

Supplementary Materials

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Materials and methods

1 Animals and hypoxia treatment

Myospalax baileyi (240–280 g) were captured around the Haibei Research Station of the Alpine Meadow Ecosystem, Chinese Academy of Sciences, in Qinghai, China (equivalent to 11.0%–13.0% O₂ at sea level; 37°39'N, 101°19'E; altitude 3000–4500 m). *Myospalax cansus* (180–240 g) were captured in Yulin, northern Shaanxi (37°06'N, 107°15'E; altitude approximately 800 m). As both species complete their life cycle in subterranean burrows (70–250 cm depth) characterized by hypoxia and hypercapnic conditions (Zeng et al., 1984; Tian et al., 2017), a comparative study was conducted. *Rhizomys pruinosus* (bamboo rat, 290–350 g) were obtained from Yangshuo, northeastern Guangxi, China (24°53'N, 110°20'E; altitude 300 m). Adult male Sprague-Dawley (SD) rats (180–200 g; certification No. SCXK20190002) were purchased from the Laboratory Animal Center of Zhejiang Academy of Medical Sciences. All animals were housed under controlled environmental conditions (21–23 °C, 12 h light/dark cycle) with free access to food and water.

For hypoxia treatment, the lab rats underwent hypobaric hypoxia exposure (10.8% or 8.0% O₂ for 8 h) in a hypobaric chamber (FLYDWC50-IIC; Avic Guizhou Fenglei Aviation Armament Co., Ltd., Guizhou, China) to mimic altitudes of 5000 m and 7000 m, respectively (Zhao et al., 2013). Lung, spleen, and liver tissues were immediately cryopreserved in liquid nitrogen and subsequently stored at –80 °C.

2 Cloning of *Mdm2* and *Mdmx* from *M. baileyi*, *M. cansus*, and *R. pruinosus*

The *Mdm2* and *Mdmx* cDNA sequences of *M. baileyi* (GenBank accession Nos. KR149455.1 and MH033626), *M. cansus* (KR149456.1 and MH033627), and *R. pruinosus* (PV700090 and PV700091) were cloned in our laboratory. Primers were designed based on various *Mdm2* and *Mdmx* sequences in GenBank: rat (XM_006241383.2 and NM_001012026.1), and blind mole rat (*Nannospalax galili*, synonym *Spalax galili*) (XM_017801625.2 and XP_008828611.1), and human (BT007935.1 and AF007111.1). To confirm the authenticity of each variant codon, we cloned genes from the lung and liver tissues of six individuals of each species.

3 Multiple alignments, phylogenetic tree, and 3D structure analysis

The sequence alignments of MDM2, MDMX, and p53 in *M. baileyi*, *M. cansus*, *R. pruinosus*, and 13 other species were generated using Multalin software (<http://multalin.toulouse.inra.fr/multalin>). The phylogenetic trees were constructed with ClustalW and MEGA7 software based on the amino acid sequences, using the neighbor-joining method (Zhao et al., 2023). The accession numbers of *Mdm2*, *Mdmx*, and *p53* genes in the phylogenetic trees are listed in Table S1. We predicted the three-dimensional (3D) structures (MDM2, MDMX, p53, and p53–MDM2 complex) using AlphaFold 3 (<https://alphafoldserver.com>) and displayed them with PyMOL.

4 Cell cultures and hypoxia treatment

The cell lines H1299 (human non-small-cell lung cancer) and HEK293T (human embryonic kidney 293 cells) were grown in medium Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Grand Island, NY, USA) or Dulbecco's Modified Eagle's Medium (DMEM; Gibco), respectively, containing 10% (volume fraction) fetal bovine serum (FBS; Gibco), 100 µg/mL streptomycin (Solarbio, Beijing, China), and 100 U/mL penicillin (Solarbio), at 37 °C in a humidified incubator with 5% (volume fraction) CO₂.

The hypoxia treatment for cells was performed using the hypoxia controller (BioSpherix, Parish, NY, USA). The cell medium was replaced at 12 h post-transfection before cells were moved to the hypoxia chamber

(0.1% (volume fraction) O₂, 24 h).

5 Quantitative real-time polymerase chain reaction

The *Mdm2*, *Mdmx*, and *p53* messenger RNA (mRNA) levels were determined by Quantitative polymerase chain reaction (qPCR). Primers were synthesized according to the gene sequences of rat, *M. baileyi* and *M. cansus* as follows:

Gene	Primer sequence (5'-3')
rat <i>Mdm2</i> -F	AGG ATG ATG AGG TCT ATC GGG T
rat <i>Mdm2</i> -R	AAG CCA GTT CTC ACG AAG GG
<i>M. baileyi</i> <i>Mdm2</i> -F	GCA AGA GCC ACA GGA AGA GA
<i>M. baileyi</i> <i>Mdm2</i> -R	GTG GCG CTT TCT TTG TCG TT
<i>M. cansus</i> <i>Mdm2</i> -F	TCT GGT GAT TGG TTG GAT CAA GA
<i>M. cansus</i> <i>Mdm2</i> -R	TCT GCA TCA CTT TCC CCT GC
<i>Mdmx</i> -F	CAA GAC CGA CTG AAG CAC GG
<i>Mdmx</i> -R	CAG CAA CAT CCC ACT CCT CAA
rat <i>p53</i> -F	GTG CAC GTA CTC AAT TTC CCT C
rat <i>p53</i> -R	TCA GAG CAA CGC TCA TGG TG
<i>M. baileyi</i> <i>p53</i> -F	GAG CGC TGC TCTGAT AGT GA
<i>M. baileyi</i> <i>p53</i> -R	TGC CTC CCA TAC AGG AGC TAT
<i>M. cansus</i> <i>p53</i> -F	GGC TCT GAC TGT ACC ACC AT
<i>M. cansus</i> <i>p53</i> -R	AGG CAC AAA CTC GAA CCT CA
β -actin-F	AGC CAT GTA CGT AGC CAT CC
β -actin-R	CTC TCA GCT GTG GTG GTG AA

6 Western blotting

Western blotting was performed to determine the MDM2, MDMX, and p53 protein levels in the tissues and cells under hypoxia. Samples were lysed in 300 μ L radioimmunoprecipitation assay (RIPA) lysis buffer (Solarbio) with 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) (Solarbio). The extracts were centrifuged at 12 000g for 15 min at 4 °C, then mixed with loading buffer and heated at 95 °C for 5 min. Protein samples were subjected to electrophoresis, then transferred to polyvinylidene fluoride (PVDF) membrane, and incubated overnight with primary antibodies against target proteins (MDM2: SPM344, Invitrogen, Carlsbad, CA, USA; MDMX: MDMX-82, Abcam, Cambridge, UK; p53: 1C12, Cell Signaling Technology, Danvers, MA, USA; green fluorescent protein (GFP)-tag and β -actin: Yeasen, Shanghai, China). Secondary antibodies were incubated at room temperature for 1 h before being visualized with enhanced chemiluminescence (ECL).

7 Plasmids

For p53-expression plasmids, the coding regions of rat, *M. baileyi*, and *M. cansus* sequences were ligated into the pEGFP-N1 expression vector. The wild-type MDM2 and MDMX genes of rat, *M. baileyi*, *M. cansus*, and mutant MDM2-F390Y (TAT→TTT to change the amino acid), MDM2-A415T (ACA→GCC) plasmids of *M. baileyi* and *M. cansus* were ligated into the pcDNA3.1+ expression vector. All fragments ligated into the vectors were sequenced to confirm authenticity.

8 Transient transfection

H1299 and HEK293T cells were plated in six-well plates for 18 h; then, the medium was replaced with complete medium containing serum and antibiotics 30 min before transfection. Transient transfection was performed using PolyJet transfection reagent (SignaGen, Laboratories, Frederick, MD, USA). Cells were

co-transfected with a mixture of MDM2, MDMX, or p53 expression plasmids. The PolyJet/DNA complex-containing medium was replaced with fresh complete medium containing serum and antibiotics 12 h post-transfection, and the cells were transferred to the hypoxia chamber. Cells were harvested 48 h post-transfection.

9 Flow cytometry

For cell apoptosis, H1299 and HEK293T cells were transfected with the indicated plasmids. After 48 h of transfection and hypoxia treatment, cells were washed with cold phosphate-buffered saline (PBS) and collected. Cells were resuspended in binding buffer, then stained with Annexin V–phycoerythrin (PE) and 7-aminoactinomycin D (7-AAD) for 15 min at room temperature, and analyzed using flow cytometry. Samples were gated on GFP-positive cells to confirm that all analyzed cells expressed the transfected p53. The p53-induced apoptosis rate was calculated as the percentage of PE⁺/7-AAD⁺ cells.

10 Statistical analysis

Statistical analysis was conducted using GraphPad Prism 8 software. The statistical significance compared with the control was determined using a two-tailed, unpaired Student's *t*-test. Comparisons between multiple groups were conducted using one-way analysis of variance (ANOVA), followed by the Tukey test.

References

- Zeng JX, Wang ZW, Shi ZX, 1984. Metabolic characteristics and some physiological parameters of mole rat (*Myospalax baileyi*) in alpine area. *Acta Biol Plateau Sin*, (3):163-171 (in Chinese).
- Tian YL, Niu YJ, Ji CP, et al., 2017. A study of the gas environment in foraging tunnels of plateau zokor (*Myospalax baileyi*) in the eastern Qilian Mountain region. *Acta Theriol Sin*, 37(2):152-161 (in Chinese).
<https://doi.org/10.16829/j.slx.201702006>
- Zhao Y, Ren JL, Wang MY, et al., 2013. Codon 104 variation of *p53* gene provides adaptive apoptotic responses to extreme environments in mammals of the Tibet plateau. *Proc Natl Acad Sci USA*, 110(51):20639-20644.
<https://doi.org/10.1073/pnas.1320369110>
- Zhao Y, Zheng ZZ, Zhang ZH, et al., 2023. Evolution of high-molecular-mass hyaluronic acid is associated with subterranean lifestyle. *Nat Commun*, 14:8054.
<https://doi.org/10.1038/s41467-023-43623-2>

Table S1 Accession numbers of *MDM2*, *MDMX*, and *p53* genes

Species	MDM2	MDMX	p53
<i>H. sapiens</i>	NM_002392.6	NM_002393.5	NM_000546.6
<i>M. musculus</i>	NM_010786.5	NM_008575.4	NM_011640.4
<i>R. norvegicus</i>	NM_001439517.1	NM_001012026.1	NM_001429995.1
<i>N. galili</i>	XM_017801625.2	XM_008830389.2	NM_001319288.1
<i>M. baileyi</i>	<u>KR149455.1</u>	<u>MH033626</u>	<u>JX998170.1</u>
<i>R. pruinosis</i>	<u>PV700090</u>	<u>PV700091</u>	<u>PV700089</u>
<i>M. cansus</i>	<u>KR149456.1</u>	<u>MH033627</u>	<u>JX998172.1</u>
<i>S. araneus</i>	XM_055118171.1	XM_055144504.1	XM_004604858.2
<i>C. cristata</i>	XM_012720533.1	XM_004685537.2	XM_004684639.2
<i>C. porcellus</i>	XM_023567664.2	XM_005005889.4	NM_001172740.1
<i>F. damarensis</i>	XM_010644210.2	XM_010613018.3	XM_010614891.3
<i>H. glaber</i>	NM_001310277.1	XM_004862148.3	NM_001310270.1
<i>M. ochrogaster</i>	XM_013350275.2	XM_005348352.3	XM_005349777.1
<i>M. oeconomus</i>	<u>KR149457.1</u>	<u>MH033629.1</u>	<u>JX998171.1</u>
<i>O. cuniculus</i>	XM_002711320.5	XM_002717573.5	NM_001082404.1
<i>O. curzoniae</i>	XM_040981975.1	XM_040985498.1	XM_041000460.1

Notes: Genes cloned in our lab are underlined.

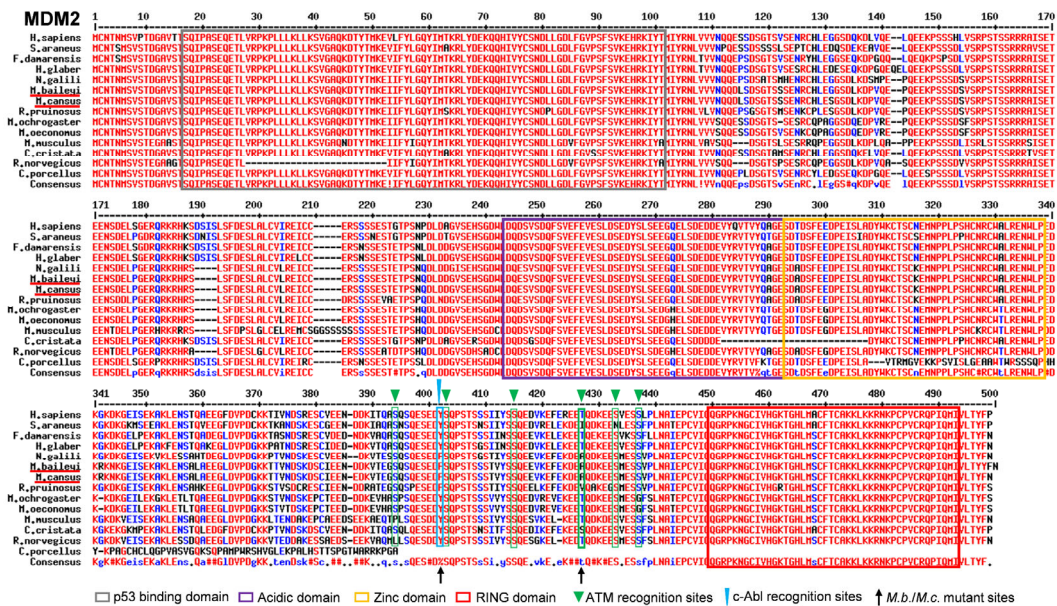


Fig. S1 Cross-species MDM2 alignments of fourteen species. MDM2 CDS sequences are highly conserved, particularly within the functional domains (color frames). The ATM-recognized (green symbol) and c-Abl-recognized (blue symbol) phosphorylation sites of MDM2 were marked. Arrows indicated mutant sites in *M. baileyi* (*M.b.*) and *M. canis* (*M.c.*).

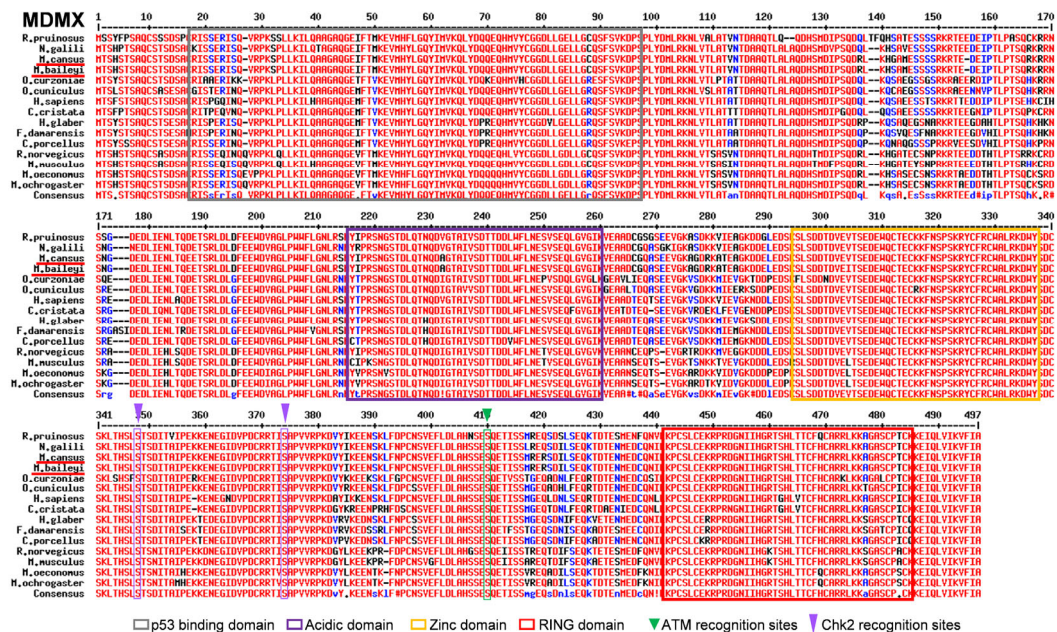
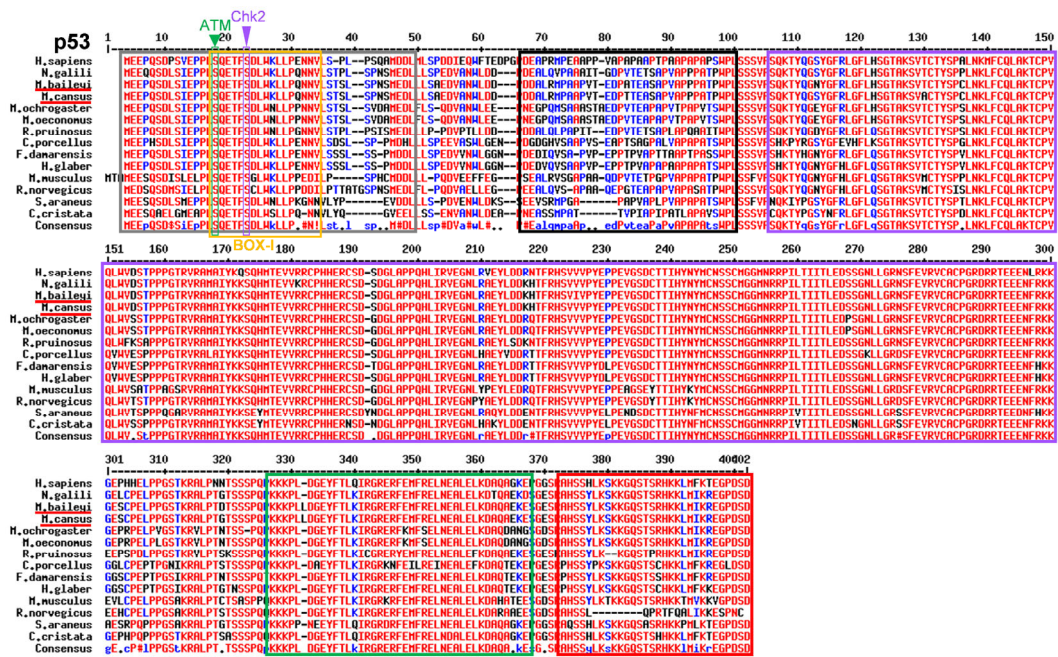


Fig. S2 Multiple sequence alignments of MDMX across 14 mammals. MDMX protein sequences are highly conserved. Functional domains are shown in different color frames. MDMX phosphorylation sites by ATM (green symbol) and Chk2 (purple symbol) under stress were marked.



□ Transactivation domain □ Proline rich domain □ DNA binding domain □ Tetramerization domain □ C-terminal domain

Fig. S3 p53 proteins are highly conserved among 14 species. Functional domains are shown in different color frames. p53 phosphorylation sites by ATM (green symbol) and Chk2 (purple symbol) under stress were marked.

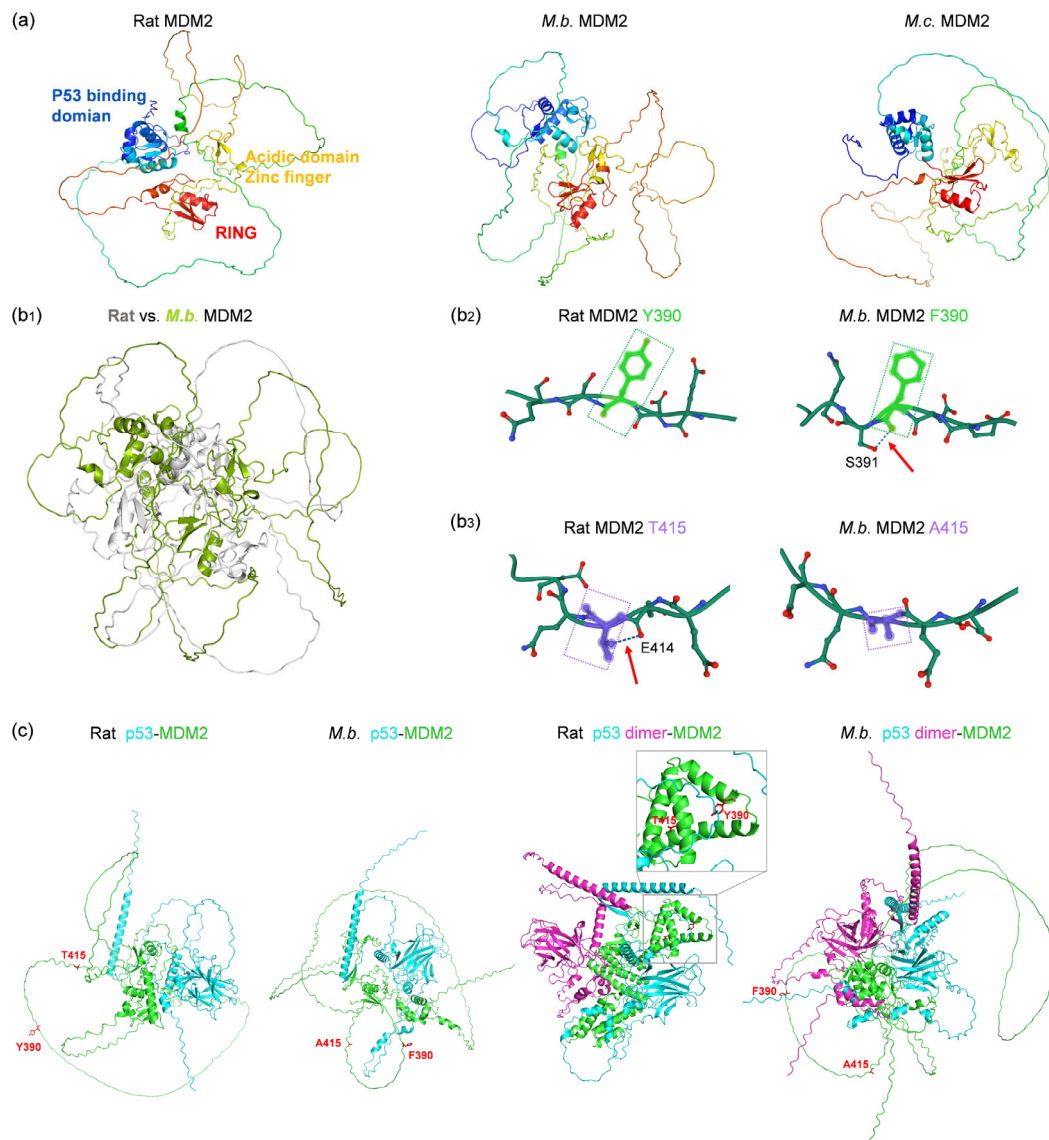


Fig. S4 Predicted 3D structures of MDM2 and p53-MDM2 complex in rats, *M.baileyi* and *M.cansus*. (a) The structures of Rat-, *M. baileyi* (*M.b.*)- and *M. cansus* (*M.c.*)-MDM2. Domains are shown in different colors. (b1) Overlay of the structure of Rat-MDM2 (gray) complexed with *M.b.*-MDM2 (green). A close-up view of the residues near MDM2 mutant sites, codon 390 (b2) and codon 415 (b3). The mutant 390 (green) and 415 (purple) residues are shown in frames. Arrows indicate the hydrogen bonds. (c) The 3D structures of the p53 monomer/dimer-MDM2 complex in rat and *M.b.* The C-terminal of MDM2 in the Rat-p53 dimer-MDM2 complex was enlarged in zoom. F390 and A415 mutation sites in red.

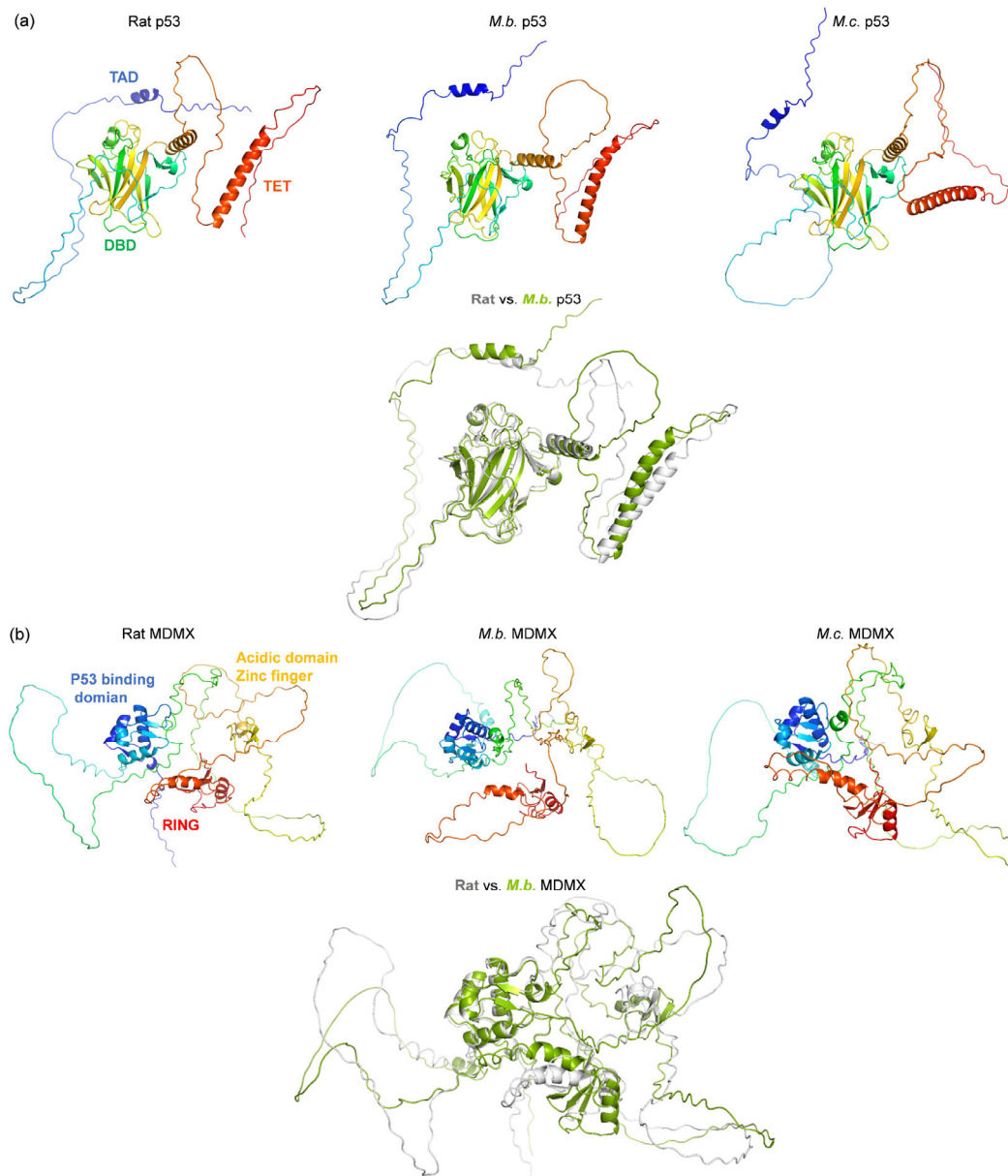


Fig. S5 Comparison of the structures of p53 and MDMX proteins in rats, *M. baileyi* (*M.b.*), and *M. cansus* (*M.c.*). (a) The overlays of the structures of Rat-p53 (gray) complexed with *M.b.*-p53 (green). (b) Structures of Rat-MDMX (gray) overlapped with *M.b.*-MDMX (green).