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ORIGINAL ARTICLE

Comparative transcriptome analysis reveals molecular adaptations underlying distinct immunity and inverted resting posture in bats

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Abstract

Understanding how natural selection shapes unique traits in mammals is a central topic in evolutionary biology. The mammalian order Chiroptera (bats) is attractive for biologists as well as the general public due to their specific traits of extraordinary immunity and inverted resting posture. However, genomic resources for bats that occupy key phylogenetic positions are not sufficient, which hinders comprehensive investigation of the molecular mechanisms underpinning the origin of specific traits in bats. Here, we sequenced the transcriptomes of 5 bats that are phylogenetically divergent and occupy key positions in the phylogenetic tree of bats. In combination with the available genomes of 19 bats and 21 other mammals, we built a database consisting of 10 918 one-to-one ortholog genes and reconstructed phylogenetic relationships of these mammals. We found that genes related to immunity, bone remodeling, and cardiovascular system are targets of natural selection along the ancestral branch of bats. Further analyses revealed that the T cell receptor signaling pathway involved in immune adaptation is specifically enriched in bats. Moreover, molecular adaptations of bone remodeling, cardiovascular system, and balance sensing may help to explain the reverted resting posture in bats. Our study provides valuable transcriptome resources, enabling us to tentatively identify genetic changes associated with bat-specific traits. This work is among the first to advance our understanding of the molecular underpinnings of inverted resting posture in bats, which could provide insight into healthcare applications such as hypertension in humans.

Key words: adaptation, bats, immunity, resting posture, transcriptome

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INTRODUCTION

During the evolutionary processes, mammals develop specialized features for adaptation to new environments. For instance, mole rats developed sharper incisors, enhanced olfaction, and elongated bodies to survive

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the harsh underground conditions (Heffner & Heffner 1993; Stathopoulos *et al.* 2014). Similarly, specialized characters such as increased lung capacity, streamlined body, and thickened skins have been shaped in dolphins to adapt to their aquatic lifestyle (Fish & Hui 1991; Reidenerg 2007). These highly specialized groups provide valuable opportunities to investigate the genetic changes underlying the development of specific traits, a central topic in evolutionary biology. The order Chiroptera, commonly called bats, comprised approximately 20% of all living mammals (Simmons 2005; Lu *et al.* 2021). Bats are widely distributed and can be found on every continent except Antarctica (Simmons 2005; Ramírez-Fráncel *et al.* 2022). This wide distribution is largely due to their specialized features, which include unique immunity, capacity for powered flight, complex social behavior, and possibly inverted resting posture (Zhang *et al.* 2013; Banerjee *et al.* 2020; Jebb *et al.* 2020; Scheben *et al.* 2020; Gutiérrez *et al.* 2021; Sun *et al.* 2021a).

Bats are well known for their strong immune defense systems that allow them to coexist with viruses (Irving *et al.* 2021; Santillán *et al.* 2021). Moreover, bats could serve as virus reservoirs for pathogenic viruses, including Hendra and Nipah viruses, Marburg virus, and SARS-like coronaviruses (Li *et al.* 2005; Calisher *et al.* 2006; Yan *et al.* 2021). During their long-term coexistence with viruses, bats may have evolved effective systems to defend them.

In addition, the vast majority of bats rest in an inverted position; they remain upside-down, which has been a mystery and attracted biologists for centuries. This upside-down position could help bats avoid predators effectively; however, the biological and physiological innovations of this behavior are complex. First, powerful hook-like claws are one modification, which are attached to the upper body via specialized tendons that enable the claws to lock tightly (Hall & Richards 2000). Second, since the upside-down position will increase blood pressure to the head, bats evolved a relatively large heart and more efficient cardiovascular system to avoid damage from this high blood pressure (Currie 2018). Finally, bats do not lose balance from hanging upside-down; instead, they have evolved to maintain their balance and negate the effects of gravity effectively (Altringham 1996).

Therefore, bats may offer us an excellent model to study disease pathology and cardiovascular disorders. Although many bat genomes have been released recently (Zhang *et al.* 2013; Pavlovich *et al.* 2018; Jebb *et al.* 2020), genomic resources of bats occupying key positions in their phylogeny are still limited. For example, the genomic data of the black-bearded tomb bat (*Taphozous melanopogon*) is absent, which is located at the basal position of the Yangochiroptera, 1 of the 2 suborders in Chiroptera. This limitation hinders comprehensive investigation of molecular mechanisms underlying the development of specialized traits in bats. Furthermore, there is currently no report on molecular evolution of the upside-down resting posture in bats.

To investigate the genetic mechanisms associated with the development of bat-specific traits such as immunity and the inverted resting posture, we sequenced the transcriptomes of 5 phylogenetically distinct bat species. Combined with the publicly available genomic data of 19 other bat species, our dataset encompasses all main basal lineages of bats (Fig. 1). First, we reconstructed the one-to-one ortholog gene dataset between bats and other mammals. Next, we performed comparative analysis to uncover molecular adaptations underlying bat-specific traits such as their extraordinary immunity and inverted resting posture. Our study provides valuable transcriptome resources for bats and also represents the first study to explore molecular mechanisms that underlie the origin of inverted resting posture in bats. Our results could also provide insight into healthcare applications, such as the study of hypertension in humans.

MATERIALS AND METHODS

Transcriptome sequencing and assembly

Five bats from phylogenetically distant clades were selected for transcriptome sequencing (Fig. 1). Total RNA was obtained from pooled samples of 7 tissues from each bat that were equally mixed (brain, liver, heart, muscle, tongue, kidney, and lung), and used for paired-end Illumina sequencing (Table S1, Supporting Information). Following sequencing, raw reads were trimmed if they contained adaptors, primers, low-quality reads, and unknown nucleotides. *De novo* assembly of the transcriptomes for each bat was performed using Trinity (trinityrnaseq-2.0.6) with default settings (Grabherr *et al.* 2011). TransDecoder (implemented in Trinity) was used to extract potential coding regions from the assembled transcripts. The longest transcript was identified and used in the following analysis. For comparative purpose, we combined our data with available genomes of 19 representative bats (Table S2, Supporting Information), such that our dataset covers all basal clades of Chiroptera (Fig. 1).

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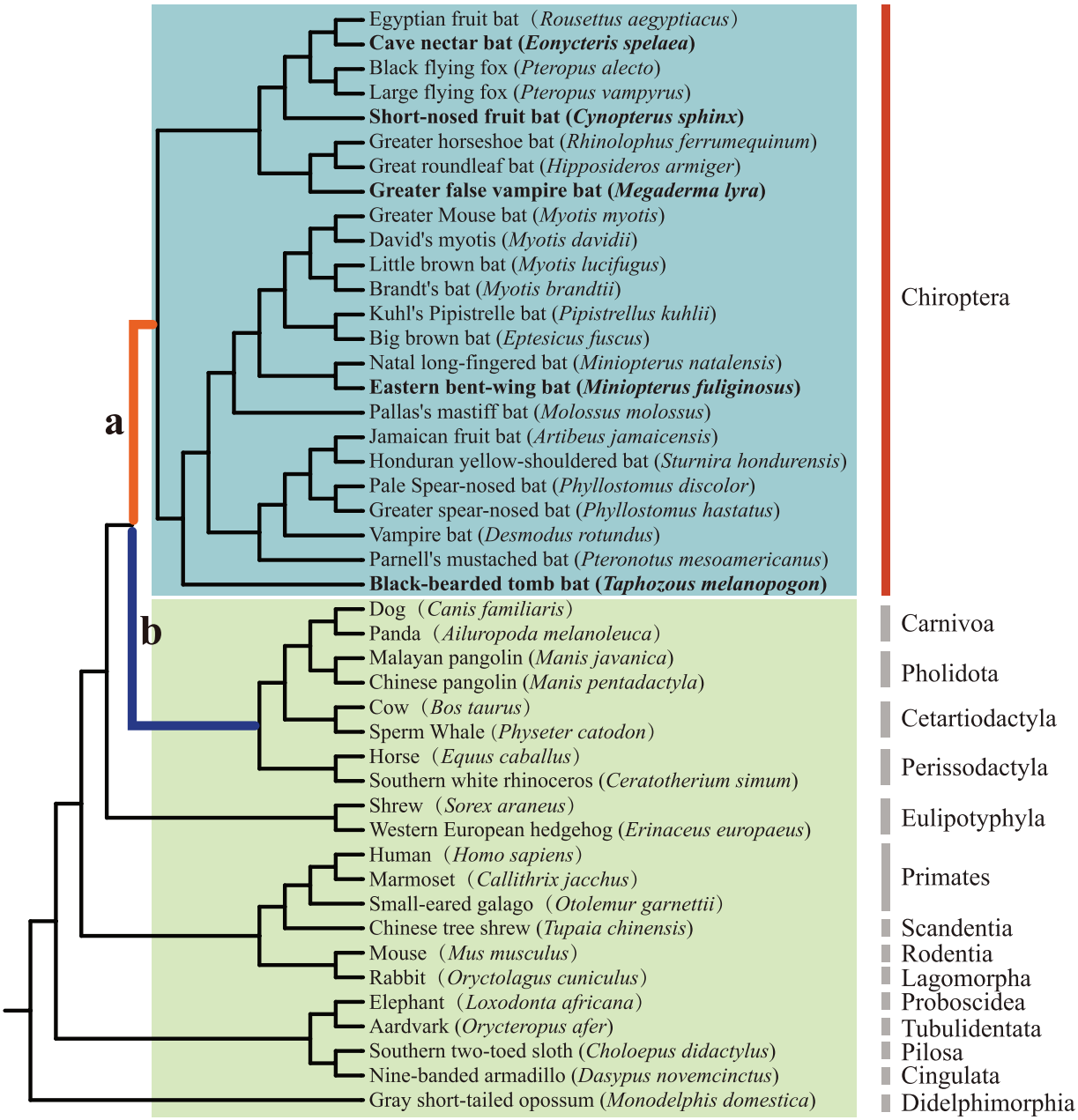

Figure 1 Phylogenetic tree reconstructed in this study. The phylogenetic tree was reconstructed with the coalescent method. Bootstrap values for each node of the tree can be seen in Fig. S2, Supporting Information. The red branch labeled with "a" represents the ancestral branch of bats; the blue branch labeled with "b" indicates the close relative of the common ancestor of bats, which was used as the control group. Newly sequenced species in the present study are marked in bold.

Identification of one-to-one ortholog genes and alignment construction

To obtain one-to-one ortholog genes, we used the reciprocal best hit method. The protein sequences of the human genome were used as a reference and to conduct a reciprocal blast with sequences of each species (Tables S2,S3, Supporting Information). The resulting ortholog clusters were further checked to determine if they met the following criteria: at least 3 Yinpterochiroptera

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bats, 5 Yangochiroptera bats, and 10 outgroup species. Following ortholog identification, the corresponding sequences for these orthologs were extracted with an in-house script. For each ortholog, multiple fasta files consisting of a maximum of 45 species were generated. All putative protein orthologs were aligned with PRANK (v.150 803) (Löytynoja & Goldman 2005). Alignments of the coding sequences were generated with pal2nal under default parameters (Suyama *et al.* 2006). Following alignment, GBLOCKS (version 0.91b) was employed to remove poorly aligned regions (Castresana 2000); aligned sequences were discarded if their length was shorter than 150 bp. In total, 10 918 one-to-one orthologs were identified for 24 bats and 21 other mammals.

Phylogenetic reconstruction

We reconstructed phylogenetic relationships of bats as previously described (Wang *et al.* 2020). Specifically, for the 1689 one-to-one orthologs, including all 45 species, the coding sequences were used as input for tree reconstruction. First, all 1689 genes were concatenated to construct the "supergene" set used to construct a maximum likelihood tree with RAxML-NG version 1.1.0 (Kozlov *et al.* 2019) under the GTR + I + G4 model suggested by ModelTest-NG (Darriba *et al.* 2019). Second, to reveal the phylogeny heterogeneity at the gene level, a maximum-likelihood tree for each gene was constructed with RAxML-NG. Third, a species tree was built with these gene trees using the coalescence method, using both MP-EST version 2.0 (Yu & Nakhleh 2015) and ASTRAL version 5.7.7 (Zhang *et al.* 2018).

Detection of genes under natural selection

To detect genes under natural selection along the ancestral branch of bats, we used the CODEML program in the PAML package (version 4.9) (Yang 2007) following a previous study (Zou *et al.* 2021), with the ancestral branch of bats setting as the foreground branch. First, the branch-site model was used to detect positively selected genes (PSGs). The PSGs were identified by comparing an alternative model assuming sites to be under positive selection on the foreground branch with a null model assuming no signals of positive selection. Next, we ran the branch model to detect rapidly evolving genes (REGs) using the same orthologous gene sets as aforementioned. We tested an alternative 2-ratio model allowing 2 different ω values in the foreground branch and background branch, respectively, and a null model assuming the same ω value in all branches. The tree topology used for the selection tests is shown in Fig. 1. In both models, likelihood ratio tests were used to calculate *P*-values. The genes with a $P < 0.05$ were considered as PSGs or REGs, respectively. Next, we performed the gene enrichment analyses for each of the 2 datasets of PSGs and the combined set of PSGs and REGs, respectively, using KOBAS-i (Bu *et al.* 2021). Pathways or GO terms were considered significant if their adjusted *P*-values were less than 0.05.

Adaptive evolution of the immune system in bats

In order to further understand our enrichment results, 103 genes in the T cell receptor signaling pathway (hsa04660) were retrieved from the KEGG database and used as a reference. These genes were then checked against our ortholog dataset, which led to the identification of 62 genes. For these genes, we first checked whether they possess a positive selection signal along the ancestral branch of bats and its control group (sister branch of the common ancestor of bats, branch b in Fig. 1) to test whether immune genes in the common ancestor of bats experienced more selection pressure than its counterpart. Moreover, we checked if other genetic changes including REGs and bat-specific mutations were available for the ancestral branch of bats.

Identification of bat-specific amino acid changes

Amino acid changes may influence the function of a protein. In addition to those genes that were found to be under selection, we searched for bat-specific amino acid changes. The bat-specific amino acids were determined by the method described in a recent work (Chen *et al.* 2019). Specifically, for each protein sequence, an amino acid site was assumed as bat-specific if this amino acid is the same in bat clade but different from that in the outgroups. Furthermore, we checked whether the mutations were located in the functional domain region of the protein by searching against Pfam (version 33) (Mistry *et al.* 2020) or InterPro (https://www.ebi.ac.uk/interpro/). The protein structure for those genes with specific amino acid changes was predicted by the Phyre2 server (Kelley *et al.* 2015).

Examination of balance-keeping and gravity-sensitive related genes

To characterize molecular adaptations underlying the upside-down resting posture in bats, we first collected

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the "balance-sensitive" genes lists. In addition to the gravity and balance sensation process, the vestibular and otoconia are also important for the sensation of gravity and balance. We, therefore, identified candidate mammalian "balance-sensitive" genes based on annotations with the GO terms, including the key words "balance," "equilibrioception," "gravity," "vestibular," "otolith," or "otoconia" from the .obo file derived from the Gene Ontology Consortium. Following the collection of these candidate GO terms and manual examination, we retrieved all genes in these terms from AmiGO2 (http://amigo.geneontology.org/, last accessed, 2022-7-1). In total, 60 candidate genes were retrieved and used for further analysis (Table S4, Supporting Information). For these candidate genes, we checked whether they were PSGs, REGs, or genes possessing bat-specific mutations. Moreover, to have a deeper understanding of functional clusters of these candidate genes, visualization of protein–protein interaction networks was performed using the STRING (v11.5) database (Szklarczyk *et al.* 2019) available at http://string-db.org/ (last accessed 2021-11-20). We tested all putative "balance-sensitive" genes and estimated their interaction scores. We retrieved combined interaction scores based on the following lines of evidence: neighborhood, gene fusion, co-occurrence, co-expression, experimental knowledge, and text mining. The resulting networks were visualized with Cytoscape (Shannon *et al.* 2003).

RESULTS

Sampling and ortholog cluster identification

We sequenced the transcriptomes of 5 phylogenetically distinct bats to identify the genetic changes associated with the development of bat-specific traits (Fig. 1 and Tables S1,S2, Supporting Information). For comparative purpose, we combined our transcriptome data with publicly available genomes of 19 other bat species (Fig. 1 and Table S1, Supporting Information), along with the genomes of 21 representative mammals from publicly available databases (Table S3, Supporting Information).

To obtain one-to-one orthologs, reciprocal blast searches were performed using human proteins as a reference. A total of 18 787 ortholog clusters were acquired. Candidate ortholog clusters were then filtered by representative species number and sequence length, which resulted in 10 918 ortholog sequences containing a maximum of 45 species.

Phylogenetic reconstruction

To uncover the molecular origin of bat-specific traits, it is critical to understand the phylogenetic position of bats within the Laurasiatheria, a long-standing and unresolved question (Foley *et al.* 2016; Doronina *et al.* 2017). Using the 1689 one-to-one orthologs among all 45 species, we reconstructed the phylogenetic relationship for Chiroptera. The concatenation methods supported Perissodactyla as the sister clade to the group of Chiroptera (Fig. S1, Supporting Information). However, considering the phylogeny heterogeneity at the gene level, the maximum-likelihood tree for each gene was constructed and then a species tree was built using the coalescence-based method of MP-EST and ASTRAL. Both methods revealed that Chiroptera is the sister clade to the group of (Perissodactyla + [Cetartiodactyla + Carnivora + Pholidota]) (Fig. 1; Figs S2,S3, Supporting Information). Ultimately, we adopted the phylogenetic tree from the coalescent method, which is more robust than the tree topology estimated from the concatenation method (Xi *et al.* 2014).

Molecular adaptations of the immune system in bats

To characterize molecular adaptations along the ancestral branch of bats, we evaluated the adaptive changes between the common ancestor of bats and other mammals using both the branch-site model and branch model implemented in PAML. A total of 233 and 682 genes were detected as PSGs and REGs, respectively (Tables S5,S6, Supporting Information). First, focusing on the PSGs, we evaluated the positive selection strength between the ancestral branch of bats and its control group. The enrichment results derived from PSGs showed that 2 immunity-related pathways of "Amoebiasis" and "T cell receptor signaling pathway" were distinguished in the ancestral branch of bats (Tables S7,S8, Supporting Information). Considering that the T cell receptor signaling pathway is vital and essential for efficient T cell development, activation, and immune tolerance (Shah *et al.* 2021), we therefore turned to the T cell receptor signaling pathway. Of the 62 genes examined in this pathway, 4 (6.5%) showed evidence of positive selection in the ancestral branch of bats (compared to zero in the control group), which is significantly higher than the background level of positive selection across the genome ($P < 0.05$) (Fig. 2a; Table S7, Supporting Information). The 4 PSGs comprise *NCK2*, playing a crucial role in shaping the T-cell repertoire and optimal T cell excitability (Roy *et al.* 2010), and *IFNG*, critical for the regulation of memory T cell differentiation (Sercan *et al.* 2010).

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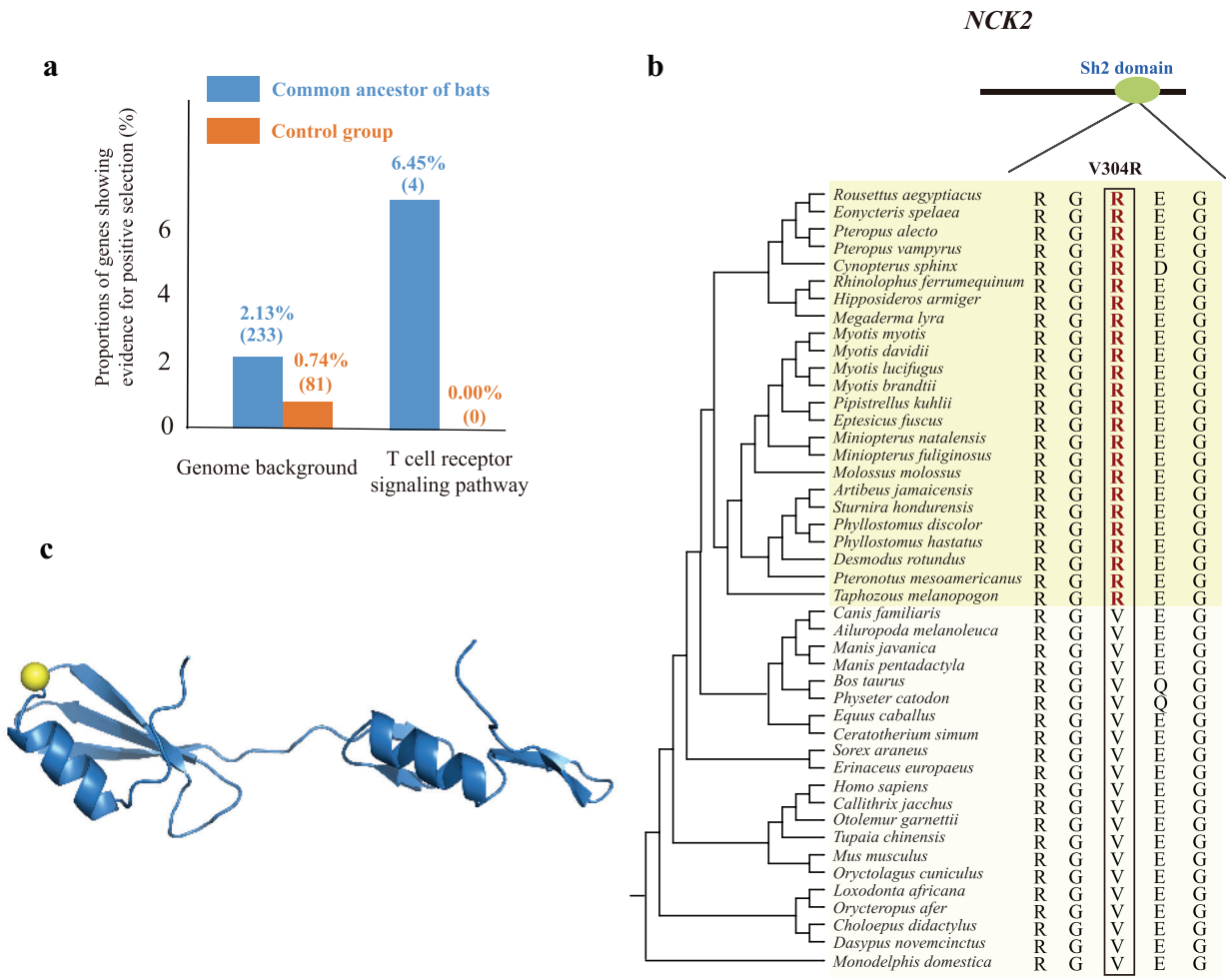

Figure 2 Adaptive changes in the immune system of bats. (a) Signals of positive selection in the common ancestor of bats and the control group. The numbers of positively selected genes are given in parentheses. (b) The bat-specific mutation (V304R) of *NCK2* is shown. (c) The 3-dimensional structure of the protein NCK2 in *Rousettus aegyptiacus*, with the mutation R304-colored in yellow and shown as a sphere.

We further found that the specific mutation in *NCK2* is located within the functional domain Sh2 (Fig. 2b,c); hence, they may affect functional activities. Moreover, to further understand adaptive changes in this pathway, we also identified 3 REGs and 3 genes exhibiting bat-specific mutations (Fig. 3). This evidence suggests that adaptive changes in the T cell receptor signaling pathway may contribute to the evolution of unique immunity in bats.

In addition to the T cell receptor signaling pathway, gene enrichment analyses based on 855 PSGs and REGs showed that these genes are primarily related to immunity, including both innate and adaptive immunity. While functional categories such as the "toll-like receptor signaling pathway" and "activation of innate immune response" (Fig. 4 and Table S9, Supporting Information) are involved in innate immunity, many GO terms and pathways involved in adaptive immunity were also identified, including the "B cell activation," "T cell activation," and "response to cytokine" (Fig. 4). These results suggest an important role of these functional processes in the unique immune system of bats.

Molecular adaptations underlying the inverted resting posture in bats

A specialized tendon and a powerful hook-like claw enable bats to hang upside-down for a long period; we thus hypothesized that genes related to bone development and morphological innovation should be the targets of

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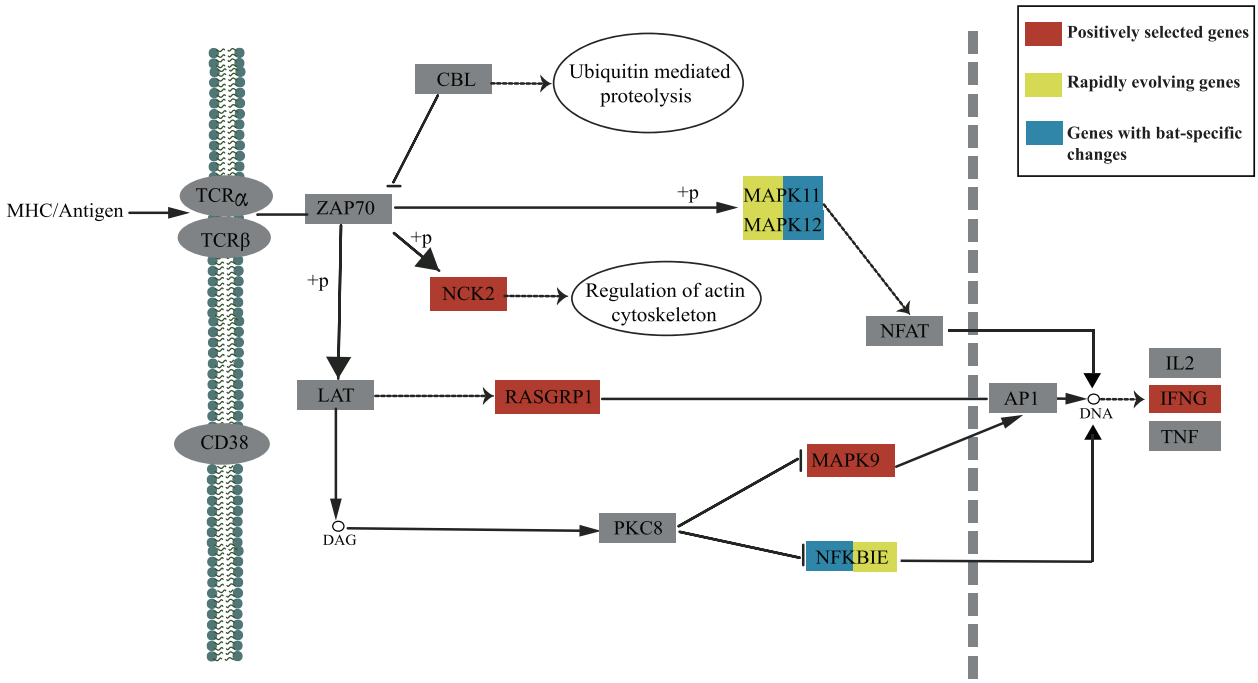

Figure 3 Diagram of the T cell receptor signaling pathway in bats. The positively selected genes are marked in red, rapidly evolving genes are marked in yellow, and genes with bat-specific changes are marked in blue.

natural selection. Indeed, enrichment terms associated with bone mineralization, such as "structural constituent of cytoskeleton" and "fibroblast migration" were identified (Fig. 4 and Table S9, Supporting Information). Genes involved in these terms included *S1PR1*, which functions in the migration of osteoclast precursor cells that regulate bone-resorbing capacity (Ishii *et al.* 2009), and *OSTF1*, knockout of which shows an increased bone mass in mouse (Vermeren *et al.* 2017), as well as *EPHA2*, which regulates bone remodeling at the initiation phase (Irie *et al.* 2009). Thus, the enrichment of bone-related functional categories may underlie the morphological innovation responsible for upside-down resting posture in bats.

In addition, many enriched terms and pathways associated with the cardiovascular system were recorded, for example, "regulation of cardiac conduction" and "heart looping" (Fig. 4 and Table S9, Supporting Information). Genes belonging to the cardiovascular category include *CASQ1*, knock down of which could lead to faster basal heart rate in mouse (Sun *et al.* 2021b). Other genes included *TBX1*, which contributes to mediating the morphogenesis of the cardiac outflow tract (Xu *et al.* 2004), and *ACE2*, which regulates the heart renin-angiotensin system (Donoghue *et al.* 2000), as well as *FGFRL1*, which shows exceptional hypertension resistance in giraffes (Liu *et al.* 2021). These genetic changes may equip bats with a highly efficient cardiovascular system to avoid damage from high blood pressure while resting upside-down.

Interestingly, bats do not become dizzy from hanging upside-down. Therefore, specialized balance and gravity sensing systems could be the targets of natural selection. In our analysis, one REG (*GCH1*) was annotated to the GO term "neuromuscular process controlling posture." To further understand how bats maintain balance and negate the effects of gravity effectively, we screened 60 "gravity-sensitive" genes and identified 3 REGs and 10 genes possessing bat-specific mutations. Finally, we performed the protein–protein interaction network and found the genes with signals of natural selection were centered in different clusters of the interaction networks based on all sources of evidence (Fig. 5).

DISCUSSION

Although efforts have been made toward understanding the mechanisms underlying bat-specific traits, knowledge of the genetic changes associated with the development of bat-specific traits is still limited (Teeling *et al.* 2018; Jiao *et al.* 2019, 2021; Jebb *et al.* 2020). One possible reason for this is that the genomes of bats occupying key positions of the phylogeny are still limited. Here, we

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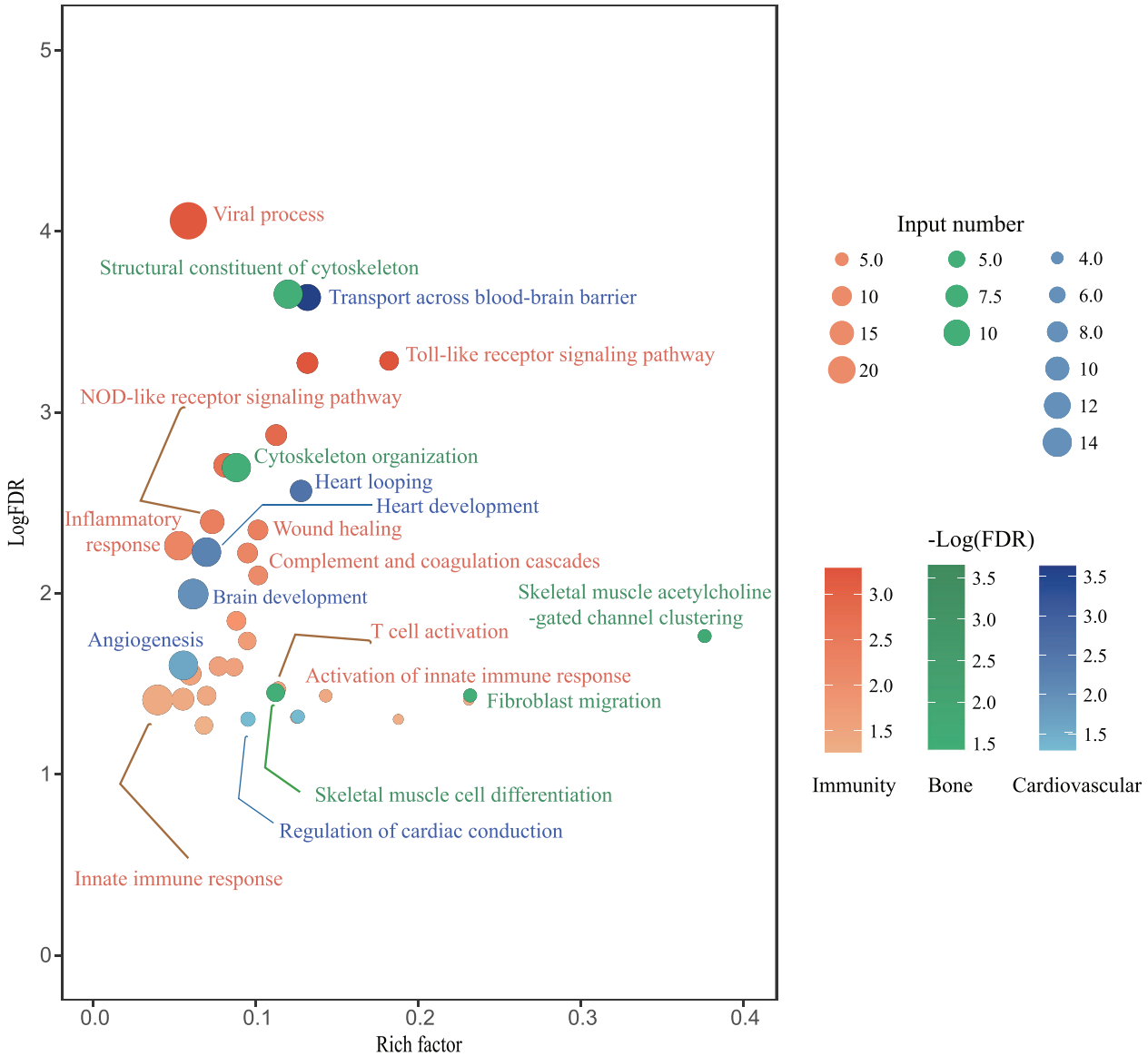

Figure 4 Scatterplots of enriched GO terms and KEGG pathways for genes under selection. Three functional categories of cardiovascular system, bone development, and immunity are shown. Rich factor is the ratio of the number of genes under selection to the number of total genes in a specific pathway. The dot size is scaled to the gene number and the dot color represents the -$\log_{10}$ (corrected *P*-value) value. Input number is the total number of PSGs and REGs that could be annotated to a specific GO term or KEGG pathway.

sequenced the transcriptomes from 5 bats that are phylogenetically important, and combined these with high-quality genomes of other 19 representative bat species to conduct a comparative analysis. Thus, our data encompassed all main basal clades of bats. Through comparative genomic analysis, we built an ortholog gene dataset, which revealed that genes under selection are involved in immunity, cardiovascular system, and bone remodeling. Together, these may contribute to molecular adaptations underlying bat-specific traits.

Bats are able to coexist with diverse viral pathogens and are thus an emerging model for studying disease tolerance (Calisher *et al.* 2006; Towner *et al.* 2009). In this study, the signal of natural selection posed on the immune system in bats is evident. First, genes under selection were significantly enriched in immunity,

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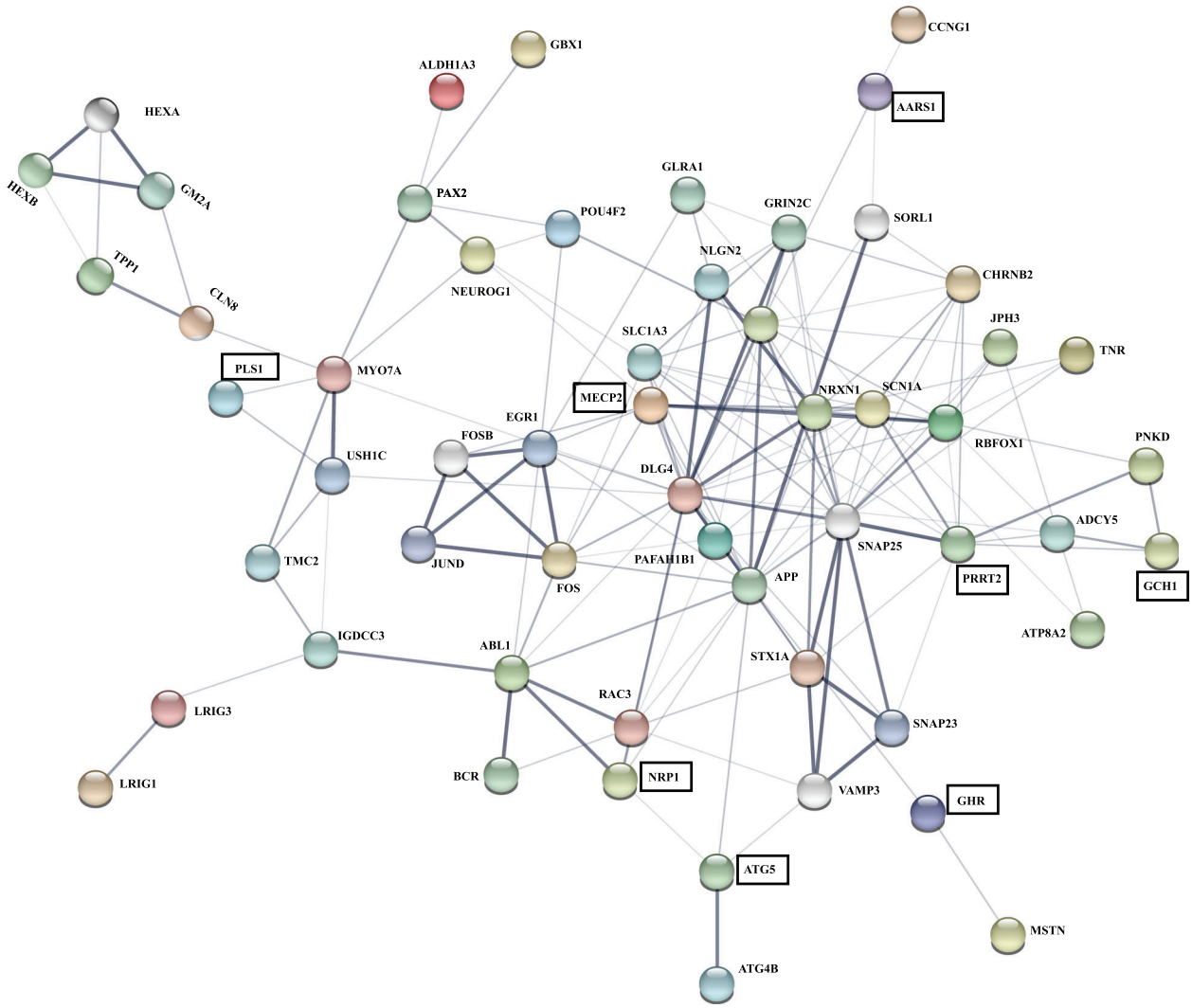

Figure 5 Protein–protein interaction network of the 60-candidate mammalian "balance-sensitive" genes. Node and edge width corresponds to the confidence of the interaction, with a wider line indicating stronger evidence. Evidence is based on the following scores: neighborhood score, fusion score, co-occurrence score of the phyletic profile, homology score, co-expression score, experimental score, database score, and text mining score. Boxed protein names indicate adaptive changes.

including both innate and adaptive immunity. Within innate immunity, 2 types of humoral immunity and cell-mediated immunity were recognized. For instance, in the aspect of humoral immunity, "complement and coagulation cascades" and "complement activation, alternative pathway" were distinguished. While in the cell-mediated immunity, the "neutrophil degranulation," "toll-like receptor signaling pathway," and "NOD-like receptor signaling pathway" were identified (Fig. 4 and Table S9, Supporting Information). For adaptive immunity, both forms of humoral immunity and cell-mediated immunity were also recorded. For example, in humoral immunity, the "B cell activation" and its activator of "antigen processing and presentation of exogenous peptide antigen via MHC class II" were found, and "T cell activation" as well as "response to cytokine" was identified in the cell-mediated immunity (Fig. 4 and Table S9, Supporting Information). Genes from these categories included *RASGRP1*, which is critical for T cell development, homeostasis, and differentiation (Priatel *et al.* 2002) and *c-REL*, which could serve as regulator for B cell and T cell division (Morrison 1980). Notably, this gene was also found to be under strong selection pressure in bats in a previous study (Zhang *et al.* 2013). Second, we found that

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immune-related genes experienced more selection pressure in bats' ancestor than in its close relative such as those in the T cell receptor signaling pathway (Fig. 2). Therefore, our study provided evidence of how natural selection shapes the unique immune system in bats. Despite these important observations, some inconsistencies were found when compared with previous studies. For example, the NF-kappa B pathway (Zhang *et al.* 2013) was significantly distinguished and hence hypothesized to relate to the origin of immunity in bats. However, the NF-kappa B signaling pathway was not significantly enriched in our study (FDR = 0.40). Instead, other immune-related pathways were significantly enriched, such as the T cell receptor signaling pathway and bacterial invasion of epithelial cells. This discrepancy may arise from methodological differences, such as ortholog identification and taxa sampling. Alternatively, these findings may indicate that the evolution of immunity in bats is complex and may involve a complicated regulation network.

Bats are also well known for their upside-down resting posture. This innovation, combined with both the morphological and physiological adaptation, likely involves many molecular adaptations: For example, the powerful claw, in which the tendons are well suited to enable the claws to lock tightly, along with the hook-like shape of the claw itself. We hypothesized this morphological innovation may be shaped by the genes that regulate bone remodeling. Indeed, we found evidence of positive selection and enrichment of functional categories for many bone-related genes (Fig. 4 and Tables S5,S6, Supporting Information). In comparison, previous studies have also used comparative genomics to identify bone-related genes in relation to morphological modifications. For example, bone development genes have been successfully identified and shown to be important for pseudo-thumb development in the panda (Hu *et al.* 2017) and the origin of hollow air-filled structure in birds (Machado *et al.* 2016). Therefore, the genes with signals of adaptive evolution identified in this study are valuable candidates, which require functional experiments for validation in the future, and have potential to explain the claw modification in bats.

In addition to the findings for the morphological innovation in bats, we detected evidence for physiological adaptations. The first line of evidence was that genes such as *CASQ1*, *TBX1*, and *ACE2* were identified as the targets of natural selection, and they were also enriched in the cardiovascular category of "regulation of cardiac conduction" and "heart looping" (Fig. 4 and Tables S5,S6, Supporting Information). These genes may help bats evolve an efficient cardiovascular system. Notably, bats have the largest relative heart size among all mammals (Canals *et al.* 2005). This large size could allow powerful cardiac muscle contraction, allowing the blood to flow back to the heart to avoid imposing high blood pressure on the brain. Moreover, bats do not become dizzy from hanging upside-down. Among the genes under selection, some were enriched in the GO term of "neuromuscular process controlling balance." Further analysis of the 60 "balance-sensitive" genes (Table S4, Supporting Information) showed that 3 REGs and 10 genes with bat-specific mutations were identified. Our protein–protein interaction network analysis revealed that these adaptive genes were centered in different clusters (Fig. 5). Although their specific functions in maintaining balance still await experimental validation, our findings provide the first genetic evidence of how bats keep balance and negate the effects of gravity effectively.

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CONFLICT OF INTEREST

The authors declare that they have no competing financial interests.

REFERENCES

Altringham JD (1996). *Bats: Biology and Behaviour*. Oxford University Press, New York.

Banerjee A, Baker ML, Kulcsar K, Misra V, Plowright R, Mossman K (2020). Novel insights into immune systems of bats. *Frontiers in Immunology* **11**, 26.

Bu D, Luo H, Huo P *et al.* (2021). KOBAS-i: Intelligent prioritization and exploratory visualization of biological functions for gene enrichment analysis. *Nucleic Acids Research* **49**, W317–25.

Calisher CH, Childs JE, Field HE, Holmes KV, Schountz T (2006). Bats: Important reservoir hosts of emerging viruses. *Clinical Microbiology Reviews* **19**, 531–45.

Chen L, Qiu Q, Jiang Y *et al.* (2019). Large-scale ruminant genome sequencing provides insights into

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their evolution and distinct traits. *Science* **364**, eaav6202.

Canals M, Atala C, Grossi B, Iriarte-Díaz J (2005). Relative size of hearts and lungs of small bats. *Acta Chiropterologica* **7**, 65–72.

Castresana J (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* **17**, 540–52.

Currie SE (2018). No effect of season on the electrocardiogram of long-eared bats (*Nyctophilus gouldi*) during torpor. *Journal of Comparative Physiology B* **188**, 695–705.

Darriba D, Posada D, Kozlov AM, Stamatakis A, Morel B, Flouri T (2019). ModelTest-NG: A new and scalable tool for the selection of DNA and protein evolutionary models. *Molecular Biology and Evolution* **37**, 291–4.

Donoghue M, Hsieh F, Baronas E *et al.* (2000). A novel angiotensin-converting enzyme–related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1–9. *Circulation Research* **87**, e1–9.

Doronina L, Churakov G, Kuritzin A et al. (2017). Speciation network in Laurasiatheria: retrophylogenomic signals. *Genome Research* **27**, 997–1003.

Fish FE, Hui CA (1991). Dolphin swimming–A review. *Mammal Review* **21**, 181–95.

Foley NM, Springer MS, Teeling EC (2016). Mammal madness: Is the mammal tree of life not yet resolved? *Philosophical Transactions of the Royal Society B* **371**, 20150140.

Grabherr MG, Haas BJ, Yassour M *et al.* (2011). Trinity: Reconstructing a full-length transcriptome without a genome from RNA-Seq data. *Nature Biotechnology* **29**, 644–52.

Gutiérrez EG, Vivas-Toro I, Carmona-Ruíz D *et al.* (2021). Socio-spatial organization reveals paternity and low kinship in the Honduran white bat (*Ectophylla alba*) in Costa Rica. *Integrative Zoology* **16**, 646–58.

Hall L, Richards G (2000). *Flying Foxes: Fruit and Blossom Bats of Australia*. UNSW Press, Sydeny.

Heffner RS, Heffner HE (1993). Degenerate hearing and sound localization in naked mole rats (*Heterocephalus glaber*), with an overview of central auditory structures. *Journal of Comparative Neurology* **331**, 418–33.

Hu Y, Wu Q, Ma S *et al.* (2017). Comparative genomics reveals convergent evolution between the bamboo-eating giant and red pandas. *PNAS* **114**, 1081–6.

Irie N, Takada Y, Watanabe Y *et al.* (2009). Bidirectional signaling through ephrinA2-EphA2 enhances osteoclastogenesis and suppresses osteoblastogenesis. *Journal of Biological Chemistry* **284**, 14637–44.

Irving AT, Ahn M, Goh G *et al.* (2021). Lessons from the host defences of bats, a unique viral reservoir. *Nature* **589**, 363–70.

Ishii M, Egen JG, Klauschen F *et al.* (2009). Sphingosine-1-phosphate mobilizes osteoclast precursors and regulates bone homeostasis. *Nature* **458**, 524–8.

Jebb D, Huang Z, Pippel M *et al.* (2020). Six reference-quality genomes reveal evolution of bat adaptations. *Nature* **583**, 578–84.

Jiao H, Zhang L, Xie HW *et al.* (2019). Trehalase gene as a molecular signature of dietary diversification in mammals. *Molecular Biology and Evolution* **36**, 2171–83.

Jiao H, Xie HW, Zhang L *et al.* (2021). Loss of sweet taste despite the conservation of sweet receptor genes in insectivorous bats. *PNAS* **118**, e2021516118.

Kelley LA, Mezulis S, Yates CM, Wass MN, Sternberg MJ (2015). The Phyre2 web portal for protein modeling, prediction and analysis. *Nature Protocols* **10**, 845–58.

Kozlov AM, Darriba D, Flouri T, Morel B, Stamatakis A (2019). RAxML-NG: a fast, scalable and user-friendly tool for maximum likelihood phylogenetic inference. *Bioinformatics* **35**, 4453–5.

Li WD, Shi ZL, Yu M *et al.* (2005). Bats are natural reservoirs of SARS-like coronaviruses. *Science* **310**, 676–9.

Liu C, Gao J, Cui X *et al.* (2021). A towering genome: Experimentally validated adaptations to high blood pressure and extreme stature in the giraffe. *Science Advances* **7**, eabe9459.

Löytynoja A, Goldman N (2005). An algorithm for progressive multiple alignment of sequences with insertions. *PNAS* **102**, 10557–62.

Lu Q, Jiao H, Wang Y, Norbu N, Zhao H (2021). Molecular evolution and deorphanization of bitter taste receptors in a vampire bat. *Integrative Zoology* **16**, 659–69.

Machado JP, Johnson WE, Gilbert MTP *et al.* (2016). Bone-associated gene evolution and the origin of flight in birds. *BMC Genomics* **17**, 371.

Mistry J, Chuguransky S, Williams L *et al.* (2020). Pfam: The protein families database in 2021. *Nucleic Acids Research* **49**, D412–9.

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Chinese Academy of Sciences and John Wiley & Sons Australia, Ltd.

Morrison DW (1980). Efficiency of food utilization by fruit bats. *Oecologia* **45**, 270–3.

Pavlovich SS, Lovett SP, Koroleva G *et al.* (2018). The Egyptian rousette genome reveals unexpected features of bat antiviral immunity. *Cell* **173**, 1098–110.

Priatel JJ, Teh SJ, Dower NA, Stone JC, Teh HS (2002). RasGRP1 transduces low-grade TCR signals which are critical for T cell development, homeostasis, and differentiation. *Immunity* **17**, 617–27.

Ramírez-Fráncel LA, García-Herrera LV, Losada-Prado S *et al.* (2022). Bats and their vital ecosystem services: A global review. *Integrative Zoology* **17**, 2–23.

Reidenerg JS (2007). Anatomical adaptations of aquatic mammals. *Anatomical Record-Advances in Integrative Anatomy and Evolutionary Biology* **290**, 507–13.

Roy E, Togbe D, Holdorf AD *et al.* (2010). Nck adaptors are positive regulators of the size and sensitivity of the T-cell repertoire. *PNAS* **107**, 15529–34.

Santillan DM, Lama T, Guerrero YG *et al.* (2021). Large-scale genome sampling reveals unique immunity and metabolic adaptations in bats. *Molecular Ecology* **30**, 6449–67.

Scheben A, Ramos OM, Kramer M *et al.* (2020). Unraveling molecular mechanisms of immunity and cancer-resistance using the genomes of the Neotropical bats *Artibeus jamaicensis* and *Pteronotus mesoamericanus*. *BioRxiv,* 2020.09.09.290502.

Sercan Ö, Stoycheva D, Hämmerling Günter J, Arnold B, Schüler T (2010). IFN-γ receptor signaling regulates memory CD8$^+$ T cell differentiation. *The Journal of Immunology* **184**, 2855–62.

Shannon P, Markiel A, Ozier O *et al.* (2003). Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Research* **13**, 2498–504.

Shah K, Al-Haidari A, Sun J *et al.* (2021). T cell receptor (TCR) signaling in health and disease. *Signal Transduction and Targeted Therapy* **6**, 412.

Simmons NB (2005). *Order Chiroptera*. In: Wilson DE, Reeder DAM, eds. *Mammal Species of the World: A Taxonomic and Geographic Reference*, 3rd ed. The Johns Hopkins University Press, Baltimore, pp. 312–529.

Stathopoulos S, Bishop JM, O'Ryan C (2014). Genetic signatures for enhanced olfaction in the African mole-rats. *PLoS ONE* **9**, e93336.

Sun C, Zhang C, Lucas JR, Gu H, Feng J, Jiang T (2021a). Vocal performance reflects individual quality in male Great Himalayan leaf-nosed bats (*Hipposideros armiger*). *Integrative Zoology*, pp. 1–10. https://doi.org/10.1111/1749-4877.12545

Sun Z, Wang L, Han L *et al.* (2021b). Functional calsequestrin-1 is expressed in the heart and its deficiency is causally related to malignant hyperthermia-like arrhythmia. *Circulation* **144**, 788–804.

Suyama M, Torrents D, Bork P (2006). PAL2NAL: robust conversion of protein sequence alignments into the corresponding codon alignments. *Nucleic Acids Research* **34**, W609–12.

Szklarczyk D, Gable AL, Lyon D *et al.* (2019). STRING v11: Protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Research* **47**, D607–13.

Teeling EC, Vernes SC, Dávalos LM *et al.* (2018). Bat biology, genomes, and the Bat1K Project: To generate chromosome-level genomes for all living bat species. *Annual Review of Animal Biosciences* **6**, 23–46.

Towner JS, Amman BR, Sealy TK *et al.* (2009). Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. *PLoS Pathogens* **5**, e1000536.

Vermeren M, Lyraki R, Wani S *et al.* (2017). Osteoclast stimulation factor 1 (*Ostf1*) KNOCKOUT increases trabecular bone mass in mice. *Mammalian Genome* **28**, 498–514.

Wang K, Tian S, Galindo-González J, Dávalos LM, Zhang Y, Zhao H (2020). Molecular adaptation and convergent evolution of frugivory in Old World and neotropical fruit bats. *Molecular Ecology* **29**, 4366–81.

Xi Z, Liu L, Rest JS, Davis CC (2014). Coalescent versus concatenation methods and the placement of *Amborella* as sister to water lilies. *Systematic Biology* **63**, 919–32.

Xu H, Morishima M, Wylie JN *et al.* (2004). Tbx1 has a dual role in the morphogenesis of the cardiac outflow tract. *Development* **131**, 3217–27.

Yan H, Jiao H, Liu Q *et al.* (2021). ACE2 receptor usage reveals variation in susceptibility to SARS-CoV and SARS-CoV-2 infection among bat species. *Nature Ecology and Evolution* **5**, 600–8.

Yang Z (2007). PAML 4: Phylogenetic analysis by maximum likelihood. *Molecular Biology and Evolution* **24**, 1586–91.

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Yu Y, Nakhleh L (2015). A maximum pseudo-likelihood approach for phylogenetic networks. *BMC Genomics* **16**, S10.

Zhang C, Rabiee M, Sayyari E, Mirarab S (2018). ASTRAL-III: Polynomial time species tree reconstruction from partially resolved gene trees. *BMC Bioinformatics* **19**, 153.

Zhang G, Cowled C, Shi Z *et al.* (2013). Comparative analysis of bat genomes provides insight into the evolution of flight and immunity. *Science* **339**, 456–60.

Zou D, Tian S, Zhang T *et al.* (2021). Vulture genomes reveal molecular adaptations underlying obligate scavenging and low levels of genetic diversity. *Molecular Biology and Evolution* **38**, 3649–63.

SUPPLEMENTARY MATERIALS

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Phylogenetic relationships among mammals inferred from the concatenated 1689 protein coding genes.

Figure S2 Phylogenetic relationships between bats and other mammals inferred from the Astral method.

Figure S3 Phylogenetic relationships between bats and other mammals inferred from the mpest method.

Table S1 Transcriptome sequencing information for the 5 newly sequenced bats used in this study.

Table S2 Bat species examined in this study. Species with newly generated transcriptome data were shown in bold.

Table S3 Taxonomic classification and data sources for outgroup mammalian species used in this study.

Table S4 Lists of candidate mammalian "balance-sensitive" genes.

Table S5 Positively selected genes (PSGs) identified in the common ancestor of bats.

Table S6 Rapidly evolving genes (REGs) identified in the common ancestor of bats.

Table S7 Significantly enriched pathways based on the PSGs identified in the common ancestor of bats.

Table S8 Significantly enriched pathways based on the PSGs identified in the control group.

Table S9 Significantly enriched pathways and GO terms based on the merged dataset of PSGs and REGs in the common ancestor of bats.

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