Codon 104 variation of *p53* gene provides adaptive apoptotic responses to extreme environments in mammals of the Tibet plateau

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Mutational changes in *p53* correlate well with tumorigenesis. Remarkably, however, relatively little is known about the role that *p53* variations may play in environmental adaptation. Here we report that codon asparagine-104 (104N) and glutamic acid-104 (104E), respectively, of the p53 gene in the wild zokor (*Myospalax baileyi*) and root vole (*Microtus oeconomus*) are adaptively variable, meeting the environmental stresses of the Tibetan plateau. They differ from serine-104 (104S) seen in other rodents, including the lowland subterranean zokor *Myospalax cansus*, and from serine 106 (106S) in humans. Based on site-directed mutational analysis in human cell lines, the codon 104N variation in *M. baileyi* is responsible for the adaptive balance of the transactivation of apoptotic genes under hypoxia, cold, and acidic stresses. The 104E *p53* variant in *Microtus oeconomus* suppresses apoptotic gene transactivation and cell apoptosis. Neither 104N nor 104E affects the cell-cycle genes. We propose that these variations in *p53* codon 104 are an outcome of environmental adaptation and evolutionary selection that enhance cellular strategies for surviving the environmental stresses of hypoxia and cold (in *M. baileyi* and *M. oeconomus*) and hypercapnia (in *M. baileyi*) in the stressful environments of the Qinghai-Tibet plateau.

ecological stress | evolution | *Apaf1* | *Noxa* | *Puma*

The regulatory mechanisms of *p53* mutation related to tumorigenesis have been widely studied and elucidated (1, 2). Notably, however, *p53* evolution and adaptation to environmental stresses have not attracted as much attention. Current studies show that *p53* is a master sensor and regulator in response to various stressors, such as DNA damage and hypoxia (3–6). Activation of *p53* by stresses results in cell-cycle arrest, DNA repair, senescence, or apoptosis in which a series of *p53* target genes are involved to maintain genomic integrity (2). The *p53* variations associated with environmental stresses have been described in the Mexican salamander axolotl *Ambystoma mexicanum* and the Israeli blind subterranean mole rat (*Spalax judaei*; hereafter, *S.j.*) (7–9).

For animals existing on high plateaus, hypoxia and cold serve as strong environmental selective pressures generating adaptive complexes to cope with these stresses. Animals that have evolved on plateaus adopt various strategies involving multiple variations to regulate a series of genes (3, 7). The zokor (*Myospalax baileyi*, Thomas, 1911; hereafter *M.b.*) and root vole (*Microtus oeconomus*, Pallas, 1776; hereafter *M.o.*) are the dominant native mammals living on the alpine meadow of the Qinghai-Tibet Plateau of China at altitudes of 3,000–4,500 m (equivalent to 11.0–13.0% O_2 at sea level). *M.b.* is genetically close to *Myospalax cansus* (Lyon, 1907; hereafter, *M.c.*), which lives in subterranean burrows at a lower altitude of about 800 m in the lowland of western China. *M.b.* and *M.c.* spend their entire life cycle at 70–250 cm underground with significantly low O_2 and high CO_2 levels in their burrows (10). Since the collision of the Indian and the Eurasian plates during the Tertiary (40–50 Mya) formed the Tibet plateau (11), small mammals living in this region have been geographically and ecologically isolated from other species and have adapted to the stressful plateau environment, contributing to the East Asian biodiversity (12–15). Our previous work demonstrated that mammals of the Qinghai-Tibet plateau are well adapted to the hypoxic environment (16–19), with particular expression patterns of HIF-1α and IGF-I and its binding protein (IGFBP-1), which mediate protection against hypoxia (20–23). Cells exposed to hypoxia succumb to *p53*-dependent apoptosis (24–27); thus mutations in *p53* are required for cell survival under selective pressures. We examined the hypothesis that plateau mammals are adapted to this environment with *p53* alterations linked to hypoxia, hypercapnia (high CO_2), and cold.

Here we report that the variations of *p53* codon 104 in three rodent species during long-term evolution and adaptation at the Qinghai-Tibet plateau reflect diverse survival strategies. The present study provides insights into the contribution of *p53* variations to native mammals' adaptation to the diverse and extreme environmental stresses of their habitats.

Results

Comparison and Phylogenetic Tree of *M.b.*, *M.c.*, and *M.o.* *p53* Sequences. The *p53* mRNAs of the subterranean *M.b.* and *M.c.* and the fossorial but above-ground–foraging *M.o.* of the Tibet plateau were

Significance

This work explores the environmental correlates of variations in codon 104 of the *p53* gene in three mammalian species: two subterranean mammals, highland- and lowland-dwelling wild zokors (*Myospalax baileyi* and *Myospalax cansus*, respectively), and one highland-dwelling aboveground species, the root vole (*Microtus oeconomus*). In *Microtus oeconomus* the codon 104E variation in *p53* suppresses apoptotic gene reactivation and cell apoptosis. In contrast, in *M. baileyi* the codon104N variation is responsible for the transactivation of apoptotic genes under three environmental stresses—hypoxia, hypercapnia (acidic stress, high CO_2), and cold temperature—that characterize its ecological niche in the Tibet plateau. We conclude that p53 in nature is adapted in structure and function in accordance with specific ecological stresses.

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cloned and sequenced. The coding regions of *M.b.* and *M.c. p53* are composed of 1,179 bp, coding a protein of 392 aa. *M.b.* and *M.c.* p53 proteins have 98% identity, and both have identities of 85%, 83%, 80%, and 95% with humans, rats, mice, and *S.j.* p53 protein, respectively. *M.o. p53* is composed of 1,176 bp and codes a protein of 391 aa, showing identities of 82%, 83%, 78%, 88%, and 88% to humans, rats, mice, *S.j*, and *M.b.* p53 protein, respectively. Phylogenetic trees based on the *p53* sequences indicated that in *M.b.*, *M.c.*, *M.o.*, and *S.j.* p53 evolved adaptively and convergently against hypoxia (Fig. 1 and Fig. S1), although *M.o.* and *M.b. p53* diverges from *M.c. p53* in terms of cytochrome *b* (Fig. 1*A*).

Multiple alignment analysis was performed using Multalin software as described (28). Compared with human *p53*, three amino acid residues of *M.b. p53* and two amino acid residues of *M.o. p53* within the DNA-binding domain (DBD) were altered. We found the mutations serine-104-asparagine (S104N), alanine-127-cysteine (A127C), and valine-215-isoleucine (V215I) within the DBD of *M.b. p53*, as well as serine-104-glutamic acid (S104E) and serine-258-proline (S258P) in *M.o. p53* (the corresponding positions in humans are 2 aa greater; i.e., codon 106). In addition to the DBD, there were a leucine insertion at position 322 in the C terminus of *M.b. p53*, an alanine-86-valine (A86V) mutation in the N terminus, and an arginine-340-serine (R340S) mutation in the C terminus of *M.o. p53*. The R340S mutation was found to be an evolutionarily positively selected site detected by using PAML 4 software (29) and was tested with the branch-site test as described (30, 31). We examined these sites because the *M.o.*-specific A86V mutation is close to the core domain and because the S104E mutation (corresponding to codon 106 in humans), which is found only in four fishes (*Barbus barbus*, *Platichthys flesus*, *Tetraodon miurus*, and *Xiphophorus hellerii*) and the squid *Loligo forbesi*, living in deep water or at the sandy bottom, resides in the core domain. The 104N of *M.b. p53* also exists in the rat, mouse, cattle, sheep, rabbit, and the Mongolian gerbil, but 104S exists in *S.j.*, *M.c.*, and human. The mutations of A127C, V215I, and leucine-322 are specific to *M.b.* (Fig. S2).

Expression of *M.b.* and *M.o.* p53 Under Hypoxia. *p53* mRNA levels under hypoxia were assessed by quantitative real-time RT-PCR. Mimicking the oxygen levels at an altitude of 7 km for 8 h induced significant increases in rat *p53* mRNA in liver (32). In contrast, it reduced *p53* mRNA in the *M.b.* liver (Fig. S3*A*). Remarkably, the p53 protein levels in both *M.b.* and *M.o.* livers were decreased in hypoxic conditions, as determined by Western blotting (Fig. S3*B*).

Codon 104 Variation Is Critical for Transcription of Apoptotic Genes in *M.b.*, *M.c.*, and *M.o.* To compare the functional characteristics of *M.b.*, *M.c.*, and *M.o.* WT *p53*, a dual-luciferase reporter assay was used in *p53*-null human non-small cell lung cancer NCI-H1299 cells and in cervical cancer HeLa cells, which have low endogenous *p53*. The *p53* target apoptotic genes *IGFBP3*, Apoptotic protease activating factor 1 (*Apaf1*), BCL2-associated X protein (*Bax*), the Bcl-2 homology 3 (BH3)-only pro-apoptotic protein (*Noxa*), and P53 upregulated modulator of apoptosis (*Puma*) and the cell-cycle arrest genes *p21* and the human homologue of mouse double minute 2 (*Hdm2*) were examined (33, 34). All rodent and human WT *p53* expression plasmids were cotransfected with reporter plasmids of these target genes, and dual-luciferase reporter assays were performed. We found that *M.b. p53* markedly activated *IGFBP3*, *Apaf1*, and *Bax* but suppressed *Noxa*. *M.c. p53* activated *IGFBP3*, *Apaf1*, *Bax*, and also *Puma*. *M.o. p53*, however, suppressed all the apoptotic gene transcriptions (Fig. 2 and Fig. S4). Endogenous expression of these apoptotic genes was tested in cells transfected with human, *M.b.*, *M.c.*, and *M.o. p53*, showing an expression pattern similar to that detected by dual-luciferase reporter assays (Fig. S4). Moreover, *p21* and *Hdm2* were not affected by human and animal *p53*, except that *p21* was suppressed by *M.c. p53* (Fig. S4). Furthermore, *M.b. p53* induced high expression of *Bcl-2*, but human, *M.c.*, and *M.o. p53* suppressed *Bcl-2* (Fig. 2). These results suggest that codon 104 variations in *p53* of *M.b.*, *M.c.*, and *M.o.* are correlated to distinct transcriptional patterns for apoptotic or antiapoptotic genes (Table S1).

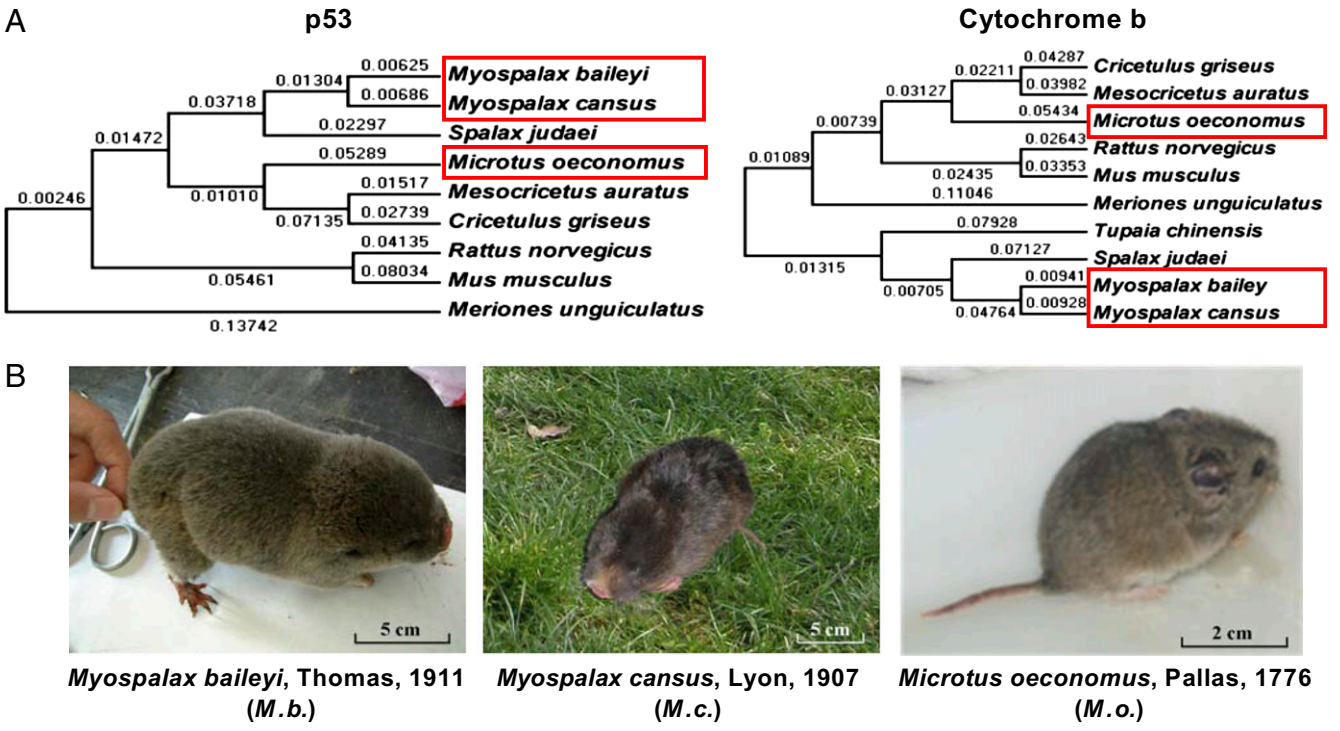

Fig. 1. (*A*) Phylogenetic trees based on the p53 and cytochrome *b* protein sequences of *M. baileyi*, *M. cansus*, and *Microtus oeconomus* as well as those of other rodents constructed using the neighbor-joining method. (*B*) Photographs of *M.b.*, *M.c.*, and *M.o.*

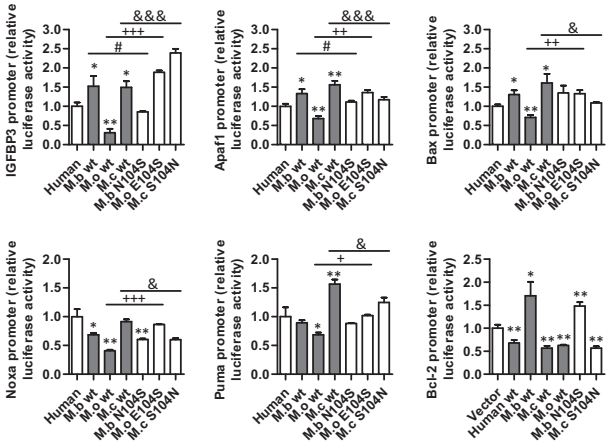

Fig. 2. Comparison of transactivation of apoptotic genes by WT *p53* and 104 mutants in *M.b.*, *M.c.*, and *M.o*. Results shown are the mean ± SD of three independent transfection assays performed in duplicate. $^{**}P < 0.01$, $^{***}P < 0.001$, WT *M.b.* and WT *M.c.* compared with human WT *p53* (unpaired *t* test). $^{\#}P < 0.05$, *M.b.* WT compared with *M.b.* 104 mutant. $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$, *M.o.* WT compared with *M.o.* 104 mutant. $^{\&}P < 0.05$, $^{\&\&\&}P < 0.001$ *M.c.* WT compared with *M.c.* 104 mutant.

Site-Directed Mutagenesis. We conducted site-directed mutagenesis to highlight the nature and functions of the mutations found in these codons in natural populations. Site-directed mutagenesis of *p53* revealed that variation in codon 104 is a key factor in the *p53* target gene transactivations seen in *M.b.* and *M.o* as compared with the other variations within the core domain (Fig. S5). Amino acid replacements in *p53* codon 104 showed that the *M.b. p53* N104S mutation down-regulated the expression of *IGFBP3* and *Apaf1*; however, the *M.c. p53* S104N mutation down-regulated the expression of *Apaf1*, *Bax*, *Noxa*, and *Puma* but up-regulated the expression of *IGFBP3*. Humanization of *M.o. p53* (E104S) up-regulated the transactivation of all apoptotic genes from a suppressed status (Fig. 2). These reversed effects suggest that the *p53* codon 104 variations in highland-dwelling *M.b.* and *M.o.* and in lowland-dwelling *M.c.* correlate to their distinct transactivational patterns for apoptotic genes and are likely to have a causal relationship for some apoptotic genes. It has been known that apoptotic activities of *p53* can be transcriptionally repressed (35–37). *Bcl-2* can be either activated to protect cells from apoptosis (38) or transcriptionally repressed by *p53* (39-41). Similar to human *p53*, *M.c.*, and *M.o. p53* transcriptionally repressed *Bcl-2*, whereas *M.b. p53* strongly activated *Bcl-2* (Fig. 2 and Table S1).

***p53* Induces Different Cell Fates in *M.b.* and *M.o.* Because of Codon 104 Variation.** To determine whether the differential transactivation of apoptotic targets by *M.b.* and *M.o. p53* altered their ability to induce apoptosis, we performed flow cytometry on HeLa cells transfected with human, *M.b.*, and *M.o.* WT *p53*. All *p53* fragments were tagged with GFP, forming *p53*–GFP fusion plasmids. GFP-fused *p53* also induces apoptosis and cell-cycle arrest (42, 43). The DNA content of cells stained with propidium iodide was analyzed using flow cytometry. WT human, *M.b.*, and *M.o. p53* induced similar degrees of G1-phase arrest (Fig. 3). However, the functional apoptotic activity of *M.b. p53* was similar to that of human *p53* (sub-G1 DNA content), whereas *M.o. p53* was defective in inducing apoptosis. Humanization of codon 104 restored its apoptotic ability (Fig. 3). Apoptosis detected using double stained method showed similar results (Fig. 4). The apoptotic function of *M.o. p53* was closely correlated with its down-regulated transactivation of the apoptotic targets *IGFBP3*, *Apaf1*, *Bax*, *Puma*, and *Noxa* (Fig. 2), suggesting that the evolutionary development of the codon 104 variation resulted in the down-regulation of the apoptotic function of *M.o. p53* (Table S1). This development allows *M.o.* to escape from hypoxia-induced apoptosis.

Variation in Codon 104 Contributes to Different *p53* Transactivation Patterns in Response to Hypoxia, Cold, and Acidic Stress in *M.b.*, *M.c.*, and *M.o.* *M.b.* and *M.o.* evolved adaptive resistance to hypoxic and hypercapnic stresses concomitant with the geological uplift of the Qinghai-Tibet plateau. Therefore, we investigated the response of *p53* target apoptotic genes to these stresses in H1299 cells transfected with WT *p53* or codon 104 mutants such as N104S for *M.b.*, S104N for *M.c.*, and E104S for *M.o*.

Under strong hypoxia stress (0.2% O_2), both WT and 104-mutant *p53* in the three mammals suppressed *Bax* and *Puma* but activated *Noxa*. However, *M.b.* WT *p53* (104N) up-regulated *Apaf1*, but its N104S mutant up-regulated *IGFBP3*; *M.c.* WT *p53* (104S) up-regulated *IGFBP3*, but its S104N mutant up-regulated *Apaf1*. Furthermore, *M.o.* WT *p53* up-regulated *IGFBP3* and *Apaf1*, but these genes were down-regulated after E104S mutation (Fig. 5, Fig. S6*A*, and Table S2). These data suggest that the variations in *p53* codon 104 in the three mammals correlate with their distinct transactivation of apoptotic genes and exhibit a functional and causal relationship in the responses of plateau-dwelling *M.b.* and lowland-dwelling *M.c.* to hypoxic stress. Down-regulation of *Bax* and *Puma* in the wild mammals benefits cell survival against apoptosis.

Under cold (30 °C) stress, both *M.b.* WT *p53* and its N104S mutant up-regulated *Bax*, *Noxa*, and *Puma* and down-regulated *IGFBP3*; *M.c.* WT *p53* up-regulated all apoptotic genes, but *IGFBP3* became down-regulated after S104N mutation. In contrast to *M.o.* WT *p53*, its E104S mutant induced down-regulation of *Puma*, *IGFBP3*, and *Apaf1* in response to cold stress (Fig. 5, Fig. S6*B*, and Table S2), suggesting that variations in the mammals' WT *p53* codon 104 are linked to their distinct transcription pattern for apoptotic genes in response to cold.

Under acidic stress, the WT and *p53* 104 mutants in the three mammals showed a similar alteration in the apoptotic target genes. However, in response to pH 6.6, WT *p53* induced activated *Bcl-2* transcription in *M.b.*, suppressed *Bcl-2* transcription in *M.c.*, and did not change *Bcl-2* transcription in *M.o.* Importantly, although Bcl-2 transcription was up-regulated by *M.b.* WT *p53*, it was down-regulated by N104S mutation. No change in response to pH 6.6 was seen with the S104N mutation in *M.c. p53* or the E104S mutation in *M.o. p53* (Fig. 6, Fig. S6*C*, and Table S2). This result suggests that WT *p53* in these species share a similar transcriptional regulation for apoptotic genes but divergent regulation for antiapoptotic genes. *M.b.* WT 104N seems to be linked specifically to *Bcl-2* transactivation under low pH, which may play protective effects against apoptosis under acidic conditions.

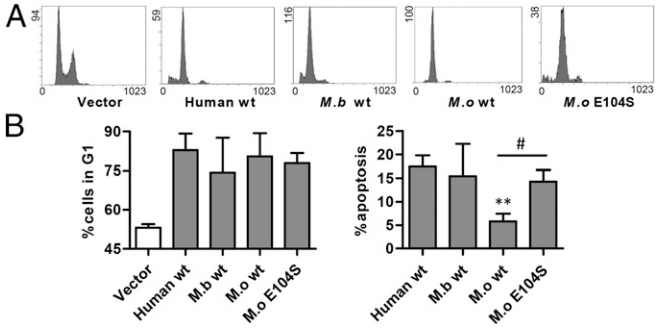

Fig. 3. Flow cytometry showing cell-cycle arrest and apoptosis induced by WT and mutated *p53* of humans, *M.b.*, and *M.o.* (*A*) Cell-cycle arrest and apoptosis induced by empty vector and WT *p53* from human, *M.b.*, and *M.o.* and the *p53* E104S mutation of *M.o.* (*B*) Histograms representing the G1 phase arrest and apoptotic rate induced by each *p53*. The apoptotic rate for cells transfected with WT *p53* was calculated as the percentage of cells with sub-G1 DNA content minus the percentage that transfected with empty vector. Results shown are the mean ± SD of four independent transfection assays, $^{**}P < 0.01$, compared with control (unpaired *t* test). $^{\#}P < 0.05$, *M.o.* E104S mutant compared with WT *p53*.

p53 Induces Different Apoptosis Rates Under Hypoxia, Cold, and Acidic Stress in *M.b.*, *M.c.*, and *M.o.* To assess WT *p53*'s induced responses of cell apoptosis, cells transfected with *M.b.*, *M.c.*, and *M.o.* WT *p53* expression plasmids were tested under hypoxic, cold, or acidic stresses using flow cytometry. The contributions of WT *p53* to apoptosis were confirmed by detecting GFP–$p53^+$ cells. All WT *p53* induced lower levels of apoptosis under hypoxia of 0.2% O_2 (Fig. 4). This reduction may correlate with the decreased *Bax* and *Puma* transcription (Fig. 5). *M.c.* and *M.o.* WT *p53* failed to reduce apoptosis, but *M.b.* WT *p53* induced down-regulation of cell apoptosis under 30 °C cold stress (Fig. 4), possibly through suppressed *IGFBP3* (Fig. 5). Furthermore, *M.c.* and *M.o.* WT *p53* failed to induce a reduction in cell apoptosis, but *M.b.* WT *p53* induced reduced apoptosis (Fig. 4) under pH 6.6 acidic stress, possibly because of up-regulated *Bcl-2* (Fig. 6). These data suggest that in the two highland-dwelling species *M.b.* and *M.o.* and in the low-land-dwelling species *M.c.* *p53* has distinct adaptive apoptotic target genes and/or antiapoptotic *Bcl-2* gene transactivation (Tables S1 and S2).

Discussion

Overview of Results. Our results suggest that the variation of *p53* codon 104 in *M.b.* and *M.o.* is the result of adaptation to the stressful environment of the Qinghai-Tibet plateau. The rising of the Qinghai-Tibet plateau started by the collision of the Indian and Eurasian plates 40–50 Mya (11). *M.b.* and *M.o.* were isolated geographically and ecologically and encountered lower temperatures and O_2 levels as the plateau rose (44). In addition to other allelic changes, we found that codon 104 differs with 104N in *M.b.* and 104E in *M.o.* We propose that these changes are adaptively related to cold and hypoxic environments. Interestingly, other than the rodent *M.o.*, only the squid *Loligo forbesi* and four fishes (*Barbus barbus*, *Platichthys flesus*, *Tetraodon miurus*, and *Xiphophorus hellerii*) have 104E (Fig. S2). Among these species, both *X. hellerii* and *L. forbesi* live in hypoxic aquatic environments (7), and *T. miurus* lives on sandy bottoms in deep water (45). *P. flesus* is also known to be moderately tolerant of hypoxia (46, 47). The homoplasy of this site shared by these species strongly suggests that the 104E in *M.o.* *p53* is related to hypoxia tolerance. In *M.b.*, however, 104N could be an adaptation coupled to hypoxia and hypercapnia at the high altitudes (~3,000–4,500 m) which *M.b.* inhabits. The two 104 variations may correlate with distinct adaptive strategies to the different ecological niches of the two plateau species. This view is supported by the present results showing that 104N in Tibetan *M.b.* has a high transactivation to apoptotic target genes and to antiapoptotic *Bcl-2*. This effect differs from the suppressed apoptotic genes and unchanged *Bcl-2* induced by *M.o.* *p53* 104E. Likewise, it differs from activated

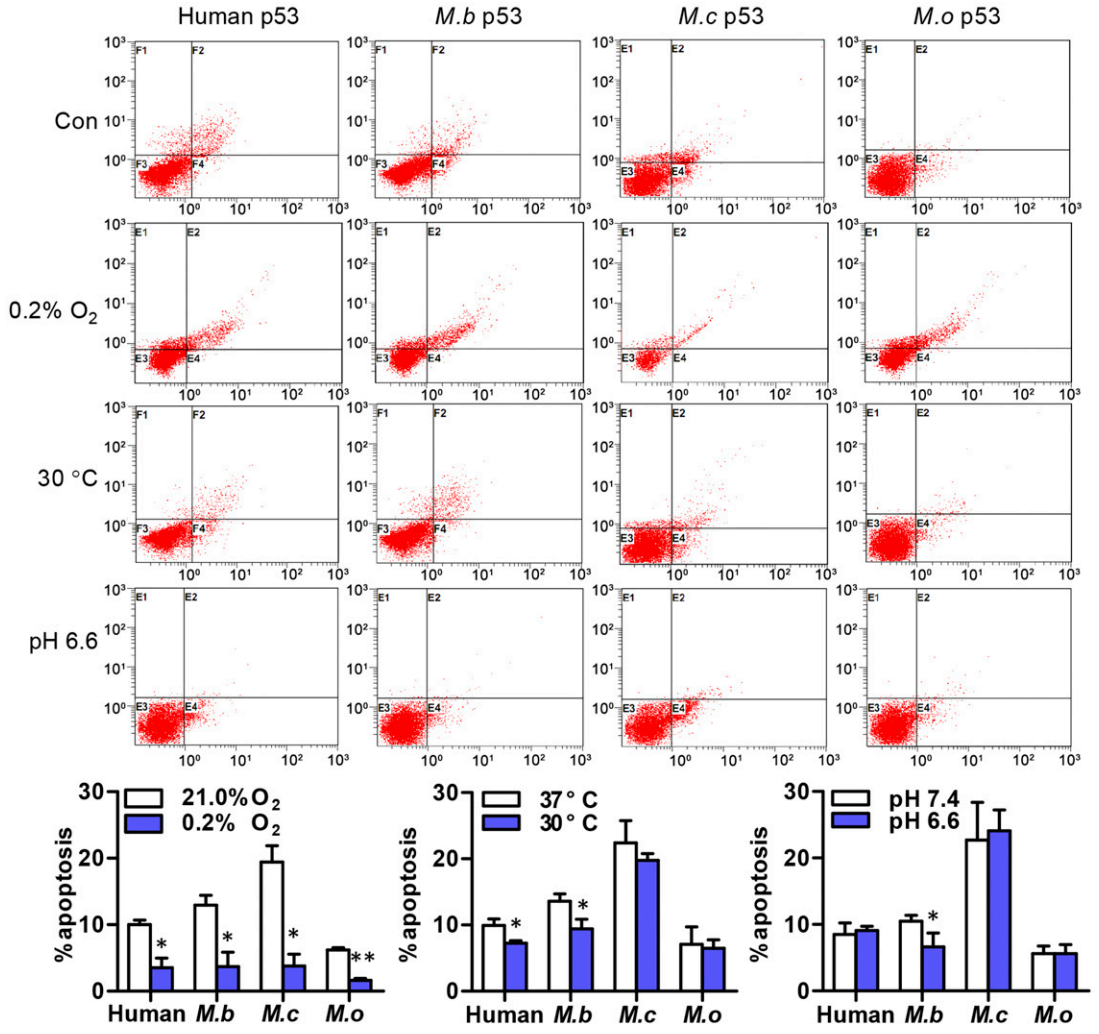

Fig. 4. *M.b.*, *M.c.*, and *M.o.* have different apoptotic *p53* variants in response to hypoxia, cold, and acidic stresses. Apoptosis induced by these *p53* genes under each stress was determined by using flow cytometry to detect GFP-$p53^+$ cells stained with Annexin V- phycoerythrin and 7-amino-actinomycin. Results shown are the mean ± SD of three independent transfection assays. $*P < 0.05$, $**P < 0.01$, stress compared with control (unpaired *t* test).

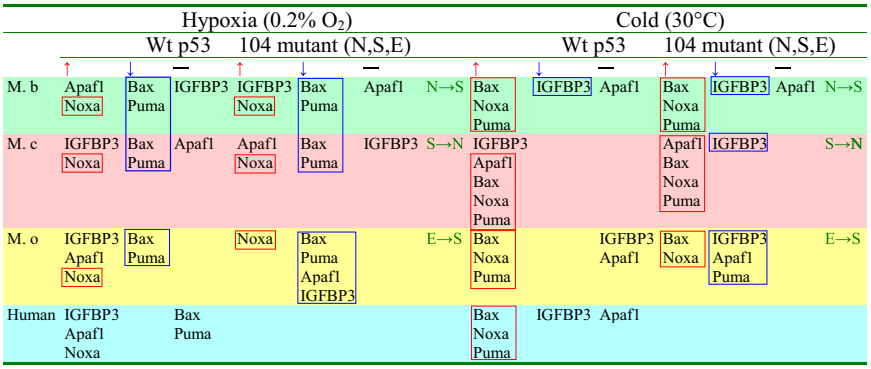

Fig. 5. Up-regulation and down-regulation of the transcription of *p53* target genes by WT *p53* and of *p53* 104 mutants under hypoxia and cold stress. ↑, up-regulation; ↓, down-regulation; –, unchanged.

apoptotic genes and suppressed *Bcl-2* induced by *p53* 104S in *M.c.*, which inhabits burrows at an altitude of <800 m in west China. It also differs from the action of *p53* in *S.j.* in Israel (7). *M.c.* is a lowland zokor and is a relative of *M.b.* However, its *p53* transactivation of apoptotic genes is stronger than that of *M.b.*, and this increased activation has been demonstrated to correlate with the codon 104S variation (Fig. 2 and Fig. S4). Therefore, the 104E and 104N variations in Qinghai-Tibet plateau's mammals appear to be associated with hypoxic and cold environments; in particular, the 104N variant seems to be associated with hypoxia and high CO_2 (Table S2).

Adaptive Environmental Correlates of *p53*. The WT *p53* variations in the two Tibetan species *M.b.* and *M.o.* are associated with cellular apoptosis and antiapoptosis induced by hypoxia. Several studies have demonstrated that the *p53* DBD is conserved and important for its functions as a tumor suppressor (2, 26). Only a few studies report that the *p53* variation is associated with environmental adaptation. In tumorigenesis, the most frequently mutated sites in the DBD are found at codons 175R, 248R, and 273R of *p53*, which are referred to as "hotspots" (48). As expected, these changes are not found in *M.b.* or *M.o.* The mutated codon 104 residing in the DBD appears to be associated with environmental fitness. In human cancers, a germ-line mutation in this codon was reported in a patient with multiple primary cancers (49). Using site-directed mutagenesis, we found that the codon 104N mutation in *M.b.* and the 104E mutation in *M.o.* *p53* are required for divergent responses of *IGFBP3* and *Apaf1* to hypoxia and cold stresses. This observation led us to analyze the 3D structure of *p53* tetramer binding to DNA and highlighting the location of codon 104. Although located in the core domain, codon 104 does not interact directly with DNA (Fig. S7); thus, it should not induce universal changes in transactivation by *p53*. The transcriptional repression of *Bcl-2* is associated with a competitive binding of *p53* against a *Bcl-2* transcriptional activator *Brn-3a* (40). The codon 104 mutation of *M.b.* *p53* did not affect the activation of *Bcl-2* (Fig. 2) but did affect the activation of *Bcl-2* under acidic stress (Fig. 6). Other sites of variation in *M.b.* *p53*, especially those within the *mSin3A*-binding domain, also seemed to contribute to *Bcl-2* activation.

The codon 104 variations in the two Tibetan species also appear to be related to the specific local environment of their niches. The high CO_2 stress in the underground burrows of *M.b.* results in a significantly higher partial pressure of CO_2 in the mammal's arterial (51.97 mm Hg) and venous (76.86 mm Hg) blood, challenging the blood-buffering system. In rats the CO_2 pressure is lower (33.68 mm Hg in arterial blood and 40.05 mm Hg in venous blood) (10, 50). Our study demonstrated that under acidic stress of pH 6.0, *M.b.* *p53* transactivation of the apoptotic target gene *IGFBP3* was decreased and that of the antiapoptotic gene *Bcl-2* was increased dramatically. However, the N104S mutation in *M.b.* resulted in enhanced *IGFBP3* and reduced *Bcl-2* activation (Fig. 6 and Fig. S6*C*). Therefore, the 104N variation is involved in the regulation of blood acidity. These data suggest that the 104N mutation in *M.b.* contributes to the animal's tolerance of a high-CO_2 environment. In addition to hypoxia and hypercapnia, the low annual mean temperature on the Tibet plateau provides an additional stress. Evidence shows that genotypic variation of *p53* is related to the average temperature of the population's environment (51). Although *M.b.* and *M.o.* live at the same altitude, *p53* in these two rodents showed markedly different responses to cold stress (Fig. 5). This difference could be explained by the two mammals' different niches. *M.o.* forages above ground, subjected to extremely low (average, −14.8 °C) ambient temperatures in winter and an average ambient temperature of 9.8 °C in summer. The temperature in the underground burrows of *M.b.* is milder and is relatively stable. This difference may have contributed to the differential evolution of codon 104 in the two plateau-dwelling rodents. The holarctic distribution of *M.o.* also suggests the species' adaptation to cold environments (52). In *M.b.* the 104N mutation of *p53* contributes to the decreased *IGFBP3* transactivation under cold and acidic stresses (Figs. 5 and 6), suggesting an elaborate modulation of *p53* to cope with multiple stresses to underground life.

Conclusions

Our study has shown an evolutionary adaptive variation in codon *p53* 104 in the two Qinghai-Tibet plateau mammals, the underground-dwelling *M.b.* (104N) and ground-dwelling *M.o.* (104E). These variations affect hypoxia, cold, and pH regulation, indicating that the *p53* variations are adaptively associated with the variable plateau niches below and above ground. Generally, these results

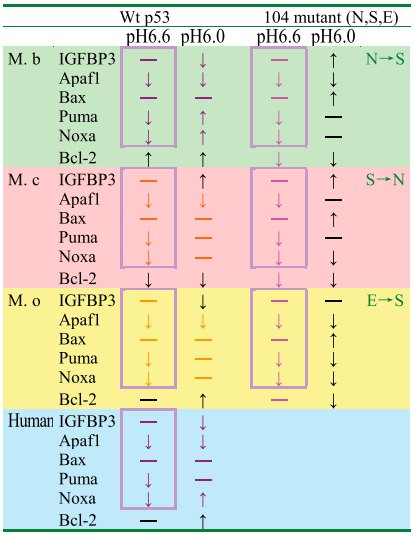

Fig. 6. Comparison of the transcription of *p53* target genes by WT *p53* and 104 of *p53* mutants under acidic stress. ↑, up-regulation; ↓, down-regulation; –, unchanged.

suggest that *M.b.* and *M.o.* are fitting model organisms for investigating the adaptive molecular evolutionary mechanisms of the interaction between gene and environment.

Materials and Methods

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. *M. cansus* (250–300 g, Fig. 1*B*) were captured from the field near Yan'an, north Shaanxi (36°36′N, 109°31′E) of China. Adult male Sprague-Dawley rats (150–200 g; Certification No. SCXK20080033) were purchased from the Experimental Animal Center, Zhejiang Academy of Medical Science. Animal protocols were approved by the IACUC of the School of Medicine, Zhejiang University and followed National Institutes of Health laboratory animal care guidelines. The methods used in this study (animal breeding, molecular cloning, sequence analysis, cell culture, plasmid construction, mutagenesis, transient transfection, dual-luciferase reporter assay, quantitative real-time RT-PCR, Western blotting, and flow cytometry) are described in *SI Materials and Methods*.

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