Lineage-specific accelerated sequences underlying primate evolution

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Understanding the mechanisms underlying phenotypic innovation is a key goal of comparative genomic studies. Here, we investigated the evolutionary landscape of lineage-specific accelerated regions (LinARs) across 49 primate species. Genomic comparison with dense taxa sampling of primate species significantly improved LinAR detection accuracy and revealed many novel human LinARs associated with brain development or disease. Our study also yielded detailed maps of LinARs in other primate lineages that may have influenced lineage-specific phenotypic innovation and adaptation. Functional experimentation identified gibbon LinARs, which could have participated in the developmental regulation of their unique limb structures, whereas some LinARs in the Colobinae were associated with metabolite detoxification which may have been adaptive in relation to their leaf-eating diet. Overall, our study broadens knowledge of the functional roles of LinARs in primate evolution.

INTRODUCTION
The genetic alterations underlying major phenotypic changes in nature remain largely unknown. Previous comparisons between human and other primate genomes have highlighted the high degree of similarity in protein-coding genes (1, 2), which is hard to reconcile with the marked differences so evident between human and nonhuman primates. Thus, King and Wilson (2) proposed that mutations in gene regulatory elements might serve to explain this paradox. Regulatory elements play a central role in many biological processes by controlling the spatial and temporal expression patterns of specific genes. Many heritable components of common disease risk fall within noncoding regions and appear to be especially enriched in regulatory regions (3, 4). Alterations of regulatory regions have fewer pleiotropic effects than changes in coding sequences and, hence, may be expected to be more likely to give rise to significant phenotypic effects (5, 6). This is also exemplified by the evolution of lineage-specific phenotypes in many species resulting from changes to regulatory elements (7, 8).

Whole-genome comparisons have allowed the identification of noncoding sequences exhibiting an elevated substitution rate across the genome in particular lineages (9), as well as thousands of human-accelerated regions (HARs) many of which have turned out to be important for brain development and cognitive functions (10, 11). The high degree of conservation of genomic elements over an extended period of evolutionary time is indicative of the functional importance of these genomic regions, whereas their accelerated evolution observed in a specific lineage suggests that these regions might have once been under strong positive selection with advantageous substitutions occurring specifically in that lineage. A previous paper referred to these lineage-specific accelerated regions as LinARs and identified the LinARs in ape genomes (12). The LinARs in other primate lineages might also have contributed mechanistically to the long-term evolution of primates and the vast phenotypic diversity apparent in extant primate lineages. However, the extent of the roles that these LinARs have played remains largely unexplored.

The search for LinARs is usually performed on genomic regions that have maintained a high level of conservation over a considerable period of evolutionary time and involves the application of a statistical test that compares the substitution rate observed in a given lineage with the expected rate in the rest of the tree (9). Therefore, the detection power and accuracy of LinARs identified are critically reliant on both the total branch length of the tree, which can affect the false-positive rate for the background evolutionary constraint signal, and the taxon sampling of the closely related lineages that influence estimates of the lineage-specific substitution rate. Here, taking advantage of the available high-quality reference genomes from 49 primate species, including 27 species that were newly sequenced and assembled with long-reads technology, we identified the LinARs in all extant primate lineages and all key evolutionary nodes across the primates and explored the functional implications of these LinARs for primate diversification.

RESULTS
Improved detection power for accelerated conserved noncoding sequences
To obtain accelerated regions for clades of interest, we first identified the highly conserved regions with multiple genome alignments across species, and then scanned for regions showing faster
evolutionary rates than neutral rates among these conserved regions using the phyloP tool (9, 13). As a result, we obtained approximately one million highly conserved elements (HCEs), together spanning ~5.5% of the human genome (fig. S1). By means of further comparison with other vertebrate genomes, we categorized these HCEs into vertebrate HCEs (29%), amniote-specific HCEs (4%), mammalian-specific HCEs (3%), and primate-specific HCEs (64%) (fig. S2). To identify the potential regulatory elements that might have contributed to lineage-specific evolution, we excluded the coding regions from these HCEs and used phyloP to screen for conserved loci that had experienced lineage-specific accelerated evolution in each evolutionary node of our primate phylogeny. In total, we identified 6.6% of HCEs that showed accelerated evolution in at least one primate branch (fig. 1 and data S1). More than 77% of these LinARs have been found to overlap with long noncoding RNAs (lncRNAs) or candidate cis-regulatory elements (cCREs) (14, 15), suggesting the functional significance of LinARs in primates.

Our updated list of LinARs in humans included 1674 regions (Fig. 2A), of which 892 sequences have been highly conserved across all vertebrates, 43 across sarcopterygians, 63 across amniotes, and 55 across mammals, whereas 621 are conserved but only in a primate-specific setting. We found that about one-third of our human LinARs (620 of 1674) overlapped with 3168 potential HARs previously identified in various sets of mammalian conserved elements (16–23). Further investigation of the alignments suggested that the disparity was mainly due to the power difference in detecting the background constraint for sequence conservation or accelerated evolution signals in humans consequent to different numbers of primate genomes being used for the analyses. Using a maximum of six primate species in the previous comparison provided insufficient power to detect the accelerated signals of the 929 human LinARs that were detected in our current comparison of 49 primate genomes (Fig. 2B). An additional 125 human LinARs were missed in previous studies because they were located within primate-specific conserved regions which were undetectable by analyses with only a few primate genomes for comparison (Fig. 2C). Conversely, various supposed HARs identified previously did not show up in our current dataset either because they turned out not to be conserved across the primates or because the level of acceleration in the human lineage was not significant when additional primate genomes were used as controls (fig. S3). More specifically, among those previously identified HARs, 2100 were artificial because the human-specific variations that detected with fewer species are in fact also appeared in other closely related primate lineages and the accelerated signal was no longer significant in human lineage when adding more primate species in analyses (fig. S4). In addition, 445 previous HARs were not detected now because these regions were not evolutionarily conserved across primate lineages (fig. S5). Moreover, a total of 66 previously detected HARs were artificial and had experienced an increased rate of evolution in the ancestral nodes of primates. Because of the low sample density for primates, the accelerated evolution signals in other evolutionary nodes could not be detected previously, but they were revealed in our analyses (fig. S6).

**Human LinARs associated with brain development**

It has been previously shown that many rapidly evolving regions in the human genome act as neurodevelopmental enhancers and play roles in neuronal gene regulatory programs (11). We found that 30% of the human LinARs located in cis-regulatory regions are functional elements annotated by the Encyclopedia of DNA Elements (ENCODE) database based on biochemical signatures of the genome (15). These human LinARs overlapped significantly with cis-regulatory elements active in the spinal cord ($P = 3.9 \times 10^{-34}$), eye ($P = 3.8 \times 10^{-31}$), and brain ($P = 5.7 \times 10^{-17}$) tissues (fig. S7). Genes associated with these LinARs were also significantly enriched in midbrain-hindbrain boundary development [Gene Ontology (GO): 0030917], rostrocaudal neural tube patterning (GO:0021903), and neuron recognition (GO:0008038) (data S2). For example, we found that the human LinAR (chr2: 235,865,048 to 235,865,565, rank23, coordinates are based on hg38) is nearest to gene GBX2 [distance to transcription start site: 303,062 base pairs (bp)], a homeobox gene involved in the normal development of rhombomeres, segments of the developing mid/hindbrain region (data S3). During gastrulation and later stages of embryogenesis, GBX2 is necessary not only for the early establishment of A/P (anterior/posterior) patterning in the neural plate but also for the normal development of the anterior hindbrain and the proper formation of the mid/hindbrain organizer in brain morphogenesis (24). Another human LinAR (chr3:2,660,287 to 2,660,641, rank140) is closest to gene CNTN4 (distance to transcription start site: 421,635 bp) which encodes a cell adhesion molecule that supports the developing nervous system by promoting neurite outgrowth and axon guidance (25).

Our collection of human LinARs included many previously detected HARs including several that have been shown to play functional roles in the human cerebral cortex (data S4) (11). In particular, it also contains many novel human LinARs with potentially important biological functions that were not found by previous studies. For instance, among the top 20 most significant human LinARs, only one (chr20: 63,102,113 to 63,102,273, rank3) overlapped with the previously detected HAR genes HAR1A and HAR1B, which have been shown to be specifically expressed in Cajal-Retzius neurons and are essential for neocortex development (26). Most of these LinARs are overlapping with the IncRNA. Thus, the top eight human-LinARs are located within seven IncRNAs (data S5), including HAR1A and HAR1B. We performed in situ hybridization (ISH) in the human motor cortex and showed that the other five of these IncRNAs were highly expressed in the human motor cortex (Fig. 2D). In addition, we found that 129 newly identified human LinARs were placed near genes known to be associated with human inherited disease, of which 105 were associated with neurological disorders (data S6). Thus, our study has expanded the list of LinARs in the human lineage that may have contributed to the evolution of uniquely human features.

**Accelerated evolution in nonhuman primate lineages**

In addition to the human LinARs, which were highly enriched within brain developmental gene regulatory elements and detection of chemical stimuli involved in sensory perception, we noted that several other evolutionary lineages closest to humans, such as the Homininae, Ponginae, and Hominidae great ape, also harbor LinARs which were found to be significantly associated with genes involved in sensory perception (a neurological process; data S2), implying that some of those LinARs residing in ancestral nodes may have contributed to the evolution of primate olfaction and other chemical senses (27). Gibbon (hylobatidae) LinARs tend to occur near genes involved in growth plate cartilage chondrocyte
Fig. 1. Landscape and characteristics of lineage-specific accelerated regions (LinARs) in primates. The number distribution of LinARs in 18 major primate evolutionary nodes (suborders and families) and 49 extant species. More than 70% of LinARs in each lineage overlapped with long noncoding RNAs (lncRNAs) or human candidate cis-regulatory elements (cCREs). The size of the circles represents the number scale of the LinARs, whereas the gray area in the pie chart represents the overlap ratio with the lncRNA or cCREs. Species pictures are copyrighted by S. D. Nash/IUCN/SSC Primate Specialist Group and are used with their permission in this study.
Fig. 2. Comparison between our identified human-accelerated regions and those of previous studies. (A) The Venn diagram depicts the overlap between the human LinARs identified in our study and previously detected HARs. The dot plot shows the distribution of conservation score $P$ values across the primates and human-accelerated signal $P$ values for human LinARs detected specifically in our study [orange corresponds to (B), pink to (C), green to previously identified HARs, and blue to shared regions]. The red lines represent the significance threshold of the $P$ values (conserved: $P = 7.24 \times 10^{-5}$, accelerated: $P = 1.45 \times 10^{-8}$). (B) A case of our newly identified human LinAR (HLinAR-Rank114). The top panel shows the branch length of the neighbor-joining tree across primate species with humans having a significantly longer branch than other lineages. The bottom panel shows the branch length of the neighbor-joining tree across the mammals, the branch length in the human lineage being no different from those in other mammalian lineages. $n$ represents the number of species. The scale bar denotes the mean number of nucleotide substitutions per site. (C) An example of our newly identified human LinAR (HLinAR-Rank18), which was not detected by previous studies because, although this region was specifically conserved in primates, it was not conserved across the mammals. (D) Results of in situ hybridization for the expression of five lncRNAs in the adult human cerebral cortex. The tissue was dissected from the motor cortex. The expression signals of all genes were detected and marked (red). The blue dot is 4′,6-diamidino-2-phenylindole staining, showing the location of the nucleus.
morphogenesis ($P = 1.7 \times 10^{-13}$, data S2), which has been shown to be related to their characteristically long bone formation (28). Genes residing close to LinARs in Simiformes and Strepsirrhini are enriched in functions related to the regulation of dendrite morphogenesis in brain development (Simiformes: $P = 1.8 \times 10^{-10}$, and Strepsirrhini: $P = 6.1 \times 10^{-5}$). While the LinARs in Cercopithecinae are highly associated with genes in the immune system ($P = 2.1 \times 10^{-10}$) and MHC class I protein complex in Hominidae ($P = 1.1 \times 10^{-12}$). For example, human leukocyte antigen C (HLA-C) is an important major histocompatibility complex class I protein that bacteria to break down plant material in multichambered stomachs (36–38). Previous studies have suggested that the Colobinae have higher microbial diversity in their guts than their closely related sister group, the Cercopithecinae (39). The fecal microbiomes of Rhinopithecus bieti (a member of the Colobinae subfamily) have broad bacterial diversity and contain a large number of glycoside hydrolases responsible for lignocellulosic biomass degradation reflecting their adaptation to a diet rich in fibrous matter (40). We found that many of the LinARs that emerged in the Colobinae ancestral lineage were associated with genes related to metabolite detoxification (table S2). These include the CYP4Z1 gene that encodes a member of the cytochrome P450 superfamily of enzymes that are important for metabolism and detoxification (41), and ASL that encodes argininosuccinate lyase, which catalyzes the reversible hydrolytic cleavage of argininosuccinate into arginine and fumarate, an essential step in the detoxification of ammonia in the liver (42).

Another example is SOD1 which encodes an antioxidant enzyme that metabolizes superoxide radicals (43). SOD1 also functions as an antimicrobial peptide that displays antibacterial, anti fungal, and anti-methicillin-resistant Staphylococcus aureus (MRSA) activity (44). We identified a Colobinae LinAR located within an intronic region of SOD1 (Fig. 4A). Although this element was highly conserved and under strong negative selection across other Simians, it accumulated multiple substitutions in the common ancestor of the Colobinae. It is of note that all these newly derived substitutions were subsequently fixed across all extant Colobinae species (Fig. 4A), implying that the neofunctionalization associated with these substitutions could have produced advantageous phenotypes that would have been rapidly subject to selective constraint in the Colobinae. The three-dimensional interaction data in six human tissues showed that this Colobinae LinAR and SOD1 were located in the same topologically associating domain (fig. S11) (45). Transcriptome comparison indicated that the SOD1 gene was widely expressed in many tissues but exhibits a lower level of activity specifically in the Colobine, black-and-white snub-nosed monkey (R. bieti), especially in the stomach, liver, and kidney, as compared to the rhesus macaque and humans (Fig. 4B). We further performed a dual-luciferase reporter gene experiment in human embryonic kidney (HEK) 293T cells and found that the SOD1-associated LinAR in R. bieti exhibited significantly lower promoter activity as compared with its counterparts in humans or rhesus macaque (Fig. 4C), suggesting that the low expression of SOD1 in the Colobinae might have been driven by this LinAR. It is possible that the low SOD1 activity in the gut of the Colobinae allows more microbes to survive, thereby assisting herbivorous digestion in this subfamily of primates. However, further confirmation in gastric cell lines will be useful in understanding the function of this LinAR.

**DISCUSSION**

Overall, our study has provided an evolutionary landscape of LinARs across the primates and broadened our understanding of the key roles played by evolutionarily accelerated regions in the acquisition of lineage-specific phenotypic innovations and adaptations. We identified hundreds of LinARs that were not found in previous studies. This suggests that increasing the phylogenetic density of genomic data by incorporating additional closely related primate species could significantly improve the detection efficiency and accuracy of LinARs, although other factors such as improving genome quality and sequence alignment quality may also
have contributed. In particular, our data allow the detection of rapidly evolving signals in a species or node within genomic regions that only experienced purifying selection constraints in the common ancestor of primates. This is important because these genomic regions may have contributed to the unique phenotypic features that have evolved in primates, such as the larger neocortex, advanced cognitive ability, and sensitive vision with depth perception (46). Many genes associated with the newly found human LinARs are functional in nervous system development. In a similar vein, the most critical LinARs function as lncRNAs which are expressed in the human brain, suggesting that these regions could have played a role in human-specific brain development. Thus, the updated list of human LinARs may contain loci associated with human-specific characteristics. Similarly, the LinARs we have described here in the gibbon and Colobinae lineages serve to demonstrate that the LinARs detected in other evolutionary

Fig. 3. Analysis of enhancer activity for two gibbon-specific accelerated regions in a lacZ reporter transgenic mouse assay. (A and B) Gibbon-specific accelerated regions downstream of two genes (DLX5 and EMX2) associated with limb development. Black rectangles represent exons, orange rectangles represent rapidly evolving regions, and arrows represent the direction of transcription. The red lines in the phylogenetic tree represent the gibbon lineage. (C) Representative E11.5 transgenic embryos obtained for two gibbon LinAR (Rank148 and Rank181) reporters. Each construct shows three embryos resulting from independent transgene integration events. The images in the left-hand panels show close-up views of forelimb and hindlimb expressions in a representative embryo for each construct, with arrows indicating the positions where limb expression is present. Right-hand panels show zoom-in figures of the shoulder. See also fig. S10.
Fig. 4. Dual-luciferase reporter gene assay for the assessment of LinAR promoter activity. (A) Sequence alignment shows a Colobinae LinAR (Rank88) located within a SOD1 intron. Black rectangles denote the exon-intron structure of SOD1, the orange rectangle represents the LinAR, and the arrow denotes the direction of transcription. (B) RNA sequencing data indicated that SOD1 gene expression was decreased in many tissues from the black-and-white snub-nosed monkey (R. bieti) as compared with humans and macaques. The expression level was normalized with quantile normalization. (C) Relative luciferase activity of reporter vectors containing orthologous sequences of this LinAR in humans, Macaca, and Rhinopithecus was measured in human embryonic kidney cells. Eight replications were performed for each experimental group. ***p < 0.001; ****p < 0.0001 (t test, calculated by GraphPad Prism software).
nodes or primate species might also help to explain some of the phenotypic innovations and adaptations that have evolved in different primate lineages.

Our study has provided a valuable resource of LinARs across primate lineages. However, more in-depth experiments are now required to investigate in detail the regulatory functions of these elements. So far, a comprehensive annotation of regulatory elements has only been performed for the human genome, which has limited our understanding of the roles of LinARs in other primate species. The initiation of an ENCODE-like project, involving, at the very least, those primate species that have been widely used as research models, such as macaques and marmosets (47, 48), will be extremely valuable in expanding our knowledge of the evolutionary roles of LinARs in these lineages. A high-throughput reporter assay system in cell lines would also be useful to investigate the functional types of these LinARs, i.e., to determine whether they play a role as enhancers or promoters. Although genetic modification remains challenging in most primate species, either due to technical obstacles or ethical reasons, the success in developing transgenic marmosets and rhesus monkeys has offered hope for further investigation of the biological roles of these LinARs during the developmental process of primates (49, 50). Last, our study has only covered 10% of extant primate species. The sequencing of high-quality reference genomes from more primate species will ultimately provide a complete picture of LinARs during primate diversification and should shed new light on how their alteration has helped to shape primate biodiversity.

MATERIALS AND METHODS

Whole-genome alignments

The soft masked versions of 50 mammalian genome assemblies, including 49 primates and the northern tree shrew (Tupaia belangeri), were used to construct pairwise genome alignments with the human genome as a reference using Lastz v.1.04.00 (51) (the parameter set ‘-step=19 -hsptresh=2200 -inner=2000 -ydrop=3400 -gapped-thresh=10000 -format=axt’ and a score matrix for the comparison of distantly related species). Twenty-seven primate genomes were newly generated in our accompanying paper. The multiple whole-genome alignments (WGA) of 50 mammals (50-way WGA) were constructed using the program Multiz v.11.2 (52).

Detection of evolutionarily conserved elements

Before identifying the conserved elements, we first filtered the above multiple alignments: At least 90% of the species were required to be present in the 50-way WGA. The hidden Markov model-based method implemented in phastCons (a package of PHAST v.1.5, ‘-msa-format MAF --branch --method LRT --mode CONACC’) in 18 major primate evolutionary nodes (orders and families) and 49 extant species. The acceleration scores were generated by mode “ACC” (‘-mode CONACC’) and were corrected to allow for multiple hypotheses using the false discovery rate (FDR; Benjamini and Hochberg) method (55). The significant acceleration at FDR-adjusted \( P \leq 0.05 \) was considered in further analyses (data S1). LinARs on the Y chromosome were removed. Annotation information for the IncRNAs and cCREs was obtained from RNAcentral (https://rnacentral.org/, release 19.0) (14) and the ENCODE database (https://encodeproject.org/) (15).

A lower total branch length of the phylogenetic tree may reduce the single base detection power, resulting in longer conserved elements. We found that some of the previously identified HARs were located in our HCE regions. However, in our phylpO test, owing to the longer conserved sequences used as input, these sites did not attain the threshold of accelerated evolution relative to that of a longer sequence background. Thus, we used phylpO to score these previous HARs in our 50-way WGA and found that 285 previous HARs were able to meet the threshold of significance (part of 620 overlapping human LinARs).

Classification of HCEs and human LinARs

We classified our HCEs and human LinARs in relation to vertebrates, sarcopterygians, amniotes, mammals, and primates using 100-way vertebrate alignment. First, phastCons was used to identify HCEs in vertebrates, sarcopterygians, amniotes, and mammals. Then, we overlapped our HCEs and human LinARs with 100-way HCEs from these four groups. The proportion of overlap was required to be more than 30%. Where our HCEs and human LinARs matched HCEs in only one taxon, we categorized these regions to that taxon. If they existed in two or more taxa, then we assigned them to their ancestral node (fig. S2). The set of 100-way vertebrate alignments for the human genome hg38 was downloaded from the UCSC website (http://hgdownload.cse.ucsc.edu/goldenPath/hg38/multiz100way/).

Human LinAR–related disease analysis

We analyzed our newly identified 1054 human LinARs in the context of human cCREs based on chromatin accessibility information generated by the ENCODE database (http://encodeproject.org). By using BEDTools v2.29.2 to find the intersections of two data sets (56), we identified 199 human LinARs overlapping marks of cCREs that were activated in human brain samples. Then, we mapped these LinARs to chromatin conformation data (HIC) from human fetal cortex (GEO accession: GSM2054568, https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSM2054568) (57) yielding 167 LinARs with chromatin interactions for 154 genes within the same topologically associating domains in the fetal cortex. We used the UCSC liftOver tool to

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convert genomic coordinates from Hg19 to Hg38 (https://genome.ucsc.edu/cgi-bin/hgLiftOver). Of the 122 (129 human LinARs) target genes corresponding to human inherited disease, 101 (105 human LinARs) genes were related to neurodevelopmental disorders recorded in the Human Gene Mutation Database (https://www.hgmd.cf.ac.uk).

Gene and tissue enrichment
To determine potential functions for LinARs in 15 major evolutionary nodes, we performed GO enrichment analysis on the Genomic Regions Enrichment of Annotations Tool (GREAT) website (http://great.stanford.edu/public/html/). The GREAT algorithm associates each human gene with a regulatory domain in the human genome (hg38) and calculates the total fraction of the genome annotated with GO terms. Statistical significance was assessed by means of the binomial test and hypergeometric test.

For tissue-type enrichment analysis, we classified human LinARs into specifically conserved primate ancestors or conserved mammalian ancestors according to whether the regions were conserved or not in the ancestral species. Regulatory elements were mapped to these accelerated regions, followed by a significant analysis of a number of overlaps. Cis-regulatory elements of humans were downloaded from the ENCODE database (https://screen.wenglab.org/) (15). P values were calculated by Fisher’s exact test and were adjusted for multiple comparisons using the FDR-controlling method of Benjamini and Hochberg in R software (p.adjust function, method = "fdr") (55).

In situ hybridization
Human RNA probes were constructed as so to correspond to five lncRNA sequences associated with human LinARs, and the RNA probes were labeled with biotin. First, the RNA probe sequence was designed. Then, the RNA probe and T7 transcript sequence were packaged into the plasmid, and the T7 transcript was used to reverse transcribe the RNA sequence in vitro, lastly combining the RNA sequence with biotin. The design and synthesis of RNA probes were completed by Shanghai MEIXUAN Biological Science and Technology Ltd. See table S3 for RNA probe sequences.

The human brain samples were derived from paraffin sections from the Li Jiali laboratory [Kunming Institute of Zoology, Chinese Academy of Sciences (CAS)], and all samples were taken from the cerebral cortices of ~70-year-old persons. Human autopsy paraffin-embedded 10-μm brain sections (tissue dissected from the motor cortex) from three individuals with no history of neurological or psychiatric disorders were used in this study. Tissues were graciously provided by the University of Pittsburgh Alzheimer’s Disease Research Center (ADRC) Brain Bank with approval from the internal review board of the Kunming Institute of Zoology, CAS. Additional frozen tissues were the generous gift of the ADRC at Washington University in St. Louis (Grant P50-AG-05681) with approval from the Ethics Committee of Kunming Institute of Zoology. Brain slices were used for ISH, and the work was performed at the Kunming Institute of Zoology, CAS being conducted under the regulations of the Human Biomedical Research Ethics Guidelines (regulated by the National Health Commission of the People’s Republic of China on 1 December 2016; approval number: 20170301). See table S4 for details of three case patients.

For hybridization, the brain slices were deparaffinized in xylene, hydrated with an alcohol gradient, and then washed in 0.1 M phosphate-buffered saline (PBS) for 10 min, 0.01 M glycine-PBS for 5 min, and 0.3% Triton X-100–PBS for 15 min, washed three times in 0.1 M PBS, and treated with proteinase K (1 μg/ml) for 30 min at 37°C. The slices were fixed in 4% paraformaldehyde (PFA) in PBS for 5 min, and then washed twice in 0.1 M PBS. The brain slices were then transferred to the prehybridization solution at 42°C for 30 min and incubated with the corresponding RNA probes at 42°C overnight. On the second day, the brain slices were first washed with hybridization buffer, and then with 4XSSC, 2XSSC, 1XSSC, 0.5XSSC, and 0.2XSSC at 37°C (20 min each time), and then washed with 0.1 M PBS: 0.2XSSC = 1:1 for 10 min at room temperature. After washing twice with 0.1 M PBS, the brain slices were incubated with streptavidin-Cy3 (Sigma-Aldrich, S6402) at a concentration of 1:200 in 0.3% Triton X-100–PBS for 3 hours at room temperature. Then, they were washed three times in 0.1 M PBS before adding 4′,6-diamidino-2-phenylindole (Invitrogen, P36931) and mounting the slide. Images were taken with a TissueFAXs cell analysis system (TissueGnostics GmbH, Austria).

LacZ in vivo reporter assay
BDF1 mouse (a cross between female C57BL/6NCrl and male DBA/2NCrl mice) care and experimental procedures were performed in compliance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Kunming Institute of Zoology, CAS (approval number: IACUC-RE-2021-12-001). Two gibbon LinARs were detected, which may be related to the development of bones, limbs, and tail. The two LinAR sequences (Rank148 and Rank181) of a gibbon were cloned into the Hsp68-lacZ reporter vector that was completed by the VectorBuilder platform. Transgenic mouse embryos were generated by pronuclear injection, and F0 embryos were collected at E11.5 and stained for LacZ activity (58). Before injection, plasmid DNA was linearized with XmnI or AhdI, followed by purification with the Gel Extraction Kit (Omega, D2500-01). BDF1 and Institute of Cancer Research (ICR) mouse strains were used as embryo donors and foster mothers, respectively. Superovulated female BDF1 mice (7 to 8 weeks old) were mated to BDF1 stud males, and fertilized embryos were collected from oviducts. The DNA was diluted to a final concentration of 2 ng/μl and injected into BDF1 zygotes, and HCZB medium was used for injection. The numbers of injected zygotes were 117 and 144, respectively. The injected zygotes were cultured in KSOM (four Yamana factors—OCT4, KLF4, SOX2, and MYC) culture medium at 37°C under 5% CO2 and 5% O2 in the air for approximately 15 hours. Thereafter, zygotes were transferred into the uteruses of ICR females. Embryos were harvested at embryonic day 11.5 in cold PBS, followed by 30 min of incubation with 4% PFA. The embryos were washed three times for 30 min with embryo wash buffer [2 mM MgCl2, 0.01% deoxycholate, 0.02% NP-40, and 0.1 M PBS buffer (pH 7.3)]. LacZ activity was detected by incubating with freshly made staining solution [1 mg/ml X-gal (Sigma-Aldrich, V900468), 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, and 5 mM EGTA (pH 7.5) in wash buffer] at 37°C for a few hours to overnight until the desired staining was achieved. Following staining, embryos were washed in lacZ buffer and stored at 4°C in 4% PFA in PBS. To be considered reproducible, bone, limb, and tail expression patterns had to be observed in at least three embryos (59). Embryos were imaged using a Nikon SMZ18 stereo microscope.
SOD1 gene expression

We downloaded RNA sequencing (RNA-seq) data from different tissues of *R. bieti* from the National Center for Biotechnology Information (NCBI) website (PRJNA248058). The genome and gene set data derived from *R. bieti* were also downloaded from NCBI (PRJNA339282, RefSeq assembly accession: GCF_001698545.2). We mapped the RNA-seq reads for each tissue to the reference genome using HISAT2 (https://github.com/infphilo/hisat2, version maintained in a 5% CO2 environment). Dual-luciferase reporter experiments

HEK293T cell lines were cultured in Dulbecco's modified Eagle's medium. All media were supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies BRL), and the cell lines were maintained in a 5% CO2-humidified atmosphere at 37°C.

HEK293T cells were seeded in the wells of a 24-well plate at a density of 1 x 10^4 cells per well 1 day before transfection. The transfection reagent ViaFect (E4981, Promega) was used according to the manufacturer's instructions. A Renilla-luciferase plasmid (PRL-TK) was cotransfected to control for transfection efficiency. SOD1-pGL3-Basic (SOD1 promoter sequences from humans, *Macaca*, and *Rhinopithecus*), and PRL-TK were then cotransfected at a total concentration of 500 ng per well (4:1 molar ratio of SOD1-pGL3-Basic and PRL-TK). Luciferase assays were performed 48 hours after transfection using the Dual-Glo Luciferase Assay System (E2920, Promega) according to the manufacturer's instructions.

Supplementary Materials

This PDF file includes:
Figgs. S1 to S11
Tables S1 to S4
Legends for data S1 to S6

Other Supplementary Material for this manuscript includes the following:
Data file S1 to S6

View/request a protocol for this paper from Bio-protocol.

REFERENCES AND NOTES


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