Supporting Information

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SI Materials and Methods

Animals and Hypoxia Treatment. *Myospalax baileyi* (250–350 g; Fig. 1*B*) and *Microtus oeconomus* (18–25 g; Fig. 1*B*) were captured from the field near Haibei Research Station of the Alpine Meadow Ecosystem, Chinese Academy of Sciences (37°39′N, 101°19′E) in Qinghai, China. The body temperature (rectum) on average is ∼35 °C (range, 33–37 °C) in *M. baileyi* and ∼36 °C (range, 34–38 °C) in *Microtus oeconomus*. The body temperature changes with fluctuations of the environmental temperature. *M. cansus* (250–300 g; Fig. 1*B*) were captured from the field near Yan'an, north Shaanxi (36°36′N, 109°31′E), China. Adult male Sprague–Dawley rats (150–200 g; certification no. SCXK20080033) were purchased from the Experimental Animal Center, Zhejiang Academy of Medical Science. Rats were maintained at room temperature in a 12 h/12 h light/dark cycle with free access to food and water. For hypoxia treatment, laboratory rats were placed in a hypobaric chamber (FLYDWC50-IIC; Avic Guizhou Fenglei Aviation Armament Co., Ltd.). The rats were subjected to 16.0%, 10.8%, or 8.0% hypoxia to mimic altitudes of 2 km, 5 km, or 7 km for 8 h. Hypoxia treatment of *M. baileyi* and *Microtus oeconomus* was carried out by placing animals in a sealed chamber filled with mixed gas of 8% O_2 and 92% N_2. Animals were killed by decapitation, and tissues were removed, immediately frozen in liquid nitrogen, and stored in −80 °C.

Cloning of *M. baileyi*, *M. cansus*, and *Microtus oeconomus* p53. *M. baileyi* and *M. cansus p53* cDNAs were cloned from mRNAs extracted from the brain or liver of *M. baileyi* (GenBank accession no. JX998170) and *M. cansus* (GenBank accession no. JX998172). *Microtus oeconomus p53* cDNA was cloned from mRNA extracted from the brain or liver of three adults (GenBank accession no. JX998171). Primers were designed based on human (NM_000546), mouse (NM_011640), and *Spalax* (AJ783406) *p53* sequences in GenBank. To confirm the authenticity of each varied codon, further *p53* was cloned from five more individuals of each species.

Multiple Alignment Tools, Phylogenetic Tree, and Evolutionary Analysis. Alignment of p53 protein sequences from *M. baileyi*, *M. cansus*, *Microtus oeconomus*, and 32 other species was generated using Multalin software (http://multalin.toulouse.inra.fr/multalin) (1). The phylogenetic tree of *p53* was constructed based on the amino acid sequences with ClustalW and Mega 4 software, using the neighbor-joining method (2). The numbers besides the branches are branch length, referring to the distance between the sequences (Figs. 1 and S1). The positive site test was performed using PAML 4 (3) software and was tested with the branch-site test as previously described (4, 5).

Cell Cultures. NCI-H1299 [human non-small cell lung cancer, American Type Culture Collection (ATCC) CRL-5803] and HeLa (human cervical cancer, ATCC CCL-2) cell lines were grown in RPMI medium 1640 (Gibco) or DMEM (Gibco), respectively, containing 10% (vol/vol) FBS, 2 mM glutamine, 100 μg/mL streptomycin, and 100 U/mL penicillin at 37 °C in a humidified incubator with 5% (vol/vol) CO_2.

Hypoxia, Cold, and Acid Treatments. The hypoxia treatment was performed using the Proox Model P110 and ProCO2 Model P120 hypoxia systems (BioSpherix). The medium was replaced 6 h posttransfection, and cells were moved to the hypoxia incubator in which the oxygen level was set as indicated. For the cold treatment, transfected cells were moved to incubators of 37 °C or 30 °C for 24 h before harvesting (6, 7). For the acid treatment, medium with 20 mM Hepes was regulated to pH 7.4, 6.6, or 6.0 by titration with 1 M HCl according to the published protocol (8).

Quantitative Real-Time RT-PCR. mRNA quantities were determined by real-time RT-PCR. Primers were synthesized according to the rat, *M. baileyi*, and *Microtus oeconomus p53* cDNA sequences as follows: rat p53-1: 5′-ATG CTG AGT ATC TGG ACG AC-3′; Rat p53-2: 5′-GAC AGG CAC AAA CAC GAA CC-3′; *M. baileyi* p53-1: 5′-CAC CTC GCT GTC ACC CAA-3′; *M. baileyi* p53-2: 5′-AGC CCA GAC GGA AAC CAT-3′; *Microtus oeconomus* p53-1: 5′-TCG CTG TCA GTT GAT GCC-3′; *Microtus oeconomus* p53-2: 5′-TTC GCC CTG GTA GGT TTT-3′0;18S rRNA-1: GTAACCCGTTGAACCCCATT; 18S rRNA-2: CCATCCAATCGGTAGTAGCG.

For endogenous *p53* target genes, primers were synthesized according to the cDNA sequences of each gene from the human: IGF-I binding protein-3 (*IGFBP3*)-F: 5′-TTCCCAACTGTGACAAGAAGG-3′; *IGFBP3*-R: 5′-GGAGAGGCTGCCCATAC-3′; Apoptotic protease activating factor 1 (*Apaf1*)-F: 5′-ACACCTTCTTGGACGACAGCC-3′; Apaf1-R: 5′-GGCGGACAACTAAGCGGGAAAG-3′; BCL2-associated X protein (*Bax*)-F: 5′-CTGACGGCAACTTCAACTG-3′: *Bax*-R: 5′-AGGAGTCTCACCCAACCAC-3′; P53 upregulated modulator of apoptosis (*Puma*)-F: 5′-CTGCTGCCCGCTGCCTACCT-3′; Puma-R: 5′-AGCGAGAGCGAGGGCTGAGG-3′; the Bcl-2 homology 3 (BH3)-only pro-apoptotic protein (*Noxa*)-F: 5′-GCAAGAACGCTCAACCGAG-3′; *Noxa*-R: 5′-GGTTCCTGAGCAGAAGAGT-3′.

Western Blotting. Western blotting was performed to determine the p53 protein levels in *M. baileyi* and *Microtus oeconomus* liver. A monoclonal antibody against p53 (1C12; Cell Signaling) was used. Samples were homogenated and lysed in 200 μL RIPA Lysis Buffer (Beyotime) with 1 mM PMSF. Extracts were centrifuged at 14,000 × *g* for 15 min at 4 °C. After the addition of 6× loading buffer, all samples were boiled at 95 °C for 5 min. Protein samples were subjected to electrophoresis. Proteins were transferred to PVDF membrane and were incubated with indicated antibodies. The samples were visualized with ECL.

Plasmids. For *p53*-expression plasmids, the coding regions of human, *M. baileyi*, *M. cansus*, and *Microtus oeconomus* WT *p53* were ligated into a pcDNA3.1$^+$ expression vector. Site-directed mutagenesis was performed using the QuikChange XL site-directed mutagenesis kit (Stratagene). The underlining in primers used to change the amino acids: *M. baileyi* 322delete: 5′-CCC AAG AAG AAG CCA CTA GAT GGA GAA TAT TTC ACC C-3′; 5′-GGG TGA AAT ATT CTC CAT CTA GTG GCT TCT TCT TGG G-3′; *M. baileyi* N104S: 5′-CCA GAA AAC CTA CCA GGG CAG CTA TGG TTT CCG-3′; 5′-CTG CCC TGG TAG GTT TTC TGG GAA GGG ACT G-3′; *M. cansus* S104N: 5′-CCA GAA AAC CTA CCA GGG CAA CTA TGG TTT CCG-3′; 5′-TTG CCC TGG TAG GTT TTC TGG GAA GGG ACT G-3′; *M. baileyi* C127A: 5′-TCA CAT GCA CGT ACT CCC CTG CCC TGA ATA AGC-3′; 5′-GCA GGG GAG TAC GTG CAT GTG ACA GAT TTG-3′; *M. baileyi* I215V: 5′-ACA CTT TTC GAC ACA GTG TGG TGG TAC CAT ATG-3′; 5′-CAC CAC ACT GTG TCG AAA AGT GTG CTT ATC-3′; *Microtus oeconomus* V86A: 5′-CGG TGA CCC CTG CAC CAG CCA CTT CCT GGC C-3′; 5′-GCT GGT GCA GGG GTC ACC GGT GCG GGG GCC

T-3′; *Microtus oeconomus* E104S: 5′-CCC AAA AAA CCT ACC AGG GCA GCT ATG GTT TCC G-3′; 5′-GCT GCC CTG GTA GGT TTT TTG GGA CGG GAC AG-3′; *Microtus oeconomus* P258S: 5′-CCA TTA TCA CAC TGG AAG ACT CCA GTG GGA AC-3′; 5′-AGT CTT CCA GTG TGA TAA TGG TGA GGA TGG GCC G-3′; *Microtus oeconomus* S340R: 5′-GTG AAC GCT TCA AGA TGT TCC GAG AGC TGA ATG AG-3′; 5′-CGG AAC ATC TTG AAG CGT TCA CGA CCA CGG ATC TT-3′; Human H179R: 5′-GTG AGG CGC TGC CCC CAC CGT GAG CGC TGC-3′; 5′-CGG TGG GGG CAG CGC CTC ACA ACC TCC GTC-3′; Human S106N:5′-CCA GAA AAC CTA CCA GGG CAA CTA CGG TTT CCG-3′; 5′-TTG CCC TGG TAG GTT TTC TGG GAA GGG ACA G-3′; Human S106E: 5′-CCC AGA AAA CCT ACC AGG GCG AAT ACG GTT TCC G-3′; 5′-TTC GCC CTG GTA GGT TTT CTG GGA AGG GAC AGA AG-3′. For human *p53* target luciferase constructs, the sequences of human *IGFBP3*, *Apaf1*, *Bax*, *Puma*, *Noxa*, *p21*, and *Hdm2* promoters were identified from the GenBank. The promoter sequences containing the *p53* response elements of the promoters were subcloned into pGL3-basic reporter plasmids to construct the reporter plasmids. The subcloned fragments range from the position upstream of response elements to a position downstream of the transcription starting site (+1). For the *Bcl-2* promoter, a fragment ~ 1,000 bp upstream of the transcription starting site was ligated into a pGL3-basic reporter vector (9). All fragments ligated into the vectors were sequenced to confirm authenticity.

Transient Transfection and Dual-Luciferase Reporter Assay. Transient transfection was performed using PolyJet transfection reagent (SignaGen Laboratories). H1299 and HeLa cells were plated in 24-well plates at 100,000 cells per well for 24 h. Then the medium was replaced with a complete medium of serum and antibiotics 30–60 min before transfection. Cells were cotransfected with a mixture of 0.36 μg *p53* expression plasmids and 0.18 μg luciferase reporter constructs. Cells were harvested 48 h posttransfection, and dual-luciferase reporter assays were performed using a dual-luciferase reporter assay system (Promega). Results were normalized to *Renilla* luciferase values (pRL-CMV vector; Promega).

Flow Cytometry. For cell-cycle arrest and apoptosis, cells were harvested and fixed in cold 70% (vol/vol) ethanol at 4 °C overnight. The next day, cells were rehydrated in cold PBS, incubated with RNase A and 50 μg/mL propidium iodide for 30 min, and then were analyzed by flow cytometry (Beckman FC 500 MCL) for DNA content (red channel). For *p53*-transfected cells, GFP-tagged *p53* expression plasmids were transfected into NCI-H1299 or HeLa cells. At 48 h posttransfection, cells were collected, fixed, stained as described above and were analyzed using flow cytometry. Samples were gated to GFP^+ cells (green channel), and the DNA content was analyzed, confirming that all the analyzed cells expressed transfected *p53*. Apoptosis induced by *p53* was calculated as the percentage of cells with sub-G1 DNA content minus the percentage that transfected with empty vector.

For apoptosis under stress, transfected cells were collected and stained with 7-amino-actinomycin D (7-AAD) for 15 min at room temperature and were incubated with Annexin V-phycoerythrin for 15 min at room temperature. Then the cells were subjected to flow cytometry. Samples were gated to GFP^+ cells and the staining of phycoerythrin and 7-AAD. Apoptosis induced by *p53* was calculated as the percentage of cells with positive phycoerythrin staining and negative 7-AAD staining.

Statistical Analysis. Statistical analysis was performed using SPSS 16.0 software. Statistical significance compared with the control was determined with a two-tailed, unpaired Student *t* test. Comparisons among multiple groups were determined by one-way ANOVA, followed by the Least Significant Difference test.

1. Corpet F (1988) Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Res* 16(22):10881–10890.
2. Saitou N, Nei M (1987) The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol Biol Evol* 4(4):406–425.
3. Yang Z (2007) PAML 4: Phylogenetic analysis by maximum likelihood. *Mol Biol Evol* 24(8):1586–1591.
4. Zhang J, Nielsen R, Yang Z (2005) Evaluation of an improved branch-site likelihood method for detecting positive selection at the molecular level. *Mol Biol Evol* 22(12):2472–2479.
5. Cao YB, Chen XQ, Wang S, Wang YX, Du JZ (2008) Evolution and regulation of the downstream gene of hypoxia-inducible factor-1alpha in naked carp (Gymnocypris przewalskii) from Lake Qinghai, China. *J Mol Evol* 67(5):570–580.
6. Shiraishi K, et al. (2004) Isolation of temperature-sensitive p53 mutations from a comprehensive missense mutation library. *J Biol Chem* 279(1):348–355.
7. Villiard E, et al. (2007) Urodele p53 tolerates amino acid changes found in p53 variants linked to human cancer. *BMC Evol Biol* 7:180.
8. Reichert M, Steinbach JP, Supra P, Weller M (2002) Modulation of growth and radiochemosensitivity of human malignant glioma cells by acidosis. *Cancer* 95(5): 1113–1119.
9. Kajimura S, Aida K, Duan C (2006) Understanding hypoxia-induced gene expression in early development: in vitro and in vivo analysis of hypoxia-inducible factor 1-regulated zebra fish insulin-like growth factor binding protein 1 gene expression. *Mol Cell Biol* 26(3):1142–1155.

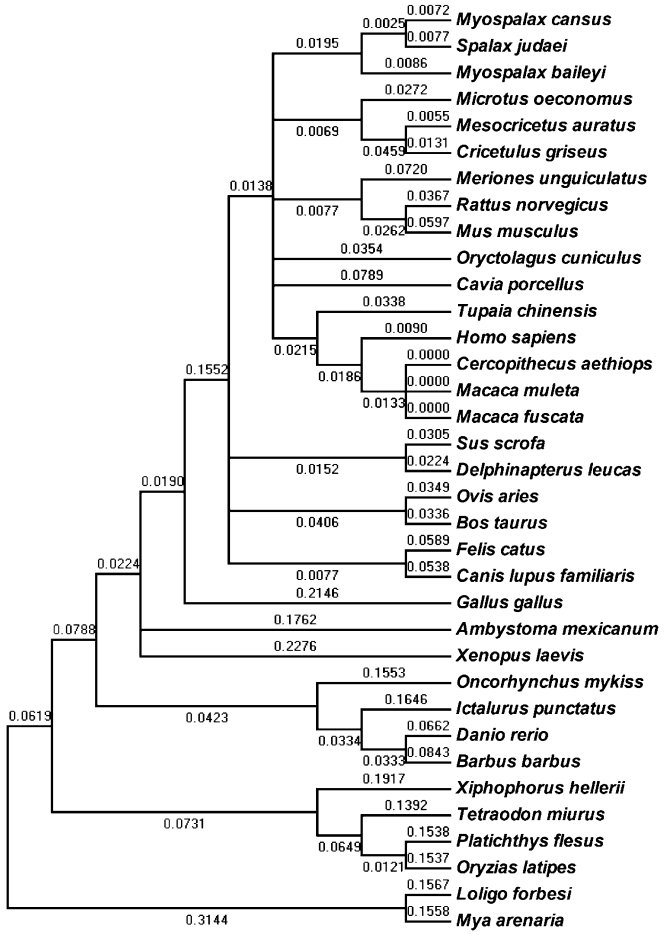

Fig. S1. Molecular phylogenetic trees based on the amino acid sequence of *p53* from *M. baileyi*, *M. cansus*, and *Microtus oeconomus* together with 32 other species with bootstrap test (500 replications).

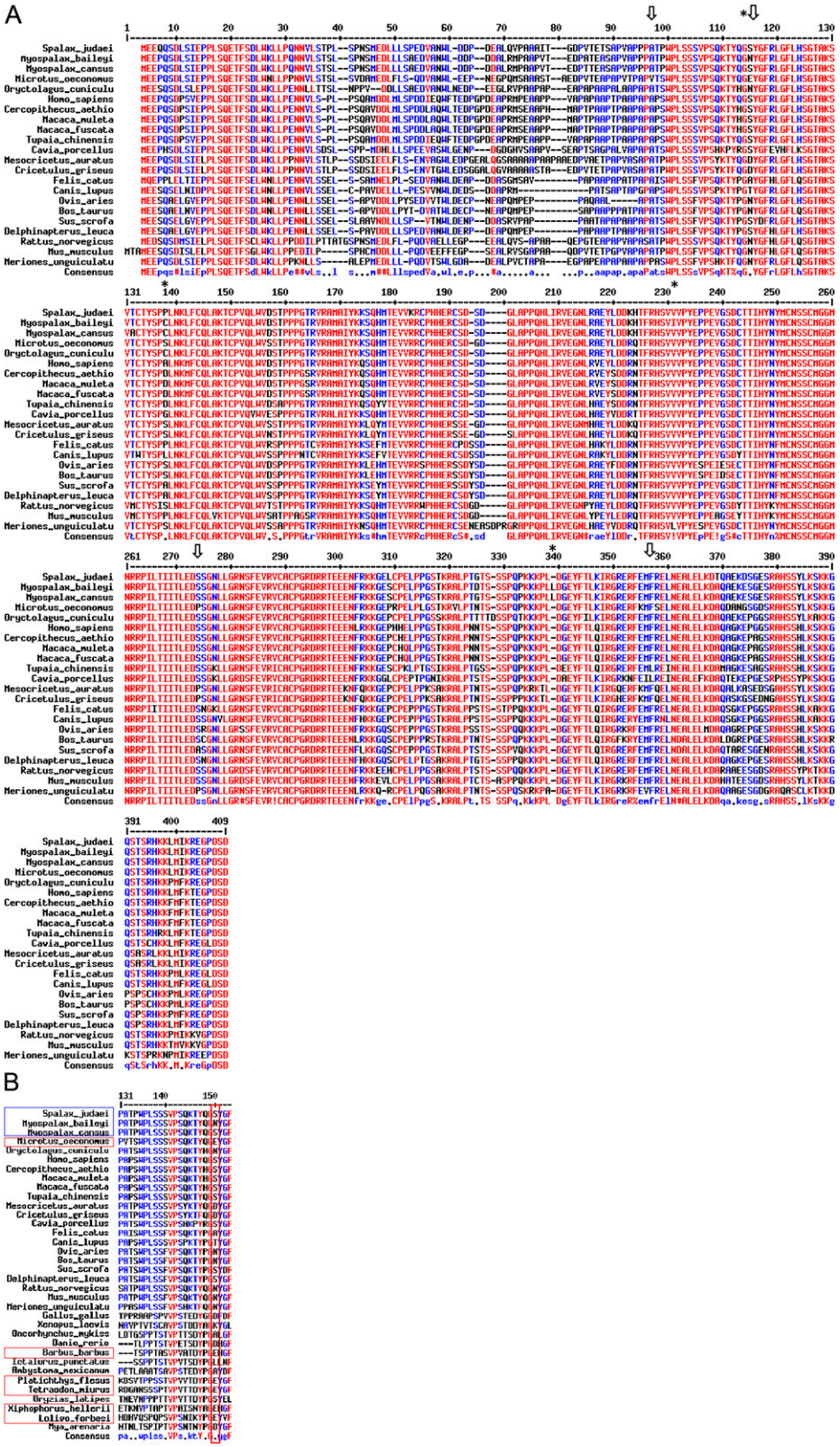

Fig. S2. *p53* sequence alignment. (*A*) Alignment of mammal *p53* sequences. (*B*) Alignment of *p53* sequences of 35 species around the region containing the codon relative to human codon 106. All *p53* sequences of species other than *Myospalax baileyi*, *Myospalax cansus*, and *Microtus oeconomus* are from GenBank.

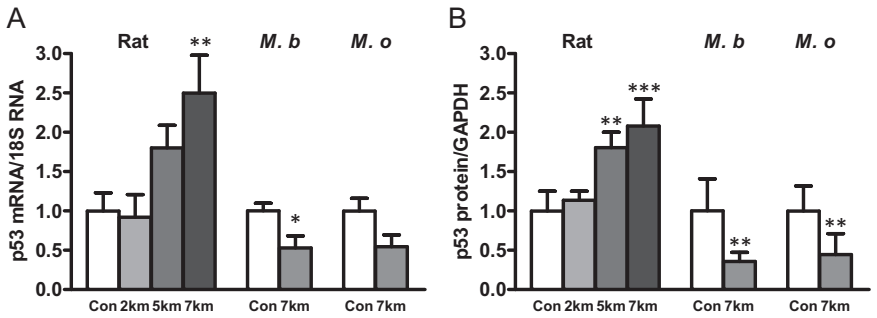

Fig. S3. *p53* mRNA expression and protein in *M. baileyi* (*M.b.*) and *Microtus oeconomus* (*M.o.*) liver under hypoxia. (*A*) *p53* mRNA and (*B*) protein levels in rat, *M.b.*, and *M.o.* liver under hypoxia treatment (2 km: 16.0% O_2, 5 km: 10.8% O_2, 7 km: 8.0% O_2). Results are mean ± SEM for mRNA and mean ± SD for protein ($n = 6$). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired *t* test) compared with control.

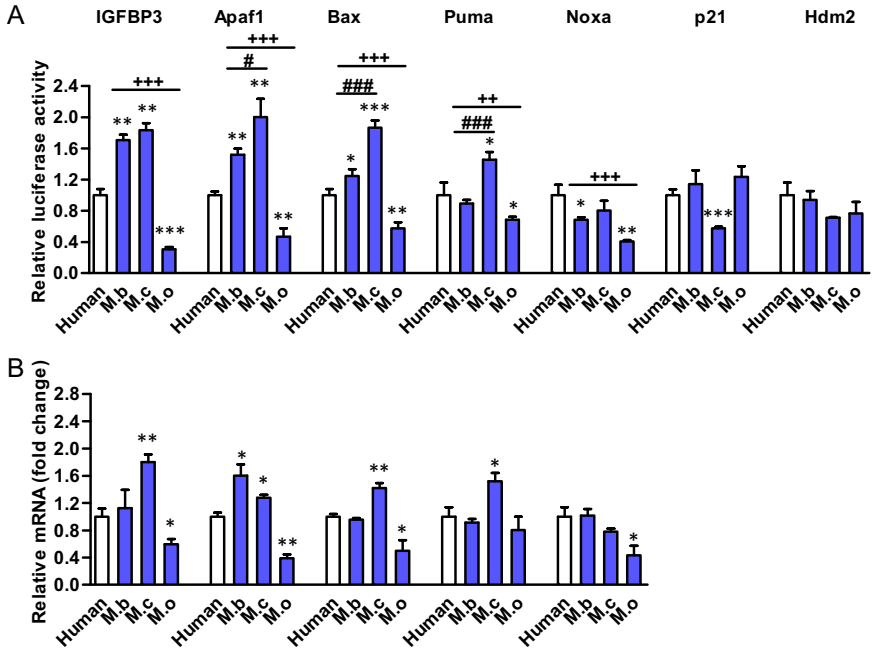

Fig. S4. Differential transactivation of human, *M. baileyi* (*M.b.*), *M. cansus* (*M.c.*), and *Microtus oeconomus* (*M.o.*) *p53* target genes. (*A*) Transcription of *p53* target genes was determined by dual-luciferase reporter assay. Results shown represent mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired *t* test) compared with human WT *p53*. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$, *M.b.* vs. *M.c.* WT *p53*. $^{++}P < 0.02$, $^{+++}P < 0.001$ *M.b.* and *M.o.* mutants vs. WT *p53*. (*B*) Expression of endogenous *p53* target genes induced by human, *M.b*, *M.c*, and *M.o.* as determined by quantitative real-time PCR. Results shown represent mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ (unpaired *t* test) compared with human WT *p53*.

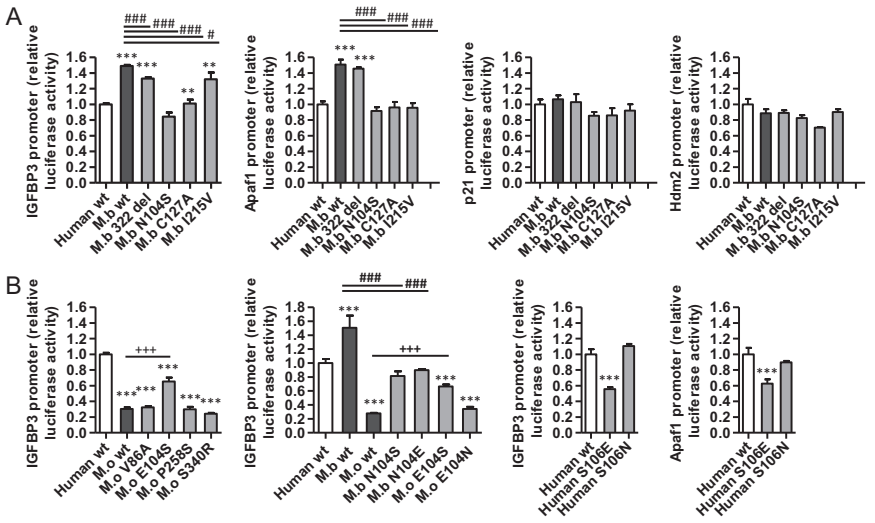

Fig. S5. Effects of site mutations on *p53* transactivation. (*A*) The transactivation of WT and site-directed mutant of *M. baileyi* (*M.b.*) *p53* to *IGFBP3*, *Apaf1*, *p21*, and *Hdm2* promoters. (*B*) Effects of *Microtus oeconomus* (*M.o.*) *p53* site mutations and mutual mutations of 104N and 104E on target gene *IGFBP3* transcription. Results shown are the mean ± SD of three independent transfection assays. $^{**}P < 0.01$, $^{***}P < 0.001$ (unpaired *t* test) compared with human WT *p53*. $^{\#}P < 0.05$, $^{\#\#\#}P < 0.001$ compared with *M.b.* WT *p53*. $^{+++}P < 0.001$ compared with *M.o.* WT *p53*.

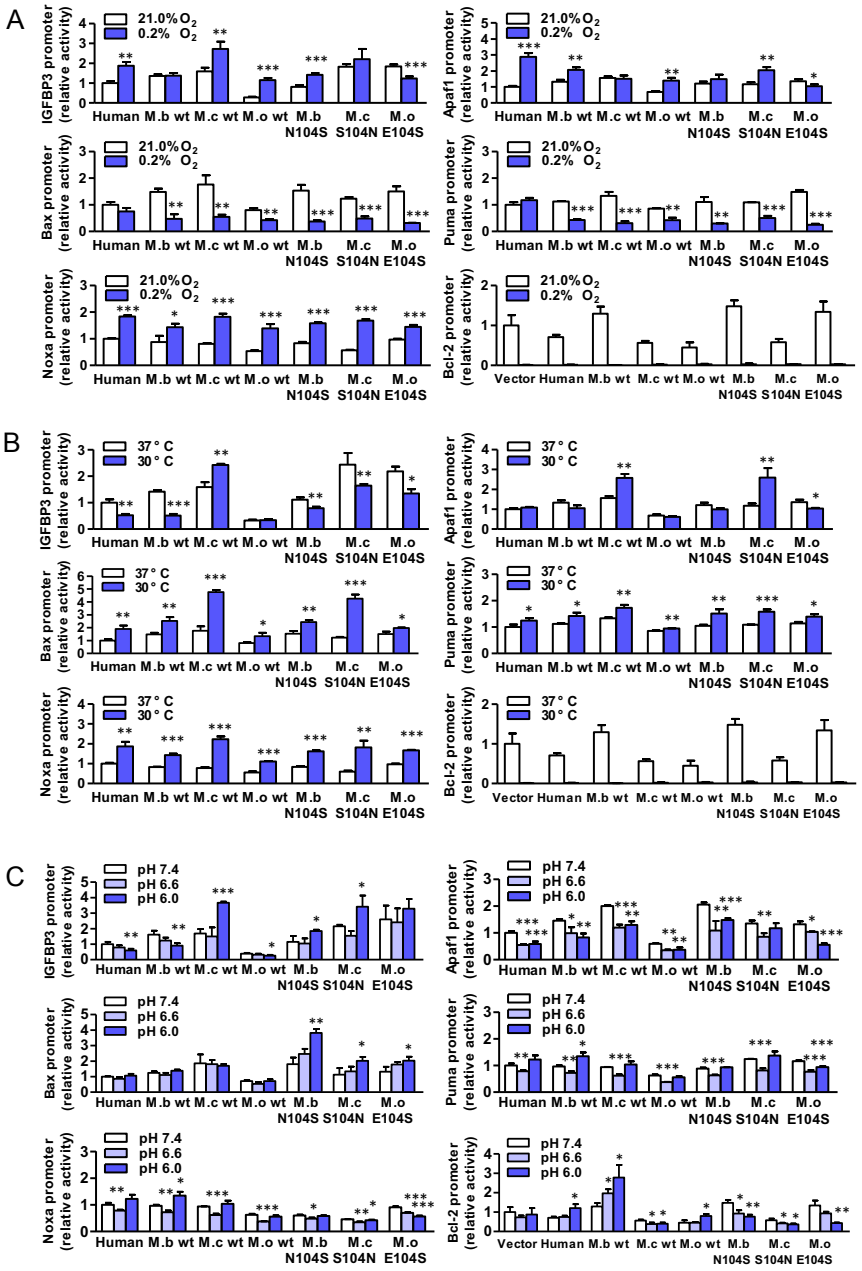

Fig. S6. Transactivation of human, *Myospalax baileyi* (*M.b.*), *Myospalax cansus* (*M.c.*), and *Microtus oeconomus* (*M.o.*) *p53* and their mutants toward apoptosis target genes under (*A*) hypoxia (0.2% O_2), (*B*) cold (30 °C), and (*C*) acidic stresses. At 6 h posttransfection, cell medium was replaced, and cells were moved to an incubator with 0.2% O_2 or 30 °C for 18 h for hypoxia or cold challenge, respectively. For acidic stresses, medium was replaced with medium with pH 6.6 or pH 6.0 for 18 h. Results shown represent mean ± SD. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ (unpaired *t* test) compared with control.

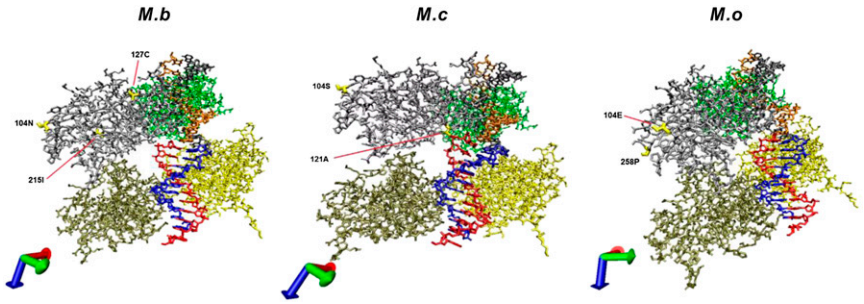

Fig. S7. 3D structure of a tetramer of *p53* binding to DNA double strands. The corresponding location of the variant sites 104N, 127C, and 215I in the *Myospalax baileyi* (*M.b.*), 104S and 121A in *Myospalax cansus* (*M.c.*), and 104E and 258P in *Microtus oeconomus* (*M.o.*) *p53* core domain is highlighted in yellow. The model is from Kitayner et al. (1), (Protein Data Bank ID: 2ATA). The presentation was created using VMD software and rendered using POV-Ray software (coloring method: Chain; drawing method: Bonds).

1. Kitayner M et al. (2006) Structural basis of DNA recognition by p53 tetramers. *Mol Cell* 22(6):741–753.

Table S1. Summarized differential responses of *p53* from *Myospalax baileyi*, *Myospalax cansus*, and *Microtus oeconomus* in cells cultured under normal conditions

Species (*p53* codon 104 mutation)	Apoptotic genes	*Bcl-2*	Apoptosis
Myospalax baileyi (104N)	↑	↑	–
Myospalax cansus (104S)	↑	↓	↑
Microtus oeconomus (104E)	↓	↓	↓

↑, up-regulation; ↓, down-regulation; –, unchanged.

Table S2. Summarized differential responses of *p53* from *Myospalax baileyi*, *Myospalax cansus*, and *Microtus oeconomus* in cells cultured under stress conditions

Species	Hypoxia			Acidic stress		Cold		
(*p53* codon 104 mutation)	*IGFBP3*	*Apaf1*	Apoptosis	*Bcl-2*	Apoptosis	*IGFBP3*	*Apaf1*	Apoptosis
Myospalax baileyi (104N)	–	↑	↓	↑	↓	↓	–	↓
Myospalax cansus (104S)	↑	–	↓	↓	–	↑	↑	–
Microtus oeconomus (104E)	↑	↑	↓	–	–	–	–	–

↑, up-regulation; ↓, down-regulation; –, unchanged.