,000.

For caspase 3, small intestine was prepared and samples were loaded on a 12% gel. The primary antibody, rabbit anti-caspase 3 antibody specific for the pro and active form of caspase 3 (sc-7148; Santa Cruz) was used at a dilution of 1:200. Secondary antibody was an HRP-conjugated, mouse anti-rabbit antibody (A-1949; Sigma)used at 1:30,000 dilution.

For p21, fibroblasts were prepared and loaded on a 12% gel. The primary antibody was a mouse monoclonal IgG1 antibody SX118 (556430; BD Biosciences). Secondary antibody was a goat anti-mouse IgG, Fab-specific, and HRP-labeled (A2304; Sigma). Fibroblast samples were denatured at 95 °C for 5 min prior to loading. Samples (60 μg/well) were loaded on a 10% SDS-PAGE gel. These samples were run in 10 μL at a protein concentration of 1 mg/mL (10 μg) and were loaded on a discontinuous 8-15% SDS-PAGE gel.

TUNEL Analysis. Paraffin-embedded small intestine was sectioned at 5 μm. For detection of apoptotic cells, the DermaTACS In Situ Apoptosis Detection Kit for skin cells and tissues (4829-30-K; Trevigen, Inc.) was used as described. The kit is based on DNA end-labeling using TdT and modified nucleotides. Detection of incorporated molecules is achieved using a chromogenic substrate with a horseradish peroxidase detection system and with biotinylated anti-BrdU antibody. The tissue was then counterstained with red counterstain C (4800-30-19; Trevigen Inc.).

Cell Cycle Analysis. Fibroblast cell cultures were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin, and streptomycin at 37 °C in humidified 5% CO_2 incubator. Cell concentrations for all methods were at least 1 × 10^6cells/mL. Cells were fixed and treated with PI dye (P-4170; Sigma) in concentration 20 μg/mL for 20 min at room temperature in the dark. Flow cytometry analysis was carried out using an EPICS

CL-MCL Beckman Coulter Analyzer at 488 nm for excitation and 615 nm for detection.

Alternatively, for maintaining live cells, the vital dye Vybrant DyeCycle Orange (V35005; Molecular Probes) was used at a concentration of 2 μL/mL at 37 °C for 15 min, protected from light. For fluorescent cell sorting, a MoFlo High-Speed Sorter (DakoCytomation) was used at 488 nm for excitation and 585 nm for detection. Samples were collected for G0/G1, S, and G2/M stages of the cell cycle. Cells were washed twice with 1× PBS and then spread onto slides and dried. After fixation in cold methanol, cells were washed in 1× PBS and permeabilized using Triton-X100. The cells were then stained for p53 expression using the mouse monoclonal anti-p53 antibody DO1 (sc126; Santa Cruz) at a dilution of 1:50 and the secondary antibody was Alexa Fluor350 goat anti-mouse (A21049; Molecular Probe) at a concentration of 1:100.

Comet Assays. Cells were grown under standard conditions and then trypsinized and washed in sterile PBS. Approximately 5,000 cells were mixed in 0.5% low melting point agarose made in PBS and loaded onto an agarose-coated slide (1.5% normal melting point agarose). Cells suspensions were cooled at 4 °C and then added to lysis buffer [2.5M NaCl, 100 mM EDTA, 10 mM Tris (pH 10.0), and 1% SDS] and incubated at 4 °C overnight. The following day slides were rinsed with H_20 and then placed in a gel box with prechilled (4 °C) unwinding buffer (300 mM NaOH and 1 mM EDTA) for 20 min in a cold room. After the incubation period, the power supply was set to 25 V and 300 mA by adjusting the buffer. After electrophoresis, slides were washed with neutralization buffer (0.4 M Tris-HCl, pH 7.5) and then stained with ethidium bromide solution (20 μg/mL) and a coverslip was applied. Comets were evaluated using a Leica fluorescent microscope. At least 100 nuclei were scored per experiment. For neutral comet assays 1× TBE was used in place of the alkaline solution. In experiments where radiation was used, cells were exposed to 10 Gy of γIR and then immediately mixed with the low melting point agarose. In studies using DMSO, cells were grown in 1% DMSO for 24 h before harvest or X-irradiation (1 Gy).

1. Hrbek T, de Brito RA, Wang B, Pletscher LS, Cheverud JM (2006) Genetic characterization of a new set of recombinant inbred lines (LGXSM) formed from the inter-cross of SM/J and LG/J inbred mouse strains. *Mamm Genome* 17:417–429.

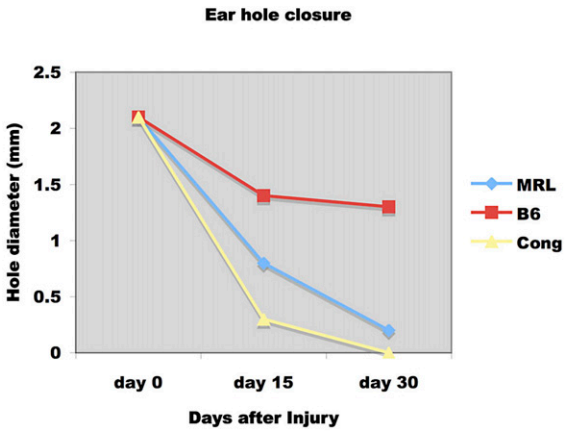

Fig. S1. Ear-hole closure in healer congenic mice. Through-and-through 2-mm ear hole punches were made in the ear pinnae of normal nonhealer B6 mice and healer MRL and congenic mice (five mice each). Hole diameters were measured on both sides on days 15 and 30. Congenic healers showed a faster rate of hole closure than the MRL mouse itself.

Table S1. Cell cycle analysis

	G0/G1 phase	S phase	G2/M phases
Nonhealers			
3T3 B6	65.6 ± 8.9*	13.5 ± 3.3	17 ± 4.5
B6	66.6 ± 7.9	15.2 ± 4.9	13.9 ± 1.3
SM/J	70.5	13.0	13.7
RI line 33	64.8 ± 12.1	10.6 ± 5.7	4.6 ± 2.5
Healers			
MRL	46.9 ± 10.6	19.1± 3.9	25.5 ± 8.2
Congenic	35.6 ± 4.3	6 ± 2.4	53.9 ± 4.3
LG/J	51.0	9.6	38
RI line 6	21.5 ± 17.3	8.7 ± 4.9	64.1 ± 17.7

*The percentage of cells in the G0/G1, S, and G2/M cell cycle phase based on the amount of DNA per cell is shown for each strain tested. The average and standard deviation of two to five experiments is presented.

Table S2. Comet analysis of healer and nonhealer ear-derived fibroblasts

I. Comets in normal ear fibroblasts	Conditions		
Mouse strains	Alkaline Normal	Alkaline X-irradiated	Neutral Normal
Healers			
MRL/MpJ	84.5 ± 4.2	100*	55 ± 7.1
LG/J	86.7 ± 7.6		35
Congenic	70 ± 5		15
Nonhealers			
C57BL/6	4.3 ± 7.5	100	
SM/J	8.8 ± 5.3		
II. Comets with DMSO treatment			
	Alkaline w/o DMSO	Alkaline plus 1% DMSO (24 hr)	
HeLa (1 Gy)	61	0	
MRL	75	30	

*Number of comets/100 cells counted