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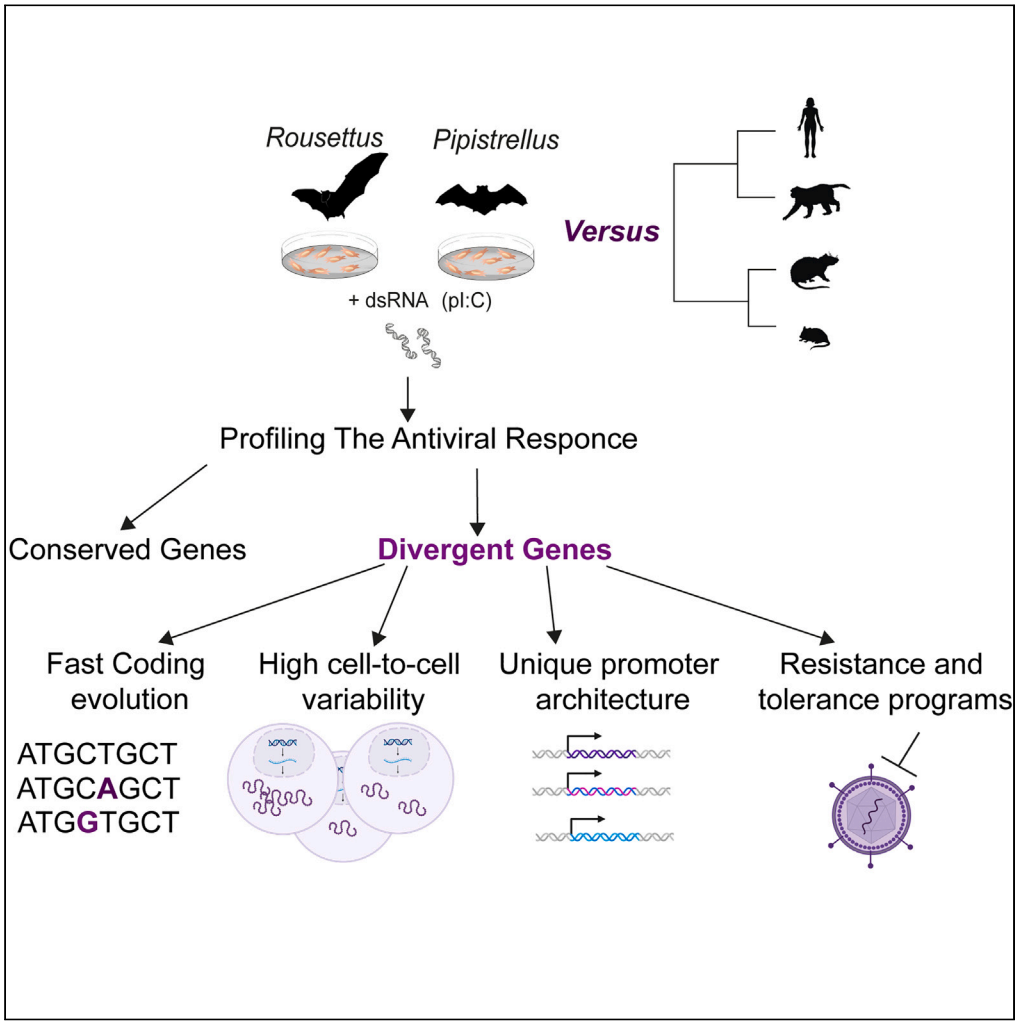

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Highlights

Comparison of transcriptional response to dsRNA in two bat species

Largely conserved antiviral response with a set of transcriptionally divergent genes

Divergence between bats is related to divergence between other mammals

Divergence is related to specific functional and regulatory characteristics

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Comparison of antiviral responses in two bat species reveals conserved and divergent innate immune pathways

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SUMMARY

Bats host a range of disease-causing viruses without displaying clinical symptoms. The mechanisms behind this are a continuous source of interest. Here, we studied the antiviral response in the Egyptian fruit bat and Kuhl's pipistrelle, representing two subordinal clades. We profiled the antiviral response in fibroblasts using RNA sequencing and compared bat with primate and rodent responses. Both bats up-regulate similar genes; however, a subset of these genes is transcriptionally divergent between them. These divergent genes also evolve rapidly in sequence, have specific promoter architectures, and are associated with programs underlying tolerance and resistance. Finally, we characterized antiviral genes that expanded in bats, with duplicates diverging in sequence and expression.

Our study reveals a largely conserved antiviral program across bats and points to a set of genes that rapidly evolve through multiple mechanisms. These can contribute to bat adaptation to viral infection and provide directions to understanding the mechanisms behind it.

INTRODUCTION

Among mammals, bats display unique life histories and adaptations, including powered flight, extreme longevity relatively to their body mass and echolocation. In addition, bats have been shown to display subclinical symptoms when infected with several viruses that can cause severe disease in humans.[1–4] In some of these cases, bats were confirmed to be the natural reservoirs of these viruses.[1–5] For example, the Egyptian fruit bat, *Rousettus aegyptiacus*, is thought to serve as a reservoir of the highly pathogenic Marburg virus,[6] based on field collection from wild bats[7–9] and on a series of ecological and experimental studies.[8,10–15] In addition, infection of *Rousettus* with the related Ebola virus is asymptomatic, although the exact reservoir species is not yet known.[12,16,17]

Several coronaviruses—severe acute respiratory syndrome coronavirus (SARS-CoV), SARS-CoV-2 and Middle East respiratory coronavirus (MERS-CoV)—that recently transferred to humans, are also thought to originate in bats, although other mammals likely acted as proximate reservoirs for human infection.[18–21] Furthermore, direct and indirect bat-to-human spillovers of two henipaviruses—Nipah virus and Hendra virus—lead to infections with high-mortality rates in humans,[22–24] leading to concerns of potential future pandemics from henipaviruses. The unique lifestyles of bats, including flight, rapid changes in body temperature, and crowded colonies in closed environments such as caves, were all suggested to facilitate bat adaptation to these viruses.[2]

The links between bat and human pathogens and the often asymptomatic or mild infections observed in bats, have led to a great interest in bat viriome and immunity, and to the notion that bats disproportionately contribute to emerging zoonotic viruses that have crossed to humans.[25] However, a recent survey of virus-reservoir relationships challenged this notion, when controlling for the size of the bat clade.[26] Bats are the second largest mammalian order after rodents, comprising ~22% of known mammalian species, and have adapted to diverse ecological niches across the planet.[27,28] Thus, the clade's size and large diversity as well as bat occurrence across the globe, have contributed to facilitating zoonotic transfers of bat viruses to humans and other mammals.

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Regardless of this, a series of studies focusing on specific bat species, immune pathways and viral infections revealed unique adaptions in the bat immune system. These include the constitutive expression in uninfected cells and tissues of the black flying fox (*Pteropus alecto*) of IFNα—an antiviral cytokine that is usually only upregulated following infection.[29] Other innate immune-related genes, including known interferon-stimulated genes, were shown to be highly expressed in uninfected cells of several bat species.[30–32] High expression of various innate immune genes may point to greater resistance to viral disease, by rapid inhibition of infecting viruses.[5,33] In contrast, tolerance to disease—a mechanism by which viruses and other pathogens replicate in host cells without leading to an excessive immune response and thereby decreasing tissue damage, was also suggested to be related to mild symptomatic infection of certain bat species.[1,5,15] For instance, bats were shown to have a dampened inflammasome activation in comparison with other mammals through various mechanisms, including the loss of the PYHIN gene family locus that encodes for inflammasome DNA sensors,[34] and the reduction of inflammation and apoptosis mediated by the inflammasome sensor NLRP3.[35] In addition to these mechanisms, certain antiviral and immune gene families were shown to expand or to contract in bats or in specific branches of the bat clade and to display signatures of positive selection in their coding sequences when compared between bat species, including type-I interferons and important antiviral proteins APOBEC3,[29,36,37] PKR,[38] and tetherin.[39]

Here, we chart the transcriptional landscape of the antiviral innate immune response in a comparative framework, by triggering this response in primary cells from two bat species. We focus on representatives of the major bat subordinal clades[40]—the Egyptian fruit bat, *Rousettus aegyptiacus* and the insectivore bat *Pipistrellus kuhlii* (Kuhl's pipistrelle). Various aspects of *Rousettus* innate and adaptive immunity have been studied,[13,41–46] due to its asymptomatic infection of filoviruses, as well as its association with infection with other viruses.[47,48] In contrast, antiviral immunity of *Pipistrellus*, a distantly related bat to *Rousettus*, remains poorly characterized, despite studies that suggested the presence of several Alpha- and Beta-coronaviruses (including closely related variants to MERS-CoV) in its population in Europe and the Middle East.[49–54] The fact that *Pipistrellus* is common in agricultural and urban areas and can be in close contact with humans, underlines the importance of studying its viriome and immune system.

We triggered the innate immune response—an expression program that involves the upregulation of cytokines and chemokines, restriction factors that inhibit viral replication and gene related to apoptosis and to the regulation of this response,[55,56] in skin fibroblasts from both bat species. Fibroblasts play key roles in infected tissues,[57,58] and, due to their robust response, are often used as experimental models to study the innate immune response across species[59–61] and between human individuals.[62,63] We profiled the transcriptional response in both species and employed a comparative genomics approach to find transcriptionally conserved and divergent innate immune genes between the two bat species. We then analyzed the regulatory and functional characteristics of these conserved and divergent genes, and compared the results in bats with analogous results in primates and rodents. We also studied innate immune gene upregulation in individual bat and human cells using single-cell RNA-seq.

Our study thus maps the evolutionary landscape of bat innate immune response, links functional and regulatory features of innate immune genes with their transcriptional and coding sequence evolution, and points to innate immune genes that diverged in bats—divergence that may play a role in their adaptation to viral infection.

RESULTS

An *in vitro* stimulation system to characterize bat antiviral response

To study the evolution of the antiviral innate immune response program, we grew skin fibroblasts from wing biopsies of two bat species—the Egyptian fruit bat, *Rousettus aegyptiacus* and the insectivore bat *Pipistrellus kuhlii* (Kuhl's pipistrelle). We triggered an antiviral response using polyinosinic:polycytidylic acid (poly(I:C)), a synthetic double-stranded RNA (dsRNA) sensed by various sensors, such as TLR3, MDA5, and IFIH1. Unlike viral infection that can lead to a virus-specific response and where the virus can antagonize the host immune system, dsRNA leads to a general antiviral response, unmodulated by viruses. We profiled the resulting transcriptional changes using RNA-seq from 10 to 12 biological replicates (*Rousettus*: 6 females and 6 males; *Pipistrellus*: 3 females and 2 males, each in two biological replicates) between stimulation and control (see Figure 1A and Table S1 for a detailed list of samples). We used the same stimulation conditions (in terms of time and concentration of dsRNA transfection) as we did previously, with an analogous fibroblast system from primates and rodents.[59] For analysis of cell-to-cell variability in gene

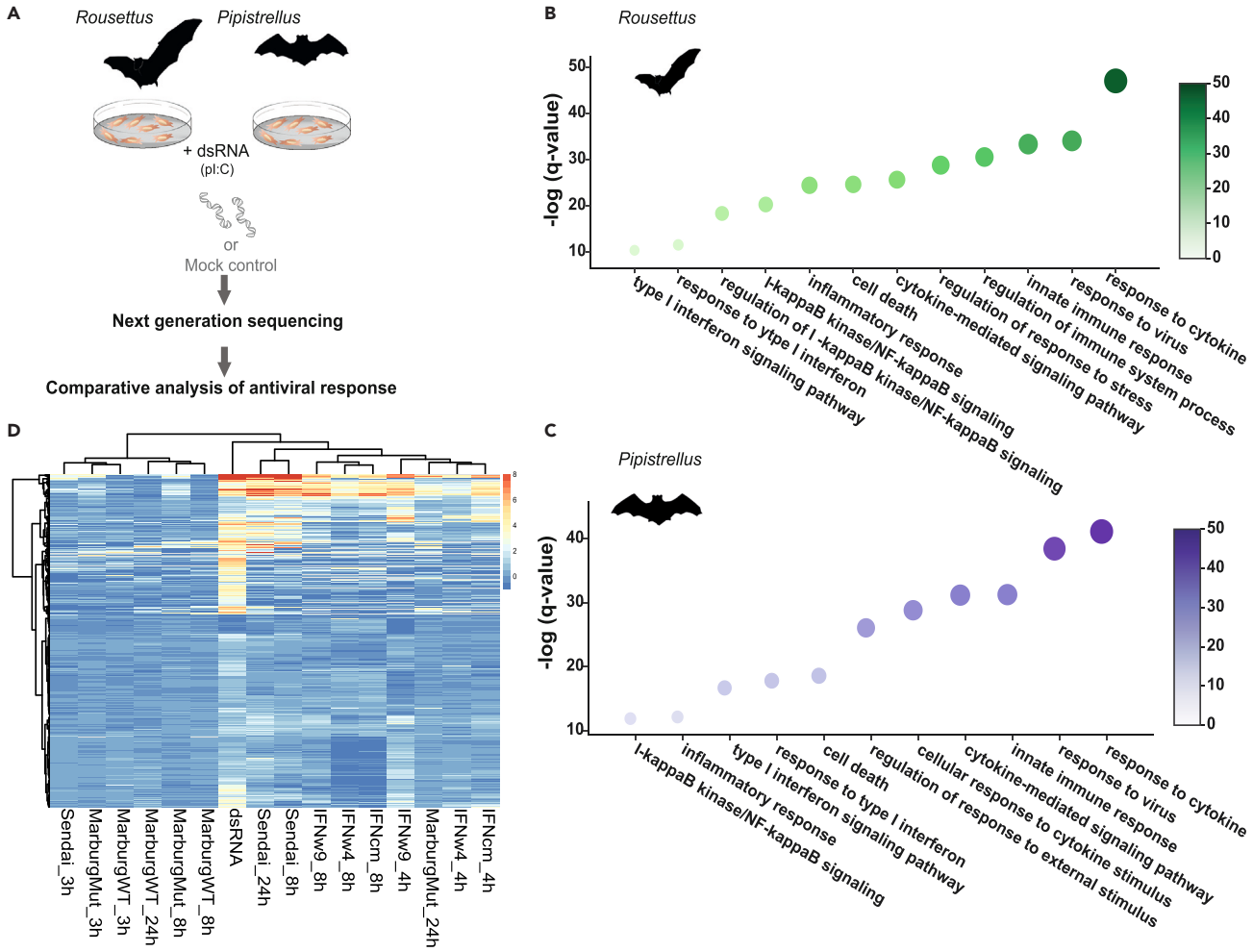

Figure 1. Characterization of dsRNA-stimulated genes in of cells from *Rousettus* and *Pipistrellus*

(A) System overview: dermal fibroblasts derived from wing biopsies from two bat species were cultured and stimulated with dsRNA and control, followed by profiling of the response using bulk RNA-seq, gene quantification and differential expression analysis using edgeR.

(B and C) Go term enrichment analysis of genes upregulated in response to dsRNA-stimulus in (B) *Rousettus* and (C) *Pipistrellus*. Selected non-redundant terms are shown in a decreasing order of significance (FDR-corrected p-values are shown). Dot colors and sizes denote p-values. Detailed analyses, including all enriched terms appear in Tables S4 and S5, respectively.

(D) Heatmap of $\log_2$(fold change) of dsRNA-stimulated *Rousettus* genes across different immune stimuli with different types of IFN and in response to infection with Sendai virus and Marburg virus.

expression before and after stimulation, we also profiled transcriptomes of single cells from *Rousettus* and human, as detailed in the relevant sections below.

PCA performed on samples of each of the bat species separately suggests that in *Rousettus* the major source of variation is treatment, while in *Pipistrellus*, the major source of variation is sex followed by treatment (Figure S1). PCA combining samples from both species (using orthologous genes, as detailed in the following text) suggests that the major source of variation is species, followed by sex and treatment (Figure S2).

DsRNA-regulated bat genes are enriched with innate immune functions

We first studied the transcriptional response of fibroblasts to stimulation in each of the bat species separately by performing differential expression analysis between control and stimulation using edgeR[64] (and excluding lowly expressed genes, keeping 17,898 and 19,741 genes in *Rousettus* and *Pipistrellus*, respectively). We observed that 968 and 840 genes in *Rousettus* and *Pipistrellus*, respectively, are upregulated in

response to stimulus (logFC>0 and FDR-corrected p-value<0.01)), while significantly fewer genes are downregulated (logFC<0 and FDR-corrected p-value<0.01)—61 and 81 genes in the respective species. Both numbers and fractions of differentially up- and downregulated genes from the overall-expressed genes are similar to those observed in analogous dsRNA stimulations of dermal fibroblast cells in primates and rodents.[59] For example, in dsRNA stimulation of human fibroblasts, there are 1,255 and 161 upregulated and downregulated genes, respectively (see detailed list of bat genes with their DE values in Tables S2 and S3). In both bat species, we observe a strong upregulation of antiviral and inflammatory cytokines and chemokines, including IFNB, CXCL10, and CCL5, as well as other genes involved in the antiviral response, such as transcriptional factors and signal transducers—IRFs and STATs, and various restriction factors, including SAMHD1 and ADAR.

We next used g:Profiler[65] to characterize pathways enriched in the set of upregulated genes in each bat species. We observed that in both bats the upregulated genes are involved in pathways known to be related to the primary and secondary waves of the antiviral response,[59] including terms such as "response to virus" and "response to cytokine", as well as other associated processes such as "cell death" and "inflammatory response" (see a set of non-redundant terms for each species in Figures 1B and 1C and the full list of enriched terms in Tables S4 and S5). These pathways are similar to those observed to be enriched in dsRNA stimulation of human and mouse fibroblasts.[59,61,63]

To further compare the genes upregulated in our dsRNA-stimulation system with other related systems, we used available data of a *Rousettus* cell line (RoNi/7.1-immortalized kidney cells), where cells were either stimulated with interferons (IFNs) or infected with wild type or mutant strains of RNA viruses (see Figure 1D, Table S6 for full list of datasets; Table S7 for gene-expression values).[10,42] The transcriptional profile of response to dsRNA from our experiments clusters with Sendai virus infection and with stimulations with the set of IFNs. Late Sendai virus infections (8 and 24 h) were more similar to dsRNA stimulation (of 4 h) in comparison with earlier infection (2 h), likely reflecting differences in kinetics of RNA recognition between the two systems. Interestingly, wild-type Marburg virus infections cluster separately from dsRNA- and IFN stimulation as well as from most Sendai virus infections.

We next looked at gene expression across individual cells within the fibroblast population using the single-cell transcriptomics data of human and *Rousettus*. For this, we profiled single-cell transcriptome of thousands of *Rousettus* and human cells, with and without dsRNA stimulation using Chromium Single Cell 3′ gene expression v.3.1 (see STAR Methods). We observe that the regulatory architecture of cytokine expression previously reported by us and others[66–70] is recapitulated in our human and *Rousettus* cells: several types of cytokines, such as IFNs, are only expressed in a few stimulated cells in both species, whereas others, such as chemokines from the CXCL (chemokine (C-X-C motif) ligand) family, are more widely expressed (Figure S3).

Finally, we tested whether the use of skin samples from different regions in the body may affect our results. We observed that the upregulated genes and their level of upregulation are highly similar between fibroblasts derived from two different skin regions in *Rousettus* (p-value<10^{-293}, Figure S4).

Together, our aforementioned analyses suggest that dsRNA-stimulation of bat dermal fibroblasts leads to a strong upregulation of genes involved in conserved antiviral pathways, similar to other mammalian species, including primates and rodents.

Transcriptional response divergence between bat species and between bats and other mammals

We next focused on the overall similarity in transcriptional response to dsRNA between species, by comparing the response level to dsRNA between orthologous genes across bats and other mammals. For this, we first inferred the set of orthologs between the two bat species as well as between these bats, two primate species (human and macaque) and two rodent species (mouse and rat), using the eggNOG program[71] (see analysis details in STAR Methods). The resulting gene orthology table between the two bats is available as Table S8, and the one-to-one orthology table for the six species is available as Table S9.

We focused on a set of 2,865 one-to-one orthologous genes that are differentially expressed in response to dsRNA in at least one of six mammalian species (the two bat species from our current analysis, human, rhesus macaque, mouse, and rat). We performed a correlation analysis between their fold change in response to dsRNA stimulation (see differential expression results based on edgeR[64] analysis for all 6 species in Table S9; we note that all following correlation results stem from this set of 2,865 genes). We observed a significant correlation between the two bat species (Figure 2B, Spearman's rank correlation $\rho = 0.427$, P-value$<10^{-27}$), corresponding to an overall similar response between the two bat species. However, when performing the correlation analysis on the same set of genes using a similar fibroblast dsRNA-stimulation system in primates and rodents (human versus macaque; mouse versus rat), we observed a higher correlation in transcriptional response between the two primates as well as between the two rodents (Figure 2, $\rho = 0.648$ and 0.529, respectively). When comparing human versus mouse transcriptional response, we observe a similar correlation to that observed between the two bat species ($\rho = 0.441$ between human and mouse versus $\rho = 0.427$ between the two bats). Similar trends are observed when we compare the correlation in orthologous gene expression between the pairs of species compared previously (Figure S5). This seemingly low correlation between the two bat species is in agreement with the fact that these two bat species belong to the two major bat suborders, Yangochiroptera and Yinpterochiroptera, whose last common ancestor is predicted to have existed ~60–70 million years ago[40,72–74] (while primates and rodents split approximately 90 million years ago[75,76]). This finding is consistent with the notion that different bat species, while having a largely conserved genetic program upregulated in response to viral infection, may still differ in the level of upregulation of specific genes and that there may be high diversity across bat species in their response to viruses. The characteristics of the most divergent antiviral genes between the two studied bat species are the focus of the subsequent sections of our analysis.

Transcriptionally divergent bat innate immune genes display specific evolutionary and regulatory characteristics

To study the characteristics of transcriptionally conserved versus divergent bat antiviral genes, we focused on the same set of 2,865 differentially expressed genes from the previous analysis. These genes were partitioned into three equally sized groups, displaying high, medium, and low levels of divergence in transcriptional response to dsRNA between the two bat species (see STAR Methods for details on the metrics used to calculate gene's divergence level. The divergence scores are found in Table S7).

We first compared the rate at which the genes in the three groups evolved in coding sequence. Thus, we asked whether antiviral genes with different levels of transcriptional divergence in response to dsRNA, also evolved at different rates in their coding sequences. This was done (1) by considering the ratio of substitution rates at non-synonymous and synonymous sites (dN/dS) in orthologs from a set of 18 bat species[77] (Figure 3A), and (2) by comparing the sequence similarity between the orthologs of the two bat species (Figure 3B) (per-gene values are available in Table S7). In both measures, we observe that innate immune genes with transcriptionally divergent response tend to have higher coding sequence divergence than the group of innate immune genes with conserved response (Figures 3A and 3B, p-value = 0.017 and 1.5 × 10-16, respectively, one sided Mann-Whitney test).

Previous work suggested that gene variability in expression between species (as well as between different conditions) is associated with different promoter architectures[59,78]: CpG Islands (CGIs), a major element in core promoters of around half the mammalian genes, are thought to be associated with homogeneous transcription between conditions and between species of nearby genes. In contrast, TATA-box elements that are relatively rare in mammalian promoters are associated with variable transcription of their regulated genes.[79] We assessed the presence of both elements (available in Table S7) in core promoter regions of *Rousettus* genes (see STAR Methods) and observed that genes with CGI promoters are significantly more conserved in their transcriptional response to stimulus than genes that lack CGIs in their promoters. In contrast, genes with TATA-box elements in their promoters exhibit greater transcriptional response divergence than those genes without TATA-box (Figure 3C, p-value<0.001 in both comparisons, one sided Mann-Whitney test).

We next studied whether gene transcriptional response divergence between species is linked with gene expression variability across individual cells from the same species. For each gene expressed in the single-cell data that is upregulated in response to dsRNA (based on our differential expression analysis shown previously), we estimated cell-to-cell variability in expression across individual cells in a manner that takes into account their mean

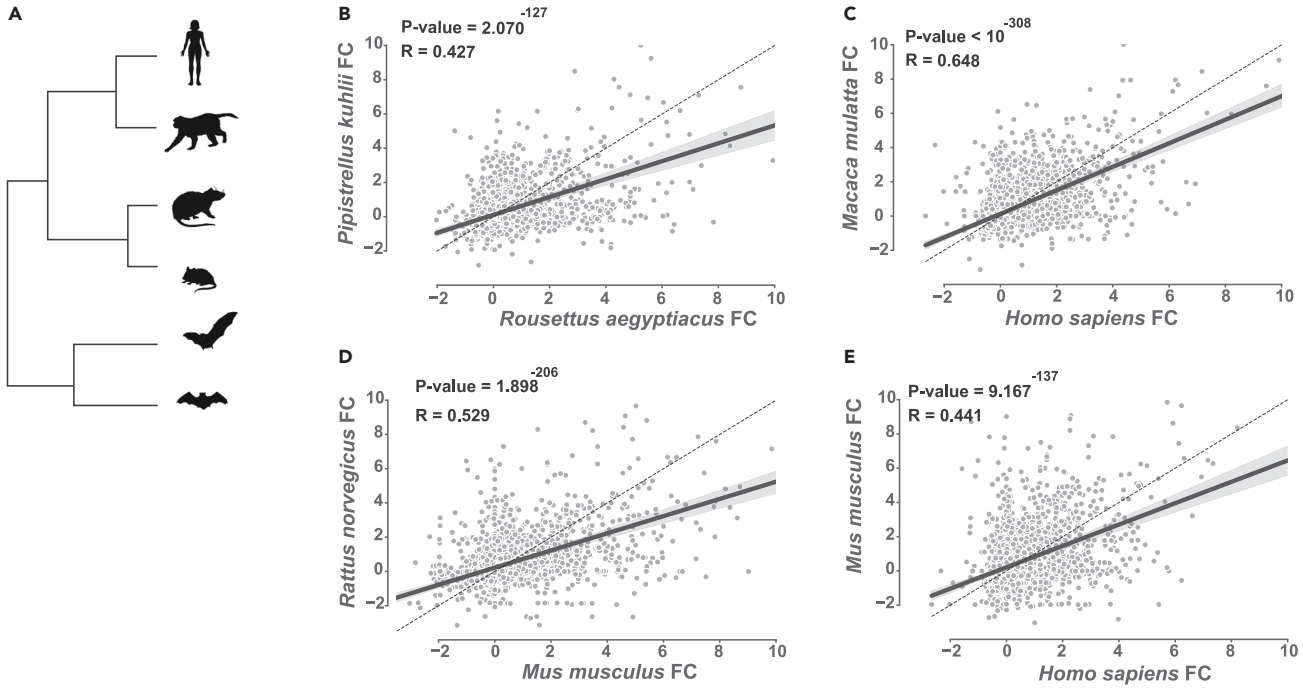

Figure 2. Correlation of response across species

(A) A schematic phylogenetic tree of the six studied species: human, macaque, mouse, rat, and the two bat species. The two bat species, belonging to Yinpterochiroptera and Yangochiroptera, are estimated to have a last common ancestor (LCA) ~60–70 million years ago (MYA), whereas primates and rodents split ~90MYA.

(B–E) Spearman's rank correlation of gene fold change in response to dsRNA, in 2,865 one-to-one orthologous genes between (B) *Rousettus* and *Pipistrellus*, (C) Human and macaque (estimated LCA – 28MYA[96]), (D) Mouse and rat (estimated LCA – 13MYA[96]), and (E) Human and mouse. Genes included are dsRNA-regulated (FDR-corrected<0.01) in at least one of the six species appearing in figures B–E (2,865 genes).

expression level, using the distance to median (DM) approach.[80] This was done separately for stimulated and unstimulated cells and for both human and *Rousettus* cells (data available in Table S7). We observed that in all tested sets of cells, the group of genes displaying high-transcriptional divergence between the two bat species also displays high cell-to-cell variability in expression (high-DM values), which is significantly higher than the variability observed in the group of low-divergence between species (low-DM values) (p-value<0.001 in both cases, one sided Mann-Whitney test, Figures 3D, 3E, and S6).

Thus, innate immune genes diverging in transcriptional response between bat species tend to vary between individual cells within the same species and have also undergone rapid evolution at the coding sequence level compared with innate immune that display conservation in their transcriptional response between bat species.

Characteristics of *Rousettus*-specific and *Pipistrellus*-specific antiviral genes

So far, we compared bat antiviral genes with high, medium, and low levels of transcriptional divergence in response to dsRNA between the two bat species, using a continuous divergence measure. Next, we asked whether there are defining characteristics for genes significantly more upregulated in one of the two species with respect to the other bat species—i.e., *Rousettus*- and *Pipistrellus*-specifically upregulated genes. For this, we used the top 20% of genes (2,433 genes out of all differentially expressed genes appearing as one-to-one orthologs in both bat species) that are most highly upregulated in *Rousettus* with respect to *Pipistrellus*, as well as the opposite group, essentially taking the two extremes of the group of high-divergence level. These two subgroups will be termed for simplicity *Rousettus*- and *Pipistrellus*-specific genes (see STAR Methods for the detailed procedure).

By looking at enriched pathways using g:Profiler[65] and by contrasting *Rousettus*- and *Pipistrellus*-specific genes, we observe that *Rousettus*-specific genes are associated with various developmental processes,

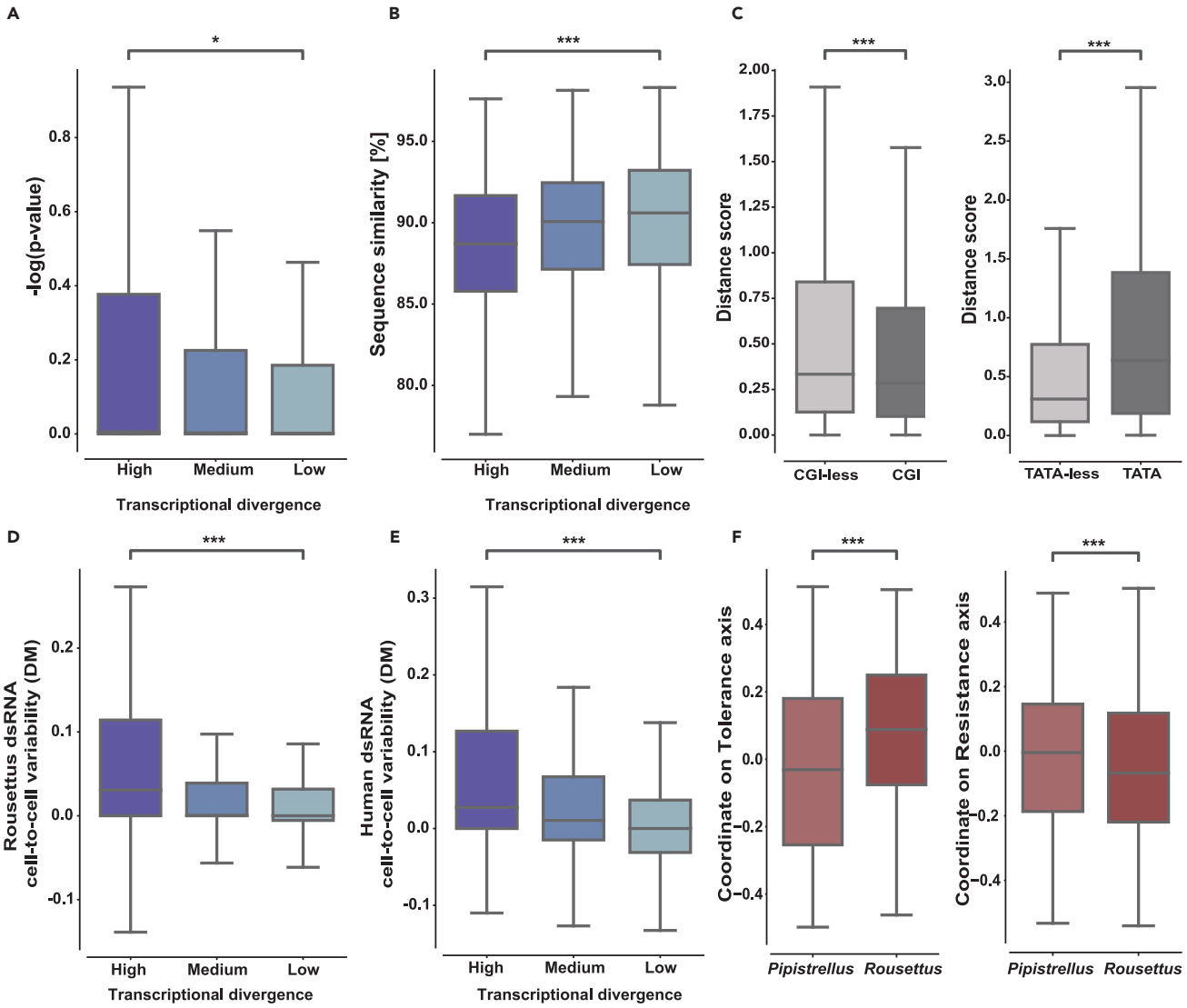

Figure 3. Regulatory and evolutionary characteristics of conserved and divergent dsRNA-stimulated genes between *Rousettus* and *Pipistrellus*

(A and B) Coding sequence divergence versus transcriptional divergence: Genes were partitioned into three groups based on divergence in response to dsRNA stimulation between the two bat species (termed high, medium, and low transcriptional divergence), and coding sequence divergence was compared between these three groups: (A) Distributions of P-values of test for positive selection across bat orthologs (using the dN/dS values from a previous analysis[79]), and (B) sequence similarity (precent of identity) between the two bat species, are shown for the three gene groups (1,899 and 2,728 genes with available data in A and B, respectively). High- and low-divergence groups are compared using a Mann-Whitney test.

(C) Divergence in response to dsRNA between the bat species in genes with and without CpG Island (CGI) in their promoters, and with and without TATA-box in their promoters (896 CGI and 1,969 CGI-less genes; 107 TATA and 2,758 TATA-less genes). Groups are compared using a Mann-Whitney test.

(D–E) Using single-cell RNA-seq, the distribution of cell-to-cell variability in gene expression, as measured using the distance to median approach (DM), is shown for each of the three groups mentioned in (A), for (D) dsRNA-stimulated *Rousettus* cells and (E) stimulated human cells (1,980 and 2,578 genes with computed DM values, respectively). High- and low-divergence groups are compared using a Mann-Whitney test. See Figure S3 for analysis of unstimulated *Rousettus* and human cells.

(F) The distribution of measures for tolerance and resistance are shown for genes that are highly upregulated in response to dsRNA in *Rousettus* versus *Pipistrellus*, or the opposite (1,928 and 1,949 genes, respectively). The distributions of *Rousettus*-specific and *Pipistrellus*-specific genes are compared using a Mann-Whitney test. (* = $p < 0.05$, *** = $p < 0.001$).

including "anatomical structure morphogenesis" and "tissue development". *Pipistrellus*-specific genes are associated with "Wnt signaling pathway" and "lipid metabolic process" and various processes associated with protein degradation including "regulation of proteolysis" and "deubiquitination" (see Tables S10–S15). The enrichment of the aforementioned pathways that are not "core antiviral pathways" is consistent

with the notion that significant evolutionary changes would occur in a large number of genes belonging to pathway at the "periphery" of the antiviral response.

We next asked whether these *Rousettus*- and *Pipistrellus*-specific genes are associated with expression programs that underlie "disease tolerance" (containment of infection while avoiding an excessive immune reaction) and "disease resistance" (effective inhibition of infection). Previous works suggested that different species of bats may display either greater disease tolerance or greater resistance to virus infections with respect to other mammals due to their unique lifestyle.[5,15] The extent of tolerance and resistance likely differs between various bat species as well as depending on the specific pathogen in question. A recent study used an array of mouse strains infected with influenza virus and profiled both the physiological response of the mice and their gene expression during infection. This has yielded distinct sets of genes that are associated with greater tolerance or resistance to infection.[81] These sets were shown to be indicative of tolerance or resistance in a spectrum of infectious diseases and conserved across species. We compared the set of *Rousettus*- and *Pipistrellus*-specific genes in terms of their association with resistance- and tolerance-associated programs (Figure 3F, additional results are found in Figure S7, detailed data are in Table S16). Interestingly, we observed that the transcriptional program generally associated with disease tolerance is significantly more prominent in *Rousettus*, while the molecular program primarily associated with resistance is significantly more active in *Pipistrellus* (p-value<0.001, one sided Mann-Whitney test, in both comparisons, see Figure 3F).

In summary, genes displaying divergence in their level of upregulation between the *Rousettus* and *Pipistrellus*, are enriched with different cellular pathways that do not represent core antiviral pathways. Furthermore, the two species differ in the activities of expression programs associated with disease tolerance or resistance.

Bat-specific duplicates of innate immune genes display transcriptional divergence across tissues and rapid coding sequence evolution at the interface with viral proteins

The aforementioned analyses focused on one-to-one orthologs across species. Gene duplication was previously suggested to be an important mechanism in the evolution of the immune system.[82] We thus asked whether antiviral genes that rapidly diverge in transcriptional response to dsRNA (as measured by us between the two bat species) and that also display rapid coding sequence evolution (as shown in Figures 3A and 3B), belong to gene families that display relatively high-duplication rates in the course of evolution. We observe that genes with a high level of transcriptional divergence do not display a significantly different duplication rate in comparison with low-divergence genes (Figure S8), suggesting that gene duplication is not significantly higher in the group of rapidly evolving bat antiviral genes as a whole.

We next searched for cases of bat-specific duplications or loss of innate immune genes, by scanning the list of dsRNA-upregulated genes in *Rousettus and Pipistrellus*, as well as those found to be upregulated in human and mouse. In addition to several antiviral genes that have been reported previously to be duplicated in bats, including the APOBEC3 family[83,84] and IFNω,[42] we also found that tetherin (BST2)—an important restriction factor against a range of enveloped viruses has experienced rapid duplication in the branch that leads to *Pipistrellus* but not to *Rousettus*. In addition, we found that PLAAT4, a phospholipase A1/2 and an acyltransferase associated with cell proliferation and differentiation, that is significantly upregulated in response to dsRNA in *Pipistrellus*, has at least two copies in *Pipistrellus* (termed LOC118707299 and LOC118707212)—both of which are significantly upregulated in response to dsRNA. Related bat species—*Rhinolophus ferrumequinum* and *Myotis lucifugus*—are predicted to have 2 and 16 PLAAT4 duplicates in their genomes, respectively (as observed by profiling the orthologs and paralogs of these genes in ENSEMBL). In contrast, *Rousettus* and a related fruit bat, *Pteropus vampyrus* seem to have no copy of PLAAT4 in their genomes, suggesting rapid changes in copy numbers of this gene in different bat families. Similarly to this loss, both IFI44 and IFI44L genes, two IFN-stimulated genes associated with antiviral activities against several RNA viruses as well as with several autoimmune diseases,[85–88] are lost in *Rousettus* and in *Pteropus vampyrus* (the latter was observed in ENSEMBL genome annotation). We note that our analysis may be affected by genome quality, annotations, and completeness, and we thus report only on cases of gene loss or duplication observed in more than a single-bat genome.

We investigated the transcriptional and coding sequence divergence between gene duplicates of a specific gene—TNFRSF14, that has seven gene copies in *Rousettus* (LOC107499783,LOC107509685,

LOC107510671, LOC107510672, LOC107510674, LOC107510998, and LOC107520019). In human cells, TNFRSF14 induces both pro- and anti-inflammatory pathways, based on binding to two different sites in its extracellular domain.[89] In addition, it serves as a receptor for entry of both human Herpes Simplex virus 1 and 2, through interaction with the herpes glycoprotein D (gD).[90] TNFRSF14 is upregulated in response to dsRNA and to IFNB in human and mouse cells (Figures 4A and 4B). However, only one of its *Rousettus* gene copies is induced in response to dsRNA stimulus. Interestingly, this copy is lowly expressed across tissues, in contrast to other *Rousettus* duplicates that display higher expression in two or more tissues. Furthermore, these paralogs seem to diverge in response to infection with Sendai and Marburg viruses (Figure 4B, right) (see a similar analysis on transcriptional divergence of APOBEC3 paralogs in Figure S9).

In addition to the observed transcriptional divergence between the *Rousettus* TNFRSF14 duplicates, we investigated the divergence in coding sequence between these duplicates. We estimated the relative divergence in amino acid substitution across the *Rousettus* TNFRSF14 duplicates in each amino acid position using Rate4site[91] (see Figure 4C and STAR Methods). We then contrasted the substitution rates of residues known to be part of the interface formed between human TNFRSF14 and herpes glycoprotein D proteins, and the other surface residues (identifying the interface residues using the CSU method,[92] as we have done previously[93]). We observed a significantly higher rate of substitution in the interface residues in comparison with the surface residues (Figure 4C). This suggests that this region, known at least in human to be a site of interactions with viral proteins, diverged more rapidly in sequence between *Rousettus* paralogs. This analysis demonstrates the potential of recent gene duplicates to diverge in both sequence and expression and potentially to contribute to bat-specific response against viruses.

DISCUSSION

Some of the unique physiological and behavioral characteristics of bats are likely linked to their co-evolution and adaptation to viral infections. Here, we took a comparative transcriptomics approach to study the expression program triggered as part of the immediate antiviral response in bats and the evolution of specific antiviral genes. We profiled this innate immune response in fibroblast cells from two bat species—Kuhl's pipistrelle (*Pipistrellus kuhlii*) and the Egyptian fruit bat (*Rousettus aegyptiacus*)—and compared this response between these two bat species as well as between bats and other mammals. In addition to the fact that these bats are evolutionarily distant, belonging to different suborders, they also differ significantly in their physiology (e.g., *Rousettus* is much larger than *Pipistrellus*), ecology, habitants, and behavior (i.e., colony size, diet, etc.). These differences in life histories suggest different exposures and adaptations to pathogens between these two bat species.

We observe that the overall innate immune response is conserved across bats, where genes are up- and downregulated in response to dsRNA in a similar manner to other mammals both with respect to the overall numbers of regulated genes and in terms of specific gene identities and cellular pathways involved in this response. When comparing the level of response across species, by looking at gene's fold change in response to dsRNA in pairs of one-to-one orthologs, we observe that the response of the two bat species is conserved, showing a significant correlation between ortholog responses in the two species. However, this level of correlation is similar to the correlation observed between primates and rodents, and lower than correlations within the rodent or within the primate clades—exemplifying the divergence between distant bat species in the level of upregulation of specific genes. These results also emphasize the importance of further studies into separate branches of the bat clade that likely evolved differently in their diverse habitants in response to different viral strains. Importantly, our study focuses on the innate immune response and its divergence between bat species. However, the adaptive immune system is also known to evolve rapidly across species. Since it plays important roles in the resolution of infection, it likely also contributes to variation between bat species in their response to viral infection.

Our focused analysis of genes that are transcriptionally divergent in their response to dsRNA between the two bat species revealed several regulatory and evolutionary trends: genes diverging in transcriptional response between the two bat species tend to also rapidly diverge in coding sequence. This suggests that innate immune genes in bats evolve concurrently through distinct evolutionary mechanisms, including changes in the level of expression and in amino acid substitutions across species. These findings are in line with our previous observations where we studied the evolution of antiviral genes in primates and rodents and showed that these transcriptionally diverging antiviral genes also diverge rapidly in coding sequence.[59]

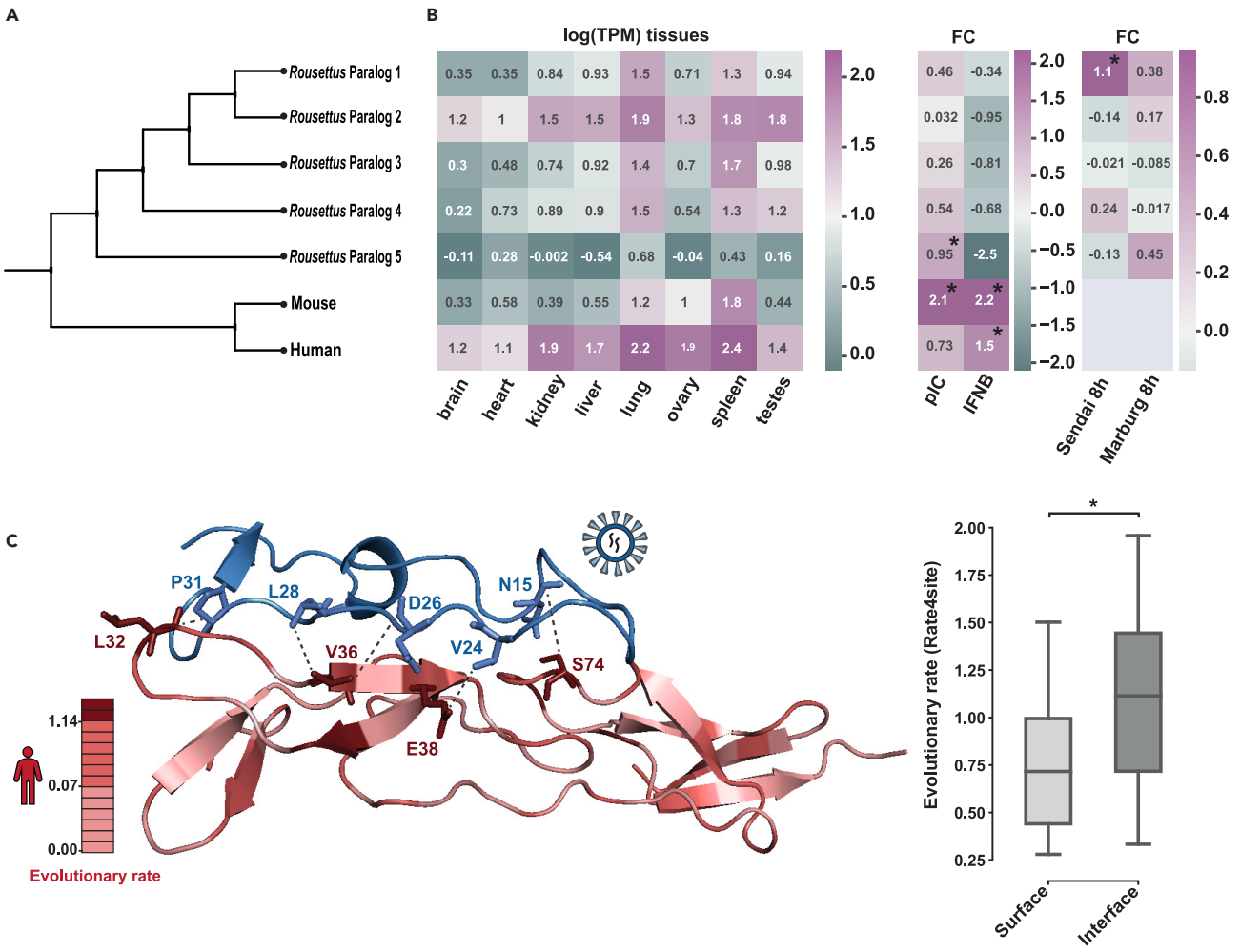

Figure 4. Duplication of TNFRSF14 in *Rousettus* and duplicate divergence in sequence and expression

(A) Reconstructed tree of TNFRSF14 of human, mouse, and five *Rousettus* duplicates.

(B) Left: TNFRSF14 gene expression across tissues of human, mouse, and five *Rousettus* duplicates; Middle: $\log_2$(Fold Change) of TNFRSF14 of human, mouse, and five *Rousettus* duplicates in response to dsRNA or IFNB, as measured in cells from human,mouse and *Rousettus* (significance of upregulation is shown within the square). Right: $\log_2$(Fold Change) of five *Rousettus* duplicates in response to infection by Sendai virus or Marburg virus.

(C) Left: Structure of human TNFRSF14 with the herpes glycoprotein D, in red and blue respectively (PDB: 1JMA). Human TNFRSF14 is colored by relative evolutionary rate of residues (as measure across all orthologs and paralogs appearing in A). Right: The distributions of evolutionary rates of residues found at the surface of TNFRSF14 (surface) compared with evolutionary rates of residues interacting with the herpes glycoprotein D (interface). Comparison is made using a Mann-Whitney test (* = $p < 0.05$).

When looking at the promoter architecture of innate immune genes in *Rousettus*, we observe that in agreement with previous findings in various mammalian systems,[78,79,94] gene transcriptional divergence between species is associated with particular promoter structures: Genes with CGIs in their promoters tend to be transcriptionally conserved between species whereas genes without such elements, and especially those with a TATA-box in their promoters, tend to be divergent in their transcriptional response between the two bat species. Furthermore, transcriptionally divergent bat genes also show high cell-to-cell variability in expression between individual cells within the same species. This is true for both *Rousettus* and human cells, and in both stimulated and unstimulated cells, as revealed by our single-cell transcriptomics of human and bat cells. The similarity observed between human and *Rousettus* cells in the set of genes found to be transcriptionally divergent (i.e., genes found to be divergent in bat cells are also divergent in human cells), suggests that the same set of innate immune genes have undergone rapid transcriptional evolution in the course of mammalian evolution while other genes involved in this response are conserved. This is likely due to different levels of regulatory constraints imposed on these genes, such as their function

(whether they have pleotropic or regulatory functions, such as transcription factors that are more conserved[59]) and the biophysical nature of their protein products—whether they are engaged in direct interactions with viral proteins and what constraints limit the evolution of the residues that interact with the viral proteins.[93]

In contrast to previous findings based on human and non-human primates,[59,82] we do not observe that the most transcriptionally divergent innate immune genes in our data belong to gene families that have undergone rapid duplication. This is in agreement with the relatively few genes known to be involved in antiviral immunity that were found to rapidly duplicate or to have been lost in the bat clade in comparison with other genes.[84]

Focusing on specific genes that are upregulated in response to dsRNA and that have relatively high-copy numbers in bats in comparison with other mammals, we found several genes and gene families previously not reported to undergo rapid gene duplication or gene loss in bats. These include both IFI44 and IFI44L genes that seem to have been lost in Pteropodidae, and PLAAT4 that shows a wide variation in paralog numbers in Vespertilionidae. We further analyzed the transcriptional and coding sequence divergence of an additional innate immune gene, TNFRSF14 that has undergone rapid duplication in the branch leading to *Rousettus*. The paralogs of this gene in *Rousettus* display distinct patterns of basal expression across bat tissues, as well as in their response to IFN and to dsRNA. Furthermore, when these paralogs were compared with the single orthologs found in human and mouse genome, we observed that the relatively high-basal expression and upregulation of the single copy of TNFRSF14 in human and mouse differ from what observed in TNFRSF14 bat paralogs, suggesting transcriptional divergence between paralogs. When looking at the substitution rate of amino acids across paralogs of bat TNFRSF14, we observe an interesting trend: The residues whose homologs in human TNFRSF14 were shown to form an interface with herpes glycoprotein D (gD) (based on the crystal structure of the human-herpes virus complex), are significantly more divergent in comparison with other surface residues of the TNFRSF14 protein. These analyses demonstrate how recent duplication events of various bat immune genes in specific branches of the bat clade, rapidly diverged through basal expression level across tissues, in transcriptional response to infection, and in coding sequence, in regions that may be involved in direct contact with viral proteins. This example, as well as recent work on functional diversification of PKR duplicates in *Myotis* species[38] and of BST2 (tetherin) duplicates in Vespertilionidae,[39] suggests that rapid duplication of specific antiviral genes has contributed to bat-specific adaptation against infection from specific viral strains or viral families.

Our work provides a comparative catalog of antiviral gene expression using a large set of individuals in two bat species. These data give insights into how different evolutionary mechanisms, including changes in gene expression in homeostasis and during infection, as well as coding sequence evolution and gene duplication, may have shaped the innate immune response of bats and contributed to bat adaptation to viral infections. In addition to providing an important resource for the community interested in bat immunity, these data have the potential to advance our understanding of pathological immune conditions in humans.

Limitations of the study

Our transcriptional response analysis is based on dermal fibroblasts. We and others have previously used this system to compare the innate immune response across species and human individuals, leveraging on the ability to grow these cells from numerous individuals and reaching a high number of cells, grown in homogeneous conditions.[59–61,95] However, these results may still require testing in and extension to *in vivo* infections of viruses. Furthermore, we studied the antiviral response in two bat species, showing that while they upregulate a largely conserved gene set, there are large differences in the level of upregulation of orthologous genes between the two species and, in addition, there is a subset of highly divergent genes. This points to the diversity of bat species and their immune responses, and to the need to study numerous bat species to gain a comprehensive understanding of the immune system across the bat clade.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

T.H. conceived and supervised the project; S.K., S.F., Y.N., M.W., G.D., Y.Y., and T.H. designed and/or performed experiments; L.S., S.K., S.F., D.S.C., G.S., E.F., A.A.A., E.C.T., and T.H. performed analysis, data curation, and visualization; T.H. wrote the manuscript with help from L.S., S.K., and S.F. All authors read the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Rousettus aegyptiacus	skin biopsies from wing & shoulder skin	Taxonomy ID: 9407
Pipistrellus kuhlii	skin biopsies from wing	Taxonomy ID: 59472
Chemicals, peptides, and recombinant proteins		
DMEM	Biological industries	01-052-1A
FBS	Rhenium	10270106
Alanyl-L-Glutamine	Biological industries	03-022-1B
Sodium Pyruvate	Biological industries	03-042-1B
Primocin	Invivogen	ant-pm-1
PenicillinStreptomycin-Amphotericin-B	Biological industries	103-033-1C
poly(I:C)	Invivogen	tlrlpic
Lipofectamin 2,000	ThermoFisher	11668027
Deposited data		
Raw and analyzed data - bulk and single-cell RNAseq	This paper	ArrayExpress: E-MTAB-10870, E-MTAB-11056
Experimental models: Cell lines		
Primary cell line of human dermal fibroblast	ATTC	PCS-201-012
Critical commercial assays		
Quick-RNA MicroPrep kit	Zymo Research	R1051
Kapa Stranded mRNA-seq Kit (24RXN)	Kapa	KK8420 ROCHE-07962193001
KAPA Unique Dual-Indexed Adapter Plate	Kapa	KK8726- 08861862001
d KAPA Adapter Dilution Buffer	Kapa	KK8721-08278539001
Software and algorithms		
Scripts for analysis	This paper	https://github.com/lilachschn/bat_gene_expression_divergence
Salmon	Patro et al., 2017[97]	https://combine-lab.github.io/salmon/
edgeR	Robinson et al., 2010[64]	https://bioconductor.org/packages/release/bioc/html/edgeR.html
eggNOG	Huerta-Cepas et al., 2019[71]	http://eggnog-mapper.embl.de/
Seurat	Hao et al., 2021[101]	https://satijalab.org/seurat/
MUSCLE	Edgar et al., 2004[109]	https://github.com/rcedgar/muscle
PhyML	Guindon et al., 2010[110]	https://github.com/stephaneguindon/phyml
Rate4site	Pupko et al., 2002[91]	https://www.tau.ac.il/~itaymay/cp/rate4site.html
g:Profiler	Raudvere et al., 2019[65]	https://biit.cs.ut.ee/gprofiler/page/apis
PyMol	PyMOL by Schrödinger	https://pymol.org/2/

RESOURCE AVAILABILITY

Lead contact

Further information and requests should be directed to the lead contact, Tzachi Hagai (tzachiha@tauex.tau.ac.il).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Original code has been deposited at GitHub and is publicly available as of the date of publication: https://github.com/lilachschn/bat_gene_expression_divergence. Please address any additional question to T. Hagai (tzachiha@tauex.tau.ac.il).

Bulk and single-cell RNA-seq data have been deposited at ArrayExpress and are publicly available as of the date of publication in ArrayExpress with the following accessions: E-MTAB-10870 and E-MTAB-11056.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Ethical compliance

Bat biopsies from two bat species - *Rousettus aegyptiacus* and *Pipistrellus kuhlii* - were collected from either naturally deceased individuals or from animals sacrificed as part of a different project, with approvals by the Israel Nature and Parks Authority (approval number 2020/4285) and the TAU ethics committee (approval number 04-20-023).

Primary dermal fibroblasts from the two bat species were extracted from wing biopsies, and in the case of *Rousettus* also from shoulder skin samples. Cells were passaged for up to 3 passages before freezing. Thawed cells were used for stimulation as described below. Further details on growth conditions are detailed below. Origin of sample and sex of each individual are mentioned in Table S1.

METHOD DETAILS

Bat dermal fibroblast extraction and growth

Bat dermal fibroblast cells were extracted from skin samples and processed in a similar manner to our previous work,[59] using mechanical and enzymatic digestion. Following extraction, we passaged cells for three passages, using a growth medium DMEM (Biological industries, 01-052-1A) supplemented with 20% FBS (Rhenium, 10270106) 2 mM L-Alanyl-L-Glutamine (Biological industries, 03-022-1B), 1 mM Sodium Pyruvate (Biological industries, 03-042-1B), Primocin (Invivogen, ant-pm-1) and Penicillin-Streptomycin-Amphotericin-B (Biological industries, 1 03-033-1C. Cells were then frozen and stored in liquid nitrogen. Individual lines that did not grow or showed signs of senescence were discarded. In the case of *Rousettus*, skin biopsies from shoulders were also collected and grown in a similar fashion. See full list of samples in Table S1. For single-cell experiments, we also used a primary cell line of human dermal fibroblast from ATTC (PCS-201-012).

Bat cell stimulation with dsRNA

Prior to stimulation, cells were thawed and grown for a few days in fibroblast growth medium. A day before stimulation, cells were trypsinized, counted and seeded into 6-well plates to reach ~70% confluence at the start of stimulation. Cells were stimulated as follows: (1) stimulated with 1 μg/ml high-molecular mass poly(I:C) (Invivogen, tlrl-pic) transfected with 2 μg/ml Lipofectamin 2,000 (ThermoFisher, 11668027); (2) mock transfected with Lipofectamin 2,000. After 4 hours, the stimulation was terminated by washing the cells in PBS. For bulk RNA-seq, cells were lysed using RNA Lysis Buffer (Zymo Research). For droplet-based single-cell RNA-seq, cells were first trypsinized and then collected to continue with library preparation according to the manufacturer's protocol for the Chromium Single Cell 3′ gene expression v.3.1 (10x Genomics), as described below.

Bulk RNA-seq

Total RNA was extracted using the Quick-RNA MicroPrep kit (Zymo Research, Cat. No. R1051). RNA concentration, purity and integrity were measured with Qubit 3.0 and TapeStation 4200. In total, 49 samples were used for subsequent library preparation and sequencing.

Libraries were produced using the Kapa Stranded mRNA-seq Kit (24RXN) (KK8420 ROCHE-07962193001) with KAPA Unique Dual-Indexed Adapter Plate (KK8726-08861862001) and KAPA Adapter Dilution Buffer (KK8721-08278539001), starting with up to 500ng of total RNA. Final cDNA amplification was performed

with 13 PCR cycles. Libraries were normalized and pooled at 10nM. Pooled samples were sequenced on an Illumina NovaSeq 6000 instrument, using the NovaSeq 6000 SP Reagent Kit (200 cycles) 20040326 and paired-end 101-bp reads.

Single-cell RNA-seq

Following trypsinization and cell count, human and *Rousettus* samples of the same treatment condition (poly(I:C) and lipofectamine) were pooled together. Cells were prepared and loaded to the Chromium controller according to the manufacturer's protocol (10x Genomics), using the Single Cell 3' Reagent Kit (version 3.1). cDNA synthesis and amplification were performed according to the protocol. Libraries were sequenced on an Illumina NovaSeq 6000 using the NovaSeq 6000 SP Reagent Kit v1.5 (100 cycles).

Read mapping to annotated transcriptome

Bulk RNA-seq

Reads were mapped and gene expression was quantified using Salmon[97] (version 1.4.0) with the following command parameters for each sample: 'salmon quant -i [index_file_directory] -l IU -1 [left_read_library/lane1] [left_read_library/lane2] -2 [right_read_library/lane1] [right_read_library/lane2] –geneMap [transcript_to_gene_file] –seqBias –gcBias -q –numBootstrap 100 –threads 8 –validateMappings -o [output_directory]'. Each sample was mapped to its respective species' annotated transcriptome, downloaded from the NCBI website (*Rousettus aegyptiacus*: Genome published by Pavlovich et al.[36]; *Pipistrellus kuhlii*: from the Bat1K project[84]). Following quantification, a count matrix was created.

In the case of *Rousettus aegyptiacus*, we mapped reads to two genome annotations – the genome published by Pavlovich et al., and the genome from the Bat1K project. Since we had higher mapping rates of reads using the first genome, we based our subsequent analyses on that mapping.

We also mapped previously published datasets of bulk RNA-seq of *Rousettus aegyptiacus* samples: (1) IFN stimulation of *Rousettus* cell lines from Pavlovich et al. (2020)[42] (run accessions SRR11148658-723), (2) Viral infection of *Rousettus* cell lines from Arnold et al. (2018)[10] (run accessions SRR7548028-63) and (3) *Rousettus* tissues dataset from the Bat1K[84] were downloaded from the NCBI Sequence Read Archive and mapped to the Rousettus UCSC transcriptome. Paired read libraries were mapped as above, but with libType A. Single read libraries were mapped as follows: 'salmon quant -i [index_file_directory] -l A -r [sample_library] –geneMap [transcript_to_gene_file] –seqBias –gcBias -q –numBootstrap 100 –threads 8 –validateMappings -o [output_directory]'.

RNA-seq data of tissues from human and mouse was used along with *Rousettus* tissue gene expression to show expression of TNFRSF14 duplicates. These data were downloaded from GTEx[98] and BodyMap[99] for human and mouse, respectively, and processed as in our previous work:[100] To achieve comparable gene expression data across species, we filtered all the pseudo autosomal expression records and merged various brain tissues by computing the mean for each gene across them, to compare with the mouse and bat brain data. For each gene in each of the eight tissues that existed in all three species, we used the mean expression between all male and female individuals as representative value of this species.

Single-cell RNA-seq

Reads were demultiplexed and quantified using Cell Ranger from Chromium Single Cell Software Suite (version 5.0.1, 10x Genomics Inc). Raw read data was demultiplexed ("unpooled") into sample-specific FASTQ files based on the sample indices. Reads were then quantified with the following command for each sample: 'cellranger count –id=[output_directory] –transcriptome=[reference_genome_directory] –fastqs=[sample_FASTQs_directory]'.

Each sample was mapped to its respective species' annotated genome, the *Rousettus aegyptiacus* genome (see above) and the human GRCh38 reference genome (ENSEMBL version 99), as well as to an index that included of both genomes (human and Rousettus). The latter was initially used to determine whether each individual cell originated in human or Rousettus. Cells were determined to be from *Rousettus*, if more than 80% of their reads were mapped to Rousettus, and human cells were determined in a similar fashion. Cells that did not meet these criteria were considered doublets and were discarded.

Following species determination for each single-cell library, all subsequent analyses were based on the separate mapping output.

Quantifying differential gene expression in response to dsRNA

Bulk RNA-seq

We quantified differential gene expression between dsRNA-treatment and control (for each species separately and, in the case of *Rousettus*, for each skin tissue separately as well) by using edgeR (version 3.32.1)[64] with rounded estimated counts from Salmon. We only kept genes that were expressed in at least 3 of the 30 *Rousettus* samples or 2 of the 19 *Pipistrellus* samples. In each of the public datasets (IFN stimulation, viral infection), we kept genes expressed in at least 3 of the respective dataset's samples. Differential expression analysis (DEA) was conducted via the edgeR exact test. P-values were corrected for multiple testing by estimating the false discovery rate (FDR). For the public datasets, separate DEAs were performed for each time point and each virus or IFN type.

PCA of stimulated and unstimulated samples within each bat species and across the two bat species was performed using RunPCA function from the Seurat package (version 4.0.1),[101] after scaling and normalization ('ScaleData', and 'NormalizeData' functions).

Single-cell RNA-seq

We filtered cells with less than 10,000 reads, and genes expressed in less than 3 of the remaining cells. We then removed cells with more than 20% mitochondrial reads. Further single-cell data analysis was done using Seurat (version 4.0.1).[101] Cell cycle phase was regressed out via Harmony.[102] Following lowly expressed gene filtering, 75% of the most variable genes were used for dimensionality reduction. Differentially expressed genes in stimulated versus unstimulated cells were estimated using Seurat's FindMarkers function.

Orthology mapping between bats, primates and rodents

To determine gene orthology relationship between species as accurately as possible, we integrated orthology annotations from ENSEMBL Compara[103] for human, rhesus macaque, mouse and rat genes, while finding orthologs between human and the two bat species using eggNOG (version 2.1.2).[71]

To obtain gene orthology using eggNOG, we ran the following command for human and mouse genomes (ENSEMBL version 99, after removal of secondary haplotypes) as well as the CDS files of the two bat species (with the genome versions mentioned above): 'emapper.py –data_dir {database_dir} –cpu 6 -i {input_cds_file} –itype CDS –output {output_name_for_results} –sensmode very-sensitive –report_orthologs –output_dir {results_dir} -m diamond -d none –tax_scope 40674 –go_evidence non-electronic –target_orthologs all –seed_ortholog_evalue 0.001 –seed_ortholog_score 60 –query_cover 20 –subject_cover 0 –override –scratch_dir {scratch dir} –temp_dir {tmp dir}'.

Genes were defined as belonging to the same orthology group (either one or more from each species), if their transcripts mapped to the same eggNOG name. Genes whose transcripts were mapped to more than a single eggNOG name were removed. The detailed eggNOG processed results for human, mouse, *Pipistrellus* and *Rousettus* (two genome annotations) are in Table S8.

From these cross-species mappings we created several tables of one-to-one orthologs – in Table S9: For cross-mammalian analysis (involving six species, as in Figures 2 and 3), we used only genes that had one-to-one orthology annotations in ENSEMBL for the primates and rodents, as well as one-to-one orthology mappings in eggNOG for human, *Rousettus* and *Pipistrellus* (10,241 genes). For cross-bat analysis we used one-to-one orthology annotations between the two bat species (13,888 genes).

Fold-change-based analysis of conservation and divergence in innate immune response

To compare the overall change in response to immune stimulation between pairs of species, we computed the Spearman's rank correlation of the fold-change between all one-to-one orthologs that were differentially expressed in response to dsRNA in at least one species (q-value<0.01 in one of the 6 species- 2,865 genes) as well as of all expressed genes (Figure 2). A similar analysis was done using the average gene expression in control samples (by taking the average TPM across all individual samples in mock stimulated conditions) – see Figure S5. In this analysis we took all 10,214 orthologous genes across the six species.

Quantifying transcriptional divergence in innate immune response between species

To estimate transcriptional divergence between the six species in response to treatment, we focused on genes that have one-to-one orthologues in all tested species. We defined a measure of response divergence between the two bat species or between bat and human, by calculating the absolute differences in the fold-change estimates across the two orthologues, for genes that are differentially expressed in at least one species, as previously done.[59,104] For example, between the two bat species, we defined: $Response\ diveregnce = \left|FC_{Rousettus} - FC_{Pipistrellus}\right|$. We classified the top third most divergent genes as high divergent genes, the medium third as medium divergent genes, and the bottom third as low divergent genes.

Defining *rousettus*-specific and *pipistrellus*-specific

We employed the following procedure to identify dsRNA-responding genes that are significantly different between the two bat species in the level of response: We computed for each of the DE genes the Response divergence value, as shown in the previous section, but without using the absolute value: $Response\ diveregnce = FC_{Rousettus} - FC_{Pipistrellus}$.

In this manner, each DE gene has a value that can be either negative or positive, depending on the fold change level in the two species. We then define the genes most responding in *Rousettus* with respect to *Pipistrellus* as the top 20% most positive genes. We define the genes most responding in *Pipistrellus* with respect to *Rousettus* as the top 20% most negative genes. For simplicity, we refer to them as *Rousettus*-specific or *Pipistrellus*-specific genes.

Promoter sequence analysis

Human and *Rousettus* CGI annotations were downloaded from the UCSC genome table browser[105] (hg38 for human), and CGI genes were defined as those with a CGI overlapping their core promoter (300bp upstream of the TSS reference position and 100bp downstream of it, as suggested previously[59,78]). Genes were defined as having a TATA box if they had a significant match to the Jaspar TATA box matrix (MA0108.1)[106] in the 100bp upstream of their TSS by FIMO[107] with default settings (we used a 100 bp window owing to possible inaccuracies in TSS annotations).

Coding sequence evolution analysis

The dN/dS ratio (non-synonymous to synonymous codon substitutions) values of *Rousettus* genes across bats and their statistical significance were obtained from a previous study that used orthologous genes from 18 bat species (Hawkins et al., 2019).[77] We computed distributions of the test statistics of positive selection, based on dN/dS values for each of the three groups of genes with low-, medium- and high- divergence in response to dsRNA (Figure 3A).

Sequence similarity analysis

To obtain the level of similarity between orthologs, we ran BLAST of *Rousettus* versus *Pipistrellus*. We selected the longest CDS for each pair of one-to-one orthologs (and the lowest TSL, in case two transcripts had the same length) of the two species. We then ran blast2seq on these the CDS sequences to obtain the similarity level.

Rate of gene gain and loss analysis

The significance (P-value) of a gene family to have undergone a high rate of gene duplication and loss (contraction) over the course of vertebrate evolution, in comparison to other gene families, was obtained from ENSEMBL.[103] These statistics are based on the CAFE tool that estimates the global birth and death rate of gene families and identifies gene families that have accelerated rates of gain and loss.[108] We calculated the distributions of P-values for the low-, medium- and high-divergence gene groups (as defined above), and plotted them as the negative logarithm values (Figure S8).

Tolerance and resistance analysis

Genes primarily associated with tolerance and resistance expression programs were taken from a previous study, which compared the transcriptional response and physiological outcomes of infection across a large set of mouse strains.[81] The association of the gene sets identified in that work with tolerance and resistance

to infection were suggested to be conserved across mammals and infectious diseases, as demonstrated by further analyses of additional systems.

We used the top 20% *Rousettus*- or *Pipistrellus*-specific genes separately, with two measures of resistance and tolerance from the above-mentioned paper, i.e., coordinates on axis T+ R (Figure 3F) and Gene-to-T or R correlation (Figure S7). The detailed T and R data appears in Table S16.

Cell-to-cell variability analysis

To quantify the biological cell-to-cell variability of genes, we used the DM (Distance to Median) approach — an established method that estimates relative cell-to-cell variability in gene expression while accounting for confounding factors such as gene expression level[80] (using a window of expression level to group genes and regress expression level) and after removal of lowly expressed genes.

TNFRSF14 evolutionary analysis

TNSFRSF14 duplicates were identified by using the orthology mapping (based on the EggNOG results) – by searching for DE genes that had 1:many orthologs relationship in at least one of the bat species. Out of seven copies that were identified we removed two copies that were either identical to a segment of another larger copy or did not include a stop codon. The remaining five copies are sufficiently different, including having non-overlapping regions, to allow Salmon to accurately distinguish between them during the quantification process.

The relative evolutionary rate of each amino acid in the TNFRSF14 protein was estimated using a multi-sequence alignment and phylogenetic tree using Rate4site,[91] as previously prefomed[93,94]: Briefly, five *Rousettus* paralog sequences and other species ortholog sequences (human, mouse and *Pipistrellus*) were aligned using MUSCLE.[109] A phylogenetic tree was constructed using PhyML,[110] and Rate4site was run using default parameters.

Interface and surface residues were inferred from the solved structure of TNFRSF14 with the herpes glycoprotein D (pdb: 1JMA [111]) using the CSU method.[92]

GO term analysis

To study the functional enrichment of genes against the background of the entire human genome we used g:Profiler program.[65] In the section of 'characteristics of rousettus-specific and pipistrellus-specific antiviral genes' we used GOrilla[112] where we tried to study the functional enrichment of *Rousettus*-specific genes against the background of *Pipistrellus*-specific genes and vice versa. The detailed results of both g:Profiler (g:GOST-fuctional profiling and g:Convert-gene ID conversion) and GOrilla programs are in Tables S10–S15.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses (Mann-Whitney test, Spearman's rank correlation and FDR-correction based on Benjamini-Hochberg procedure[113] were performed using the SciPy package in Python (version 3.9). Data in boxplots represent the distribution with lines for median, first quartile and third quartile and whisker lines extending to the furthest value within 1.5 of the interquartile range. Plots were created using matplotlib and seaborn packages.