

METHODS AND RESOURCES

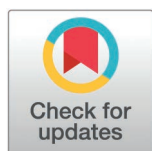
Expanding the bat toolbox: *Carollia perspicillata* bat cell lines and reagents enable the characterization of viral susceptibility and innate immune responses

Victoria Gonzalez^{1,2}, Cierra Word³, Nahomi Guerra-Pilaquinga³, Mitra Mazinani⁴, Stephen Fawcett⁴, Christine Portfors⁵, Darryl Falzarano^{1,2}, Alison M. Kell⁶, Rohit K. Jangra³, Arinjay Banerjee^{1,2,7,8,9†*}, Stephanie N. Seifert^{4‡*}, Michael Letko^{4‡*}

1 Vaccine and Infectious Disease Organization (VIDO), University of Saskatchewan, Saskatoon, Canada, **2** Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, Canada, **3** Louisiana State University Health Sciences Center-Shreveport, Shreveport, Louisiana, United States of America, **4** Paul G. Allen School for Global Health, Washington State University, Pullman, Washington, United States of America, **5** Washington State University, Vancouver, Washington, United States of America, **6** Department of Molecular Genetics and Microbiology, University of New Mexico, Albuquerque, New Mexico, United States of America, **7** Department of Biology, University of Waterloo, Waterloo, Canada, **8** Department of Laboratory Medicine and Pathobiology, University of Toronto, Toronto, Canada, **9** Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of British Columbia, Vancouver, Canada

† These authors are co-senior authors on this work.

* arinjay.banerjee@usask.ca (AB); stephanie.seifert@wsu.edu (SNS); michael.letko@wsu.edu (ML)



OPEN ACCESS

Citation: Gonzalez V, Word C, Guerra-Pilaquinga N, Mazinani M, Fawcett S, Portfors C, et al. (2025) Expanding the bat toolbox: *Carollia perspicillata* bat cell lines and reagents enable the characterization of viral susceptibility and innate immune responses. PLoS Biol 23(4): e3003098. <https://doi.org/10.1371/journal.pbio.3003098>

Academic Editor: Katherine Kedzierska, University of Melbourne at Doherty Institute, AUSTRALIA

Received: November 19, 2024

Accepted: March 4, 2025

Published: April 15, 2025

Copyright: © 2025 Gonzalez et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data availability statement: The following top-performing cell lines from this study are available through BEI Resources and distributed by ATCC: Bulk, non-clonal *Carollia perspicillata* kidney cells immortalized with SV-40 T-antigen (NR-59778) and clonal CKg9 cells (NR-59779). All other cell lines are available

Abstract

Multiple viruses that are highly pathogenic in humans are known to have evolved in bats. How bats tolerate infection with these viruses, however, is poorly understood. As viruses engage in a wide range of interactions with their hosts, it is essential to study bat viruses in a system that resembles their natural environment like bat-derived in vitro cellular models. However, stable and accessible bat cell lines are not widely available for the broader scientific community. Here, we generated in vitro reagents for the Seba's short-tailed bat (*Carollia perspicillata*), tested multiple methods of immortalization, and characterized their susceptibility to virus infection and response to immune stimulation. Using pseudotyped virus library and authentic virus infections, we show that these *C. perspicillata* cell lines derived from a diverse array of tissues are susceptible to viruses bearing the glycoprotein of numerous orthohantaviruses, including Andes and Hantaan virus and are also susceptible to live hantavirus infection. Furthermore, stimulation with synthetic double-stranded RNA prior to infection with vesicular stomatitis virus and Middle Eastern respiratory syndrome coronavirus induced a protective antiviral response, demonstrating the suitability of our cell lines to study the bat antiviral immune response. Taken together, the approaches outlined here will inform future efforts to develop in vitro tools for virology from non-model organisms and these *C. perspicillata* cell lines will enable studies on virus–host interactions in these bats.

upon request. Please refer to [S3 Fig](#) for a bright field image that depicts the morphology of the kidney cells. Raw numerical values for all graphed data are available for download as [Supporting information](#).

Funding: Research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (NIAID/NIH) under Award Numbers 1R21AI169527 (M.L. and A.B.), R 21AI156482 and P20GM134974 (R.K.J.), and Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant (RGPIN-2022-03010) awarded to A.B. This work was partly supported by an NSF Biology Integration Institute grant under award numbers NSF DBI 2021909 and 2213854 (S.N.S). V.G. is supported by an NSERC scholarship (#569587-2022). VIDO receives operational funding from the Government of Saskatchewan through Innovation Saskatchewan and the Ministry of Agriculture, and from the Canada Foundation for Innovation through the Major Science Initiatives Fund. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Abbreviations: ANDV, Andes virus; DMEM, Dulbecco's modified Eagle's medium; dsRN, double-stranded RNA; FBS, fetal bovine serum; GFP, green fluorescent protein; hTERT, human telomerase gene; HTNV, Hantaan virus; *IFIT1*, interferon-induced protein with tetratricopeptide repeats 1; *IFNβ*, interferon beta; ISG, interferon-stimulated gene; MERS-CoV, Middle Eastern respiratory syndrome coronavirus; MOI, multiplicity of infection; PBS, phosphate-buffered saline; PCDH1, Protocadherin-1; rVSV, recombinant VSV; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SEC1–2, soluble extracellular cadherin domains 1 and 2; VSV, vesicular stomatitis virus.

Introduction

Most viral outbreaks in the last century have stemmed from cross-species transmission of viruses from wildlife to humans, often facilitated by domestic animals or hosts that may amplify the virus. For example, influenza viruses can spread to humans from birds and pigs, human immunodeficiency virus originated in chimpanzees, and hantaviruses spillover from rodents. Decades of wildlife surveillance efforts have revealed that bats can carry a wide range of viruses, including rabies, Marburg, henipaviruses, sarbecoviruses (subgenus of coronavirus) found across Asia, Africa, and Europe, and orthohantaviruses [1,2]. Since 2000, viruses similar to those found in bats have spilled over into the human population and has resulted in multiple, severe viral outbreaks, including severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the resulting COVID-19 pandemic.

Despite the increasing research interests in bats and the pathogens they carry, there is a significant lack of laboratory tools for these animals. One confounding factor is the enormous diversity of bats, with over 1,480 unique species spread across six continents [3]. The first bat cell line was derived from the lungs of an adult female Mexican free-tailed bat (*Tadarida brasiliensis*) in 1965 by Kiazef and colleagues and was made available through the ATCC cell line collection that year [4]. In the 50 years since then, over 130 cell lines have been generated from 44 bat species; however, only 3 additional bat cell lines have been made publicly available to researchers (R05T, R06E, EfK3; [S1 Table](#)). Therefore, most bat species are understudied, leaving large knowledge gaps regarding how viruses transmit between bats and the animals they encounter. While some viruses are known to be carried by individual bat species, others are frequently shared between taxa [5]. Furthermore, comparative analyses show both variable and conserved components of bat immune pathways [6], which may suggest that bat-associated viruses may have evolved many mechanisms to evade the host immune response. As researchers discover novel viruses in bats, it is imperative that laboratory tools accommodate the growing need to study these viruses and their spillover potential.

Another challenge to developing bat laboratory resources includes the species-specific interactions that often occur between viruses and their hosts. While some viruses may be able to replicate in multiple species, most viruses, including those found in bats, are species-specific and do not replicate efficiently or at all with commonly available laboratory reagents. For instance, Hendra virus was observed to have higher infection efficiency in cells derived from its natural reservoir, the black flying fox (*Pteropus alecto*), compared to Nipah virus which is primarily found in the large flying fox (*Pteropus vampyrus*) [7]. Additionally, Ebola-virus and Marburg virus have similar replication kinetics in cells derived from the Egyptian fruit bat (*Rousettus aegyptiacus*), the natural host for Marburg virus [8]. Meanwhile, modeling of Ebolavirus infection in the Jamaican fruit bat (*Artibeus jamaicensis*) demonstrated systemic infection and oral shedding of the virus, but this was not observed for Marburg virus [8]. These studies emphasize the role of innate host factors and varied immune responses triggered in different bat species, where viruses may exploit or antagonize these responses in the bat species they have co-evolved with. Thus, to truly study how bat viruses interact with their natural reservoir hosts, it is essential to study these viruses in experimental systems that closely mimic their natural environments.

Development of antiviral drugs and therapeutics requires an intricate understanding of virus–host interactions, which can only be derived from laboratory experiments in either animal- or cell culture-based models. Here, we developed new bat cell lines and laboratory reagents from a panel of tissues from the Seba's short-tailed bat (*Carollia perspicillata*). These cell lines were screened for their ability to support infection by a diverse panel of viruses representative of viral genera associated with this species, including alphacoronaviruses, betacoronaviruses, and orthohantaviruses [1]. These cell lines were further developed for

routine mammalian cell culture and experiments to study bat viruses and innate immune responses. Our studies demonstrate that the resulting *C. perspicillata* cell lines are immunocompetent and mount a protective antiviral response upon double-stranded RNA (dsRNA) stimulation, making them suitable for future studies of bat-virus interactions and bat immunity. In addition, the kidney-derived cell lines are susceptible to a range of viruses, including Middle Eastern respiratory syndrome coronavirus (MERS-CoV), vesicular stomatitis virus (VSV), and orthohantaviruses. Notably, these cells support Andes orthohantavirus replication, which is closely related to other orthohantaviruses naturally found in these bats [9,10].

Results

Carollia perspicillata primary cultures are susceptible to entry by diverse viruses

We set out to develop bat-derived cell lines specifically for virological research with several criteria for the resulting product: (1) the cells should originate from a species that can be bred in captivity and studied under laboratory conditions, (2) the genome sequence for the species should be available, (3) the cells should grow under standard mammalian cell culture conditions to ensure accessibility, (4) the cells should have an intact innate immune response in order to gain insight into virus-host interactions, and (5) for virological relevance, the cells should support infection with viruses that are native to the bat species.

With the first and second points of our selective criteria in mind, we obtained tissues collected from a single, 3-year-old male *C. perspicillata* (Cp) bat from a disease-free captive colony housed in the Washington State University campus in Vancouver, Washington (Fig 1A). Brain, lung, kidney, liver, and spleen tissues were frozen until processing, and primary cells were isolated following standard protease digestion and culturing techniques (see Methods [7,11–14]). We then generated a panel of diverse viral glycoproteins representative of different viral families, entry pathways, and zoonotic origin. Additional focus was given to orthohantaviruses as close relatives of Andes orthohantavirus have been identified in *C. perspicillata* [9,10,15]. Primary Cp cells were susceptible to entry by many of the tested pseudoviruses, with the hantavirus and VSV glycoproteins mediating the strongest entry (>100-fold over background signal; Fig 1B). Notably, viral entry observed in primary cells was similar in intensity to several standard laboratory cell lines including Huh-7, Vero E6, and HEK-293T cells transduced to express coronavirus receptors, human dipeptidyl peptidase 4 (hDPP4) and human angiotensin converting enzyme 2 (hACE2) (Fig 1C), as well as an immortalized Jamaican fruit bat (*A. jamaicensis*) cell line we developed previously (S1A Fig) [16]. In contrast, several other bat cell lines that we developed in earlier studies, including immortalized kidney cells from the big brown bat (*Eptesicus fuscus*) and the Egyptian fruit bat (*R. aegyptiacus*) [14,17] were less susceptible to viral entry than the *Carollia* cells (S1B–D Figs).

Immortalization of *C. perspicillata* cell lines through diverse methods

While primary cells more faithfully mimic tissue-specific gene expression profiles, they are limited in their number of cell divisions and passages and are not a homogenous population. Not surprisingly, most molecular virology studies are performed in immortalized cell lines that divide indefinitely and can therefore be easily shared among laboratories. Several methods have been used by various groups to immortalize primary cells. Viral proteins, such as the large T-antigen from SV40, inhibit tumor suppressor proteins like TP53 and Rab and shut down inhibition of cell division that normally follows in later passage primary cells [18,14]. Similarly, the *Myotis polyomavirus* T antigen (MyPVT) has been shown to enhance DNA replication in Vero cells and immortalize *E. fuscus* kidney cells, though the mechanisms of action

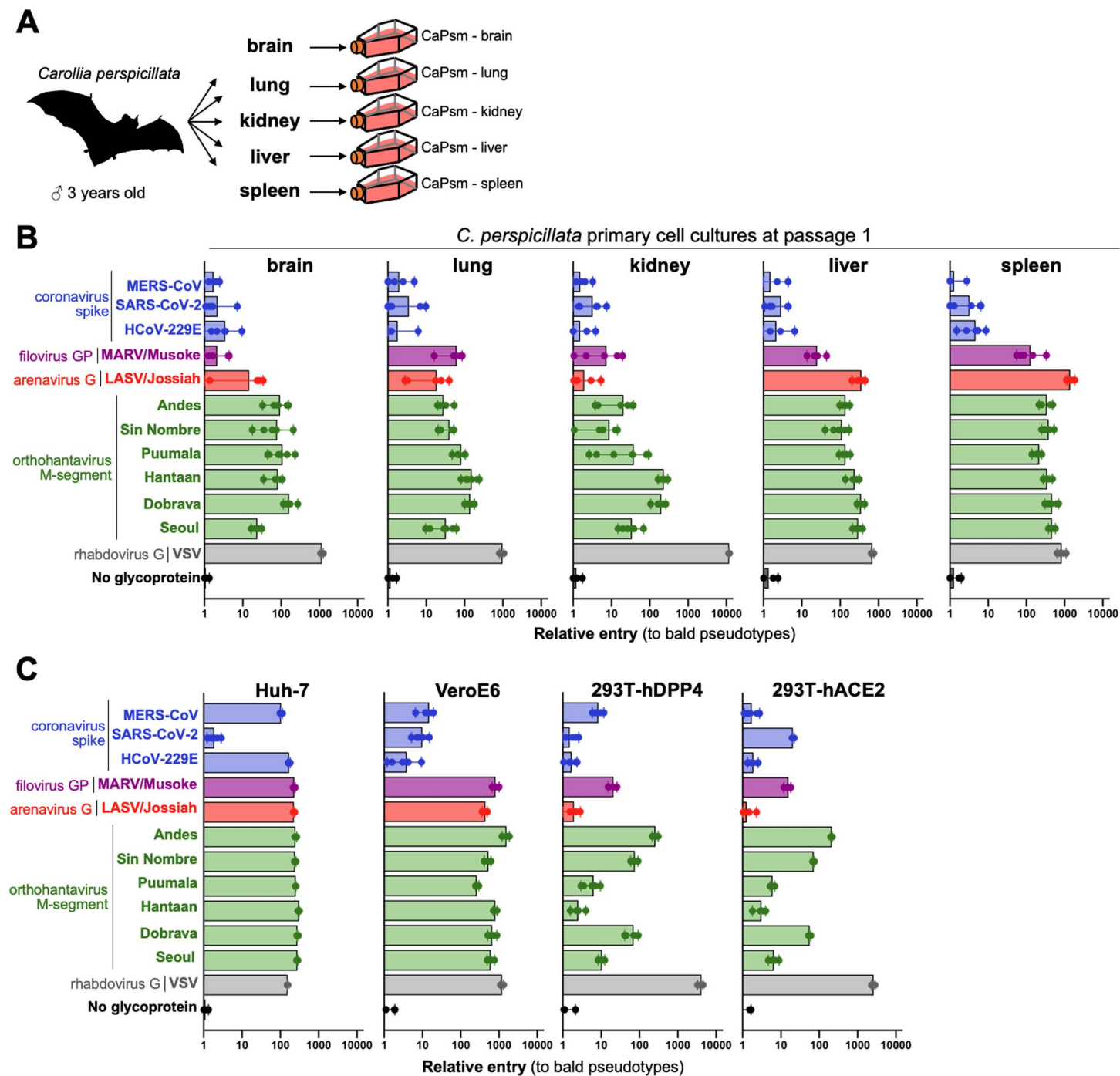


Fig 1. *Carollia perspicillata* primary cultures are susceptible to entry of diverse viruses. (A) Primary cells were isolated from brain, lung, kidney, liver, and spleen of a single *C. perspicillata* bat and (B) infected with pseudotypes bearing glycoproteins from the indicated viruses. (C) Cell entry phenotypes of common laboratory cell lines to viral entry with pseudotype panel. Shown are results for six infection replicates per virus. Bat silhouette image produced by Roberto Díaz Sibaja (PyloPic). The data underlying this figure can be found in the [S1 Data](https://doi.org/10.1371/journal.pbio.3003098.g001) file available from the journal.

<https://doi.org/10.1371/journal.pbio.3003098.g001>

are unclear [14]. Over-expression of human telomerase reduces the genomic damage from continual cell divisions and has been used to immortalize bat cells previously [7,19]. Direct knockout of the tumor suppressor, *TP53*, is yet another strategy to develop life-extended cells

that has been employed more recently with the development of Cas9-targeted gene modification [20–23]. We tested all four strategies with multiple primary cell cultures using either in-house generated or commercially obtained lentivirus transduction systems (see [Methods](#)). Notably, while some methods were effective across all cell types tested, others led to cell cycle arrest following lentiviral transduction, preventing further characterization ([Fig 2A](#)). SV40 T-antigen successfully immortalized all four cell lines, whereas MyPV T-antigen was only effective for lung and kidney cells. In addition, immortalization by TP53-guided Cas9 was successful for all cell lines except those from the lung, while hTERT could only be introduced into spleen cells.

As we and others have noted, most of the immortalized cells were less susceptible to viral entry than their parental primary cells ([Fig 2B–2E](#)) [7]. A few notable exceptions to this trend included *Cp* brain cells transduced with TP53-guided Cas9 (CaPsm-B gRNA:TP53), which showed high susceptibility (>100-fold over background) to orthohantaviruses ([Fig 2B](#)), and

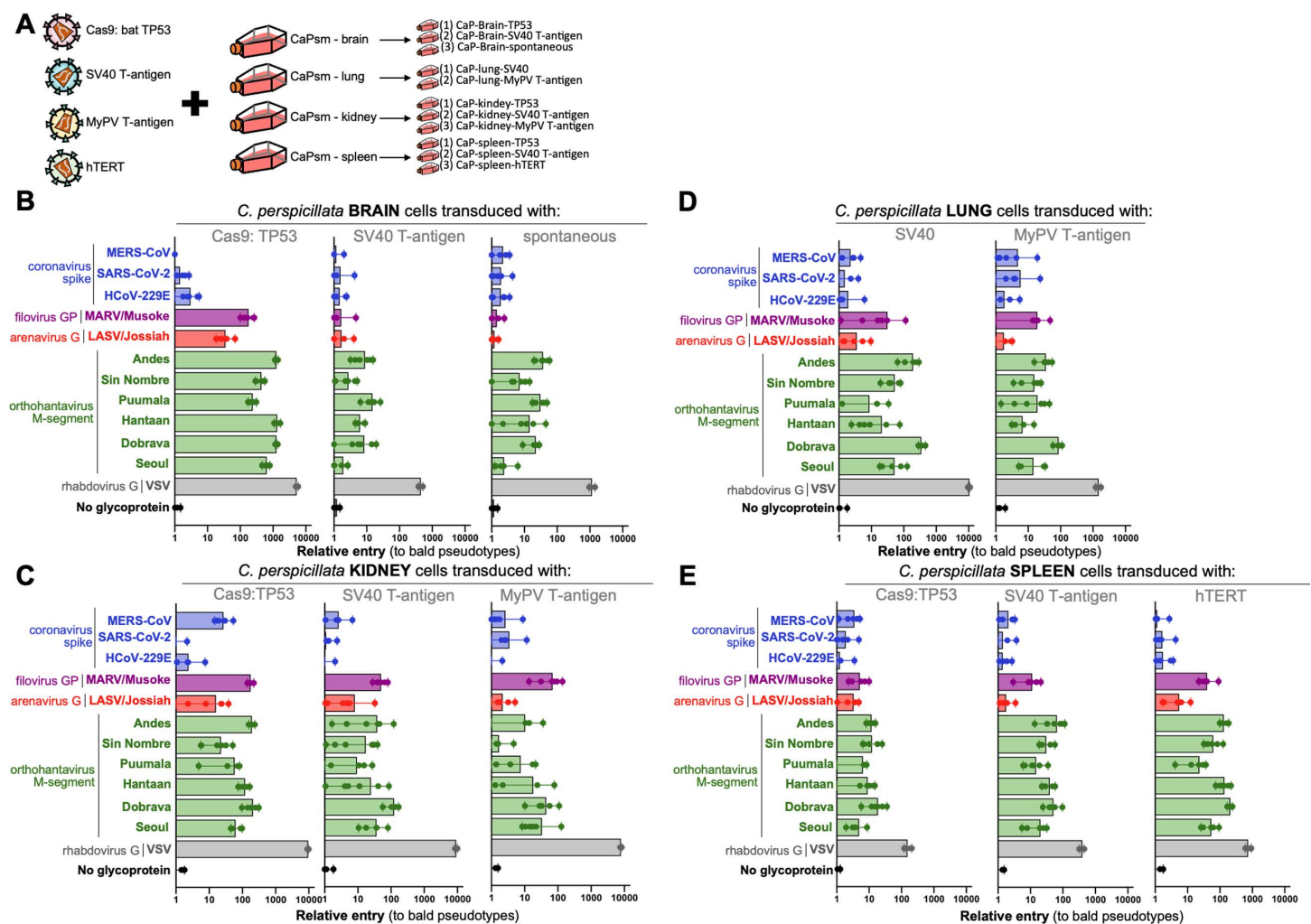


Fig 2. *Carollia perspicillata* cell lines immortalized through diverse methods are susceptible to viral entry. (A) Select primary cells were further transduced with lentiviral vectors representative of different strategies to immortalize cell lines. Transduced (B) brain, (C) lung, (D) kidney, and (E) spleen cells were screened for entry with pseudotyped particles bearing glycoproteins from the indicated viruses. Shown are the data from infection experiments performed in six replicates. The data underlying this figure can be found in the [S1 Data](#) file available from the journal.

<https://doi.org/10.1371/journal.pbio.3003098.g002>

kidney cells transduced with TP53-guided Cas9 (CaPsm-K gRNA:TP53), which showed measurable susceptibility (>10-fold over background) to MERS-CoV (Fig 2C). To test whether the cells retained their viral susceptibility over time, we performed entry assays on cells that were maintained for up to 31 passages (S2 Fig). Unfortunately, many of the transduced cells did not continually divide suggesting the approaches taken did not lead to immortalization. Three out of four of the immortalized cells evaluated broadly lost susceptibility to pseudovirus entry, with only the CaPsm-K gRNA:TP53 cell line exhibiting an increase in susceptibility to MERS-CoV spike pseudotyped virus (S2 Fig). Unfortunately, even transduced cell lines that propagated well eventually lost susceptibility to pseudotype entry. Transduced kidney cell lines retained susceptibility to pseudotype entry over multiple passages and were therefore selected for further testing and development (S2 Fig).

***C. perspicillata* cells support infection with replication-competent recombinant VSV (rVSV)**

To further assess if these primary cells were suitable for viral entry experiments, we tested a subset of primary cells for their ability to support infection with replication-competent rVSV. VSV bearing its native glycoprotein or glycoproteins from Andes and Hantaan virus efficiently replicated in kidney cells immortalized with SV40-T antigen (CaPsm-K SV40T) (Fig 3A). To assess if Andes virus entry in these cells is mediated by a known hantavirus receptor, Protocadherin-1 (PCDH1), we incubated rVSVs with soluble PCDH1 (soluble extracellular cadherin domains 1 and 2, sEC1–2) to verify if entry into CaPsm-K SV40T cells is mediated by this cellular receptor. Human osteosarcoma U2OS cells were used as a positive control. Similar to U2OS cells, a reduction in Andes virus glycoprotein-mediated infection was observed in the CaPsm-K SV40T cells (Fig 3B and 3C). Notably, Hantaan virus was not inhibited in either cell line, in agreement with previous work showing that this virus does not use PCDH1 as a receptor [24]. However, preincubation of cells with a human PCDH1-specific antibody (mAb-3305) that has been shown to block PCDH1-dependent infection of Andes virus previously [24], did not block Andes virus glycoprotein-mediated infection in CaPsm-K SV40T cells but did so in human pulmonary microvascular endothelial cells (HPMEC) (Fig 3D). To determine if this was due to the rapid recycling of mAb-3305, the PCDH1-specific antibody was preincubated with CaPsm-K SV40T cells on ice (Fig 3E). Preincubation of the CaPsm-K SV40T cells with mAb-3305 did not block infection, suggesting that this antibody may not be cross-reactive or alternatively, Andes virus can use an alternate entry receptor in these bat cells.

Immortalized cells respond to poly(I:C) stimulation

To test the immunocompetency of the *Cp* kidney cell lines, we first measured the transfectability of both primary and immortalized kidney cell cultures using poly(I:C) (surrogate viral dsRNA) labeled with rhodamine (poly(I:C)-Rho) (Fig 4A). Poly(I:C)-Rho was successfully delivered in all cell lines, with the CaPsm-K gRNA:TP53 cell line demonstrating the highest uptake of the immunostimulant (Fig 4A). As immortalization has been speculated to alter the ability of cell lines to have an intact interferon response [25], we characterized the response of our bat cells to poly(I:C) by measuring interferon beta (*IFN* β) and interferon-induced protein with tetratricopeptide repeats 1 (*IFIT1*) transcript levels using qPCR to assess the detection of dsRNA and the type I IFN signaling pathway (Fig 4B). All four immortalized kidney cell lines upregulated *IFN* β and *IFIT1* transcripts to similar levels, demonstrating the recognition of dsRNA and induction of antiviral genes. To assess whether the *IFN* β response was effective in blocking virus infection, we stimulated cells with poly(I:C) prior to infection with VSV that was engineered to express the green fluorescent protein (VSV-GFP) (Figs 4C and 4D). While VSV-GFP replicated efficiently

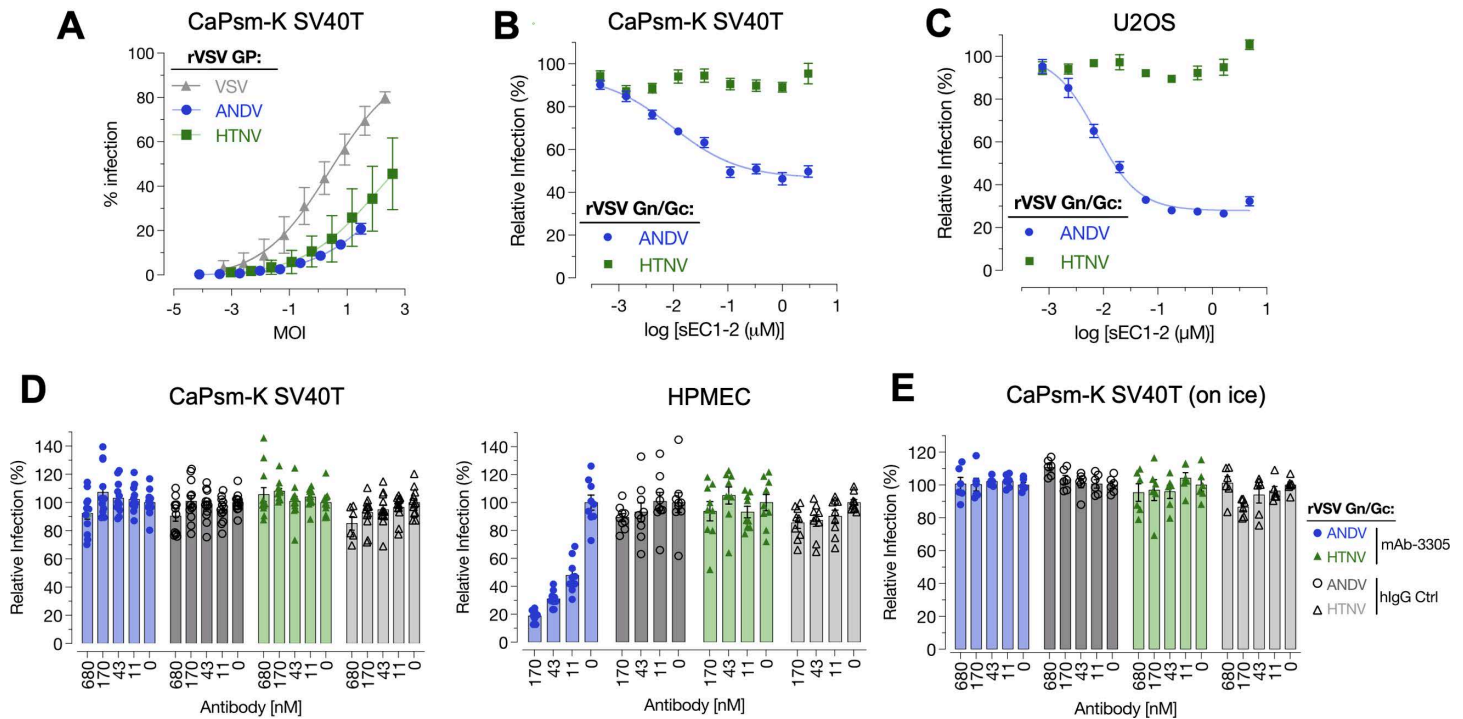


Fig 3. *Carollia perspicillata* cells support infection with replication-competent recombinant VSV (rVSV). (A) *Cp* kidney cells immortalized by SV40 T-antigen (CaPsm-K SV40T) were infected with replication-competent VSV (rVSV) pseudotyped with indicated orthohantavirus glycoproteins. Neutralization of rVSV ANDV and HTNV with soluble PCDH1 receptor (sEC1-2) on *Cp* kidney cells (B), and human U2OS cells for comparison (C). (D) Neutralization of rVSV-ANDV and HTNV on *Cp* kidney cells by preincubation of cells with an anti-PCDH1 (mAb-3305) antibody. Neutralization of rVSV ANDV by mAb-3305 on human pulmonary microvascular endothelial cells (HPMEC) is shown as a positive control. (E) To rule out the possibility that mAb-3305 fails to block rVSV-ANDV infection in *Cp* kidney cells due to rapid uptake/clearance of the antibody, the experiment was performed on ice. The data underlying this figure can be found in the [S1 Data](#) file available from the journal.

<https://doi.org/10.1371/journal.pbio.3003098.g003>

in both primary and immortalized kidney cells, CaPsm-K gRNA:TP53 cells were the least permissive. However, pretreatment with poly(I:C) completely inhibited virus replication. Immunoblotting for IFIT1, an interferon-stimulated gene (ISG), confirmed that poly(I:C) induced the expression of IFIT1 in infected and uninfected cells (Fig 4E).

Our entry results using pseudotyped virus suggested that the *Cp* kidney cell lines expressed the necessary factors for MERS-CoV entry (Fig 2C). Therefore, we repeated our poly(I:C) experiment but replaced VSV with authentic MERS-CoV (Fig 4F). Like VSV-GFP, all cell lines were permissive to infection with MERS-CoV, with the virus replicating to lower titers in CaPsm-K gRNA:TP53 cells. Stimulation with poly(I:C) prior to infection reduced MERS-CoV replication, with the most substantial reduction observed in kidney cells immortalized using MyPVT. IFIT1 analysis through immunoblotting demonstrated similar expression of IFIT1 in cells pre-stimulated with poly(I:C), with IFIT1 not induced in cells only infected with MERS-CoV (Fig 4G). Taken together, these results demonstrate that *C. perspicillata* kidney cells are immunocompetent, warranting further study of their responses to infection.

Immortalized clonal cell lines retain susceptibility to viral entry

Our entry results suggest that the *C. perspicillata* kidney cell lines transduced with TP53-guided Cas9 are susceptible to entry via the widest range of viral glycoproteins of all the cell lines developed (Figs 2C and S2). Importantly, despite immortalization, these cells also

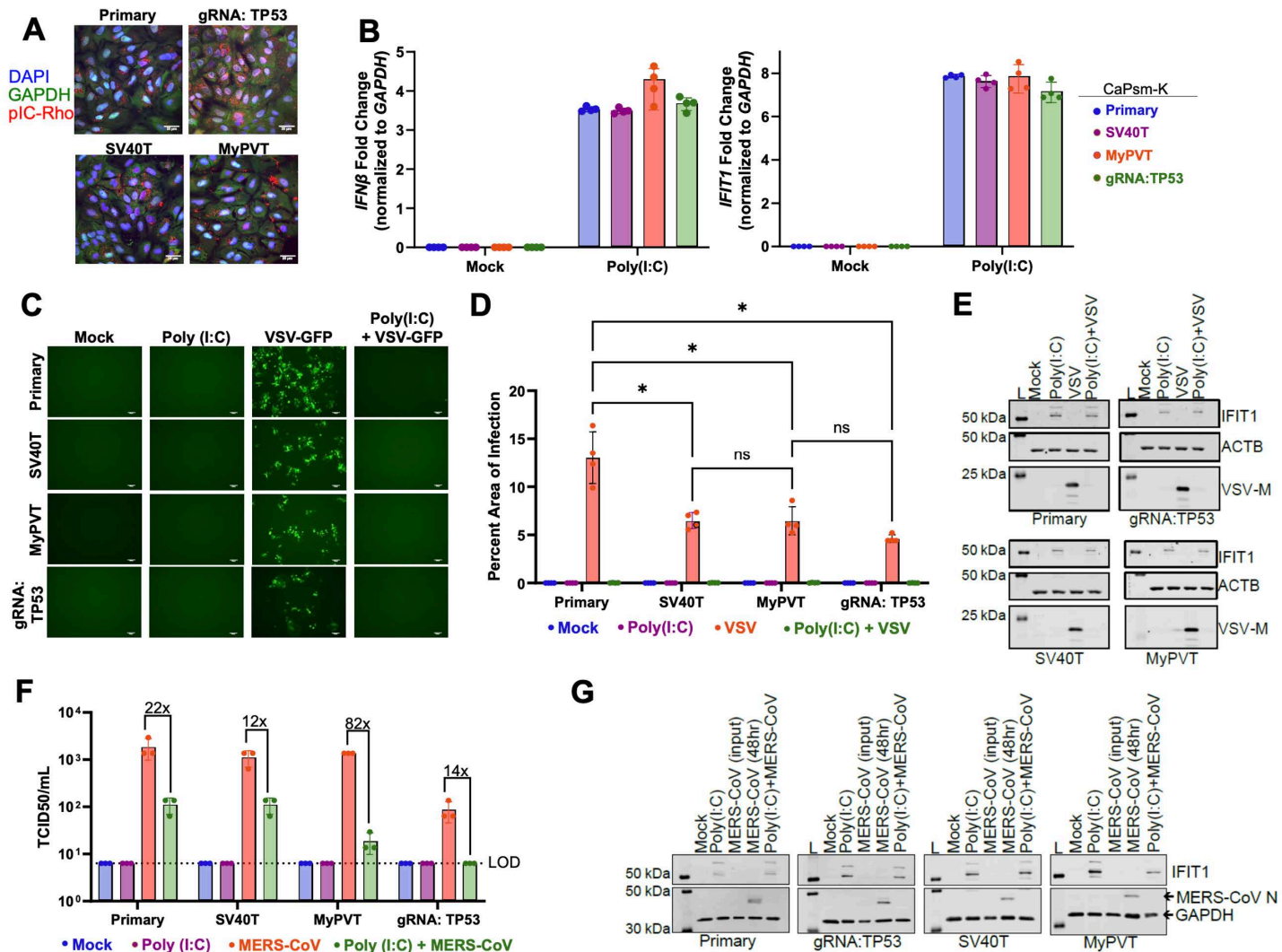


Fig 4. Immortalized *Carollia* kidney cells respond to surrogate virus infection. (A) CaPsm-K primary and immortalized cells were transfected with 10 μ g of poly (I:C) chemically labeled with rhodamine (pIC-Rho) for 16 h. Following fixation, cells were stained with antibodies against GAPDH and DAPI and visualized by confocal microscopy. Scale bars represent 25 μ m. (B) CaPsm-K cells were transfected with 100 ng of poly (I:C) for 8 h. The upregulation of *IFN- β* and *IFIT1* transcripts was assessed by qPCR. Data are represented as a mean $\pm$ SD, $n = 4$ replicates. (C) CaPsm-K cells transfected with 100 ng of poly (I:C) for 8 h were infected with VSV-GFP (MOI 1) for 16 h ($n = 4$). Viral replication was visualized by fluorescent microscopy. Scale bars represent 100 μ m. (D) Green fluorescent protein (GFP) signal was quantified using ImageJ (Two-way ANOVA with Šídák's multiple comparisons test). (E) Representative western blot of VSV-GFP infected cells, where IFIT1, VSV-M, and ACTB were probed for. L = molecular weight ladder. (F) CaPsm-K cells transfected with 100 ng of poly(I:C) for 8 h were infected with MERS-CoV (MOI 0.1) for 48 h ($n = 3$). Supernatant was collected and TCID₅₀ assay was performed to assess viral titer. (G) Representative western blot of MERS-CoV infected cells, where IFIT1, MERS-CoV nucleocapsid (N), and GAPDH were probed for. L = molecular weight ladder. The data underlying this figure can be found in the [S1 Data](#) file available from the journal.

<https://doi.org/10.1371/journal.pbio.3003098.g004>

retained their interferon response (Fig 4). We observed that later passage *Cp* TP53-guided cells were more susceptible to MERS-CoV than earlier passages (S2 Fig), and that this entry phenotype was more variable throughout the course of this study. Therefore, we wondered whether the *Cp* cells represented a mixed population of susceptible and non-susceptible cells. To help address this question, we isolated individual cells through limiting dilution plating (Fig 5A; see Methods). Twenty clones were selected from the limiting dilutions for their ability to form dense monolayers. From these 20 clones, only five retained the ability to divide from one cell

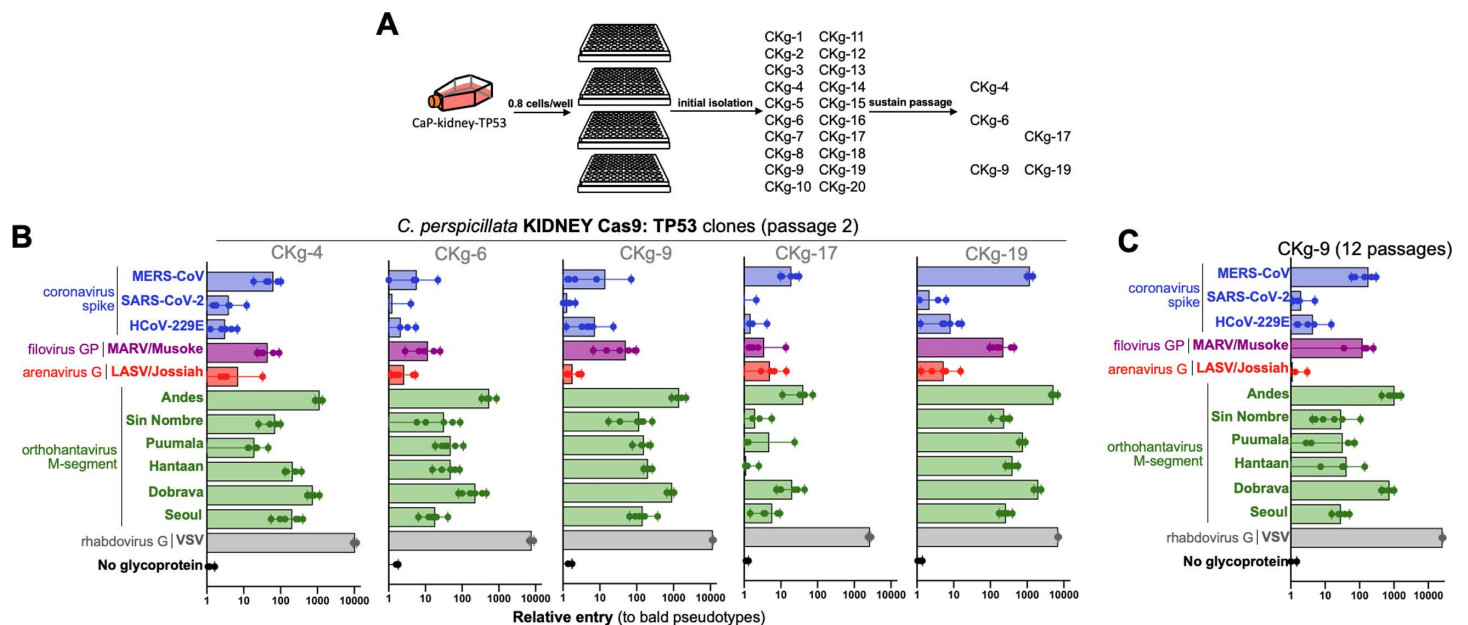


Fig 5. Immortalized clonal cell lines retain susceptibility to viral entry. (A) Select transduced cells were further plated for clonal isolation and 20 clones were propagated from single cells plated in 96-well format. (B) Clones that continuously grew were further screened for suppressibility to viral entry with pseudotypes bearing glycoproteins of the indicated viruses. (C) Clones that grew past 10 passages were re-screened for susceptibility to viral entry. Shown are the results from infection experiments performed in six replicates. The data underlying this figure can be found in the [S1 Data](#) file available from the journal.

<https://doi.org/10.1371/journal.pbio.3003098.g005>

through plating for infection. Testing the viral pseudotype panel on these cells revealed minor variation in viral susceptibility between clones and an overall improvement in entry from the heterogeneous parental population (Fig 5B). Of the five clones that were tested, only a subset continuously grew in culture, with clone-9 (CKg9) exhibiting the best growth and highest susceptibility to viral entry through continuous passage (Fig 5C).

Immortalized *Carollia* cells support whole virus replication

To further assess the suitability of these cells for virological experiments, we infected primary and immortalized *Cp* kidney cells with authentic VSV-GFP (MOI 1), MERS-CoV (MOI 0.1), and SARS-CoV-2 (MOI 0.01) for 1–72 h (Fig 6A). Upon infection with VSV-GFP, all cell lines supported virus replication, with viral titers peaking at 24 h in the primary cells. However, VSV-GFP titers did not peak until 48 h in CaPsm-K SV40T and MyPVT cells, and until 72 h in gRNA:TP53 cells. Similarly, CaPsm-K gRNA:TP53 cells were the least permissive to MERS-CoV, with titers remaining below 10^2 TCID₅₀/ml by 72 h of infection (Fig 6A). Meanwhile, similar replication kinetics were observed for infected primary and SV40T-immortalized cells, with MERS-CoV titers peaking around 10^3 TCID₅₀/ml by 72 h of infection. For cells immortalized using MyPVT, infection peaked and plateaued at 48 h. Consistent with our previous data which demonstrated that the *Cp* kidney cells do not permit entry of pseudoviruses displaying the SARS-CoV-2 spike protein (Fig 2C), infection with authentic SARS-CoV-2 was not supported in the primary or immortalized kidney cell lines (Fig 6A).

As orthohantaviruses related to Andes virus have been reported in *C. perspicillata* [9,10,15], we subsequently tested the infectivity of Andes virus in the clonal transduced CKg9 cell line and observed day-over-day increases in the viral genome suggestive of viral replication (Fig 6B). Infection of these cells with Seoul virus, another rodent-borne orthohantavirus,

allowed for detection of Seoul nucleocapsid protein in the cell lines (Fig 6C), suggesting that the Ckg9 cell line supports Seoul virus replication.

Discussion

Some bat species have been known to carry viruses that can infect people since the early 1930s, when Joseph Pawan first described transmission of rabies virus from the common vampire bat (*Desmodus rotundus*) to humans in Trinidad [26,27]. Since these initial findings, outbreaks of bat-derived viruses in humans have become an unfortunate but more frequent occurrence. The COVID-19 pandemic and subsequent identification of several closely related viruses in bats underscores the urgency of understanding bats and the viruses they carry, particularly the mechanisms underpinning asymptomatic carriage to inform the development of effective therapeutics [28–30].

Our knowledge of viruses has derived from our ability to study them in the laboratory. Despite nearly a century of bat virus research, there are very few standardized tools available for researchers to study bat viruses. Bat-derived cell lines are essential to studying molecular interactions between bats and their viruses. While several bat cell lines have been developed by various groups, most of them are not publicly available, and the few that are do not support replication of most viruses [7,11,31–33]. For example, while Chinese rufous horseshoe bats (*Rhinolophus sinicus*) harbor viruses related to SARS-CoV, primary and immortalized cell

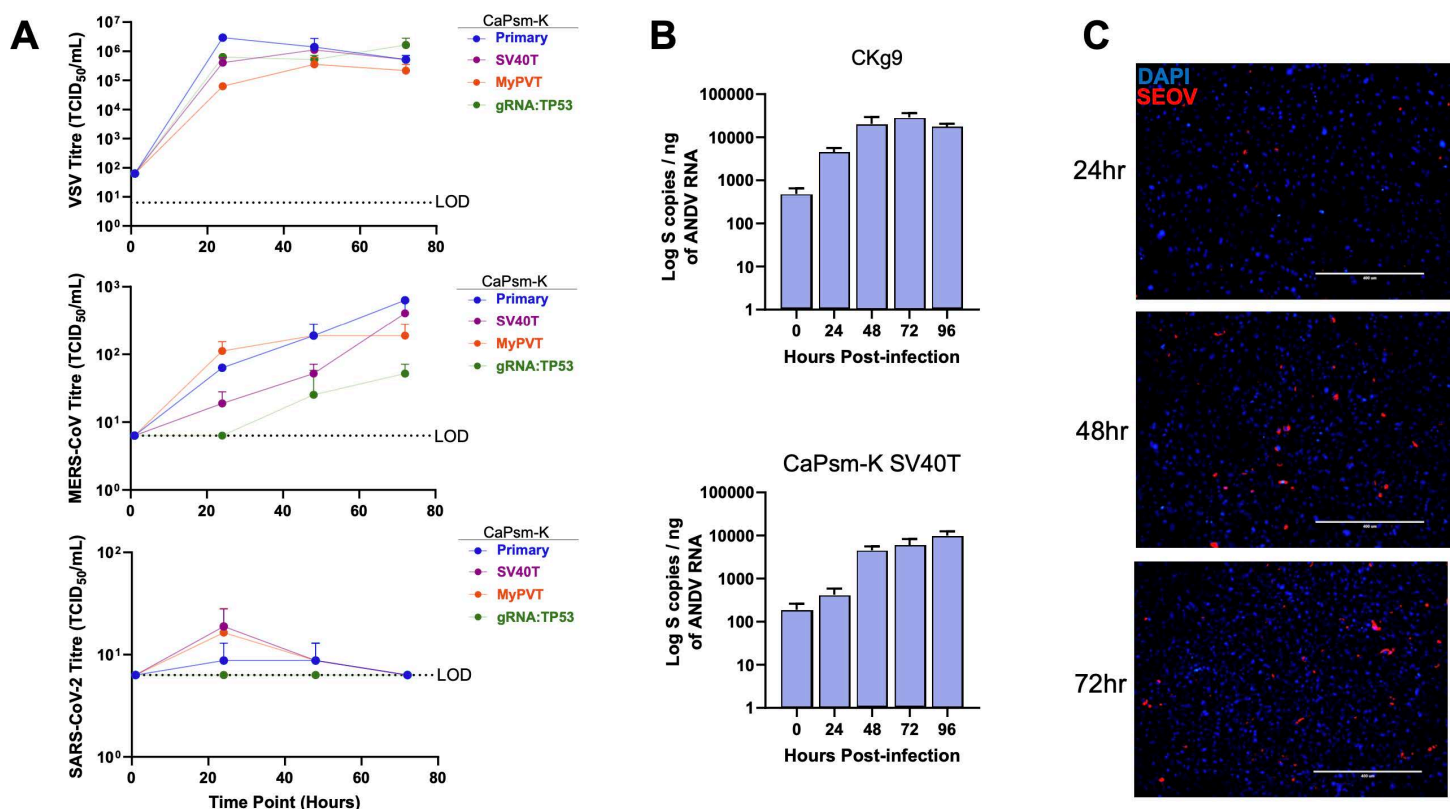


Fig 6. Immortalized *Carollia* cells support whole virus replication. (A) Primary and immortalized kidney (CaPsm-K) cells were infected with VSV-GFP (MOI 1), MERS-CoV (MOI 0.1), or SARS-CoV-2 (MOI 0.01) for up to 72h. Replication was monitored using TCID₅₀ limiting dilution assays. (B) Clonal transduced kidney cells (CKg9) cells and CaPsmK-SV40T cells infected with Andes virus were monitored by qPCR. (C) CKg9 cells infected with Seoul virus (SEOV) were monitored by end-point microscopy for Seoul nucleocapsid protein. The data underlying this figure can be found in the [S1 Data](#) file available from the journal.

<https://doi.org/10.1371/journal.pbio.3003098.g006>

cultures from these animals do not support SARS-CoV or SARS-CoV-2 infection [11,31]. Bat cell lines that do support viral replication often remain in the lab that develops them. Without common laboratory reagents available to more researchers, the long-standing question of how bats tolerate viruses that are otherwise pathogenic in other animals may remain elusive. Here, we developed new bat cell lines, tested methods to immortalize and subclone them, introduced gene disruptions, and measured innate interferon responses. Importantly, because virological studies are the goal of our cell lines, we continually assessed their ability to support viral entry and replication throughout the study and discontinued the use of cells that lost this ability. The resulting cells grow under standard laboratory conditions with accessible and cost-effective cell culture media and support viral growth and innate immune responses. Changes in cell gene expression are a major hurdle for developing cell lines. As mammalian cell cultures age, their expression profile changes over time [34]. Routine laboratory cell culture includes the use of fetal-bovine serum, which does not contain species-specific growth factors for bat cells, or other non-bovine species. In addition, immortalization of cell lines is also speculated to impact cellular process, with the success of the technique employed dependent on the cell [35]. Thus, we performed multiple immortalization techniques to account for potential impacts on cellular responses and to increase our success rate of obtaining an immortalized cell line. Our results demonstrate that immortalization with the SV40 T-antigen was compatible with all *Cp* cell lines, followed by the knockout of TP53 (Fig 2A). While we successfully obtained cell lines with extended lifespans, further work is required to confirm the processes that facilitated their immortalization. Notably, we generated 11 immortalized cell lines but observed differences in viral susceptibility between primary, early- and late-passage immortalized cells (Figs 1, 2, and S2). As these cells were all derived from the same individual, and share similar interferon response profiles, this variation in cell phenotypes—in particular specific coronavirus entry profiles—suggests shifts in receptor expression, which has been documented for coronavirus receptors in other bat cell lines [36]. Nevertheless, some cell lines retained their susceptibility and additional screening of clonal immortalized derivatives allowed us to identify cell lines that retained susceptibility to coronaviruses (Fig 2). Thus, receptor downregulation appears stochastic and not necessarily a factor of cell culture age. Taken together, these findings should be taken into consideration when developing animal derived cell lines, with the inclusion of clonal screening to identify key subpopulations of cells with desired laboratory properties.

The *Carollia* cell lines developed in this study supported entry for all the pseudotyped orthohantaviruses tested in our assays and could support infection with *bona fide* Andes and Seoul viruses (Figs 1, 3, and 6B–6C). The receptor for New World orthohantaviruses, which includes close relatives of orthohantaviruses found in *Carollia* bats, has been shown to be PCDH1 [24]. The soluble receptor (sEC1–2) experiments show that PCDH1 may mediate Andes virus entry in these cells. However, the mAb-3,305 experiments suggest that either this antibody does not recognize bat PCDH1 due to sequence divergence or bat PCDH1 may not be the only receptor required for Andes virus entry in these bat cells (Fig 3). Alternatively, sEC1–2 may have blocked Andes virus entry by steric hindrance and another unknown receptor mediates hantavirus entry in these cells. Recent work has also demonstrated the expansion of the Buritense hantavirus among *Carollia* species that circulate near human communities in Brazil [37], increasing the risk of spillover. Indeed, our study and these cell lines will provide researchers with a unique opportunity to study hantaviruses in their bat hosts.

In addition to serving as hosts for hantaviruses, *Carollia* species have been identified to host betacoronaviruses [1]. While not all *Cp* immortalized cell lines supported entry of pseudoviruses expressing the SARS-CoV-2 spike protein, many permitted the entry of pseudoviruses expressing MERS-CoV spike, with the CaPsm-K gRNA:TP53 cell line exhibiting the

highest efficiency (Fig 2C). Similarly, CaPsm-K primary and immortalized cells supported authentic MERS-CoV infection (Fig 6A), which was reduced upon pretreatment with an immune-stimulant (dsRNA) (Fig 4F and 4G). Although all CaPsm-K cells were permissive to MERS-CoV, the primary cells exhibited the highest replication, whereas none supported SARS-CoV-2. Notably, while stimulation with synthetic dsRNA induced IFIT1 expression, no induction was observed following infection with VSV-GFP nor MERS-CoV (Fig 4E and 4G). This may be due to the ability of the virus to circumvent the host innate immune system, leading to the suppression of ISGs like *IFIT1*. A similar phenomenon has been recently reported for human nasal air-liquid interface cultures infected with MERS-CoV, where detection of IFIT1, Viperin, and PKR occurred only when viral proteins that antagonize the IFN response were abrogated [38]. Therefore, our *Carollia* cell lines are susceptible to immune evasion strategies employed by some viruses, suggesting that the tolerance of bats toward virus infections may be the result of a cumulative host response that needs further research.

Many of the cell lines developed in this study may be suitable for work with filoviruses and arenaviruses (Figs 1, 2, 5, and S2). While filoviruses such as Marburg have not been found in *C. perspicillata*, these viruses have been reported in other bat species and are known to use a highly conserved receptor that facilitates filovirus entry in a broad range of mammalian cell lines [39–43]. Similarly, while Lassavirus is not known to circulate in bats, genomic material from arenaviruses has been sequenced from *C. perspicillata* bats [44].

Bats are known to carry a wide range of viruses, some of which are highly pathogenic in humans or are closely related to such viruses. Remarkably, bats do not exhibit obvious signs of disease from this viral burden, with the unexplained exception of Tacaribe virus, Rabies virus, and Lloviu virus [45–47]. This tolerance to viral infection is a significant area of research, with the goal of one day adapting these mechanisms in the form of human therapies. Cell lines developed in this study will clarify virus–host interactions in *C. perspicillata* bats using viruses that these bats are speculated to naturally harbor or be exposed to like orthohantaviruses and influenza A-like viruses (H18N11).

Methods

Biosafety and ethics statement

Bat tissues were sourced from a US-based facility at Washington State University (Vancouver, WA, USA). Collection of animal tissues used in this study was approved by Washington State University IACUC (ASAF #6588). Experiments with live, replication-competent coronaviruses were performed at the University of Saskatchewan/VIDO's BSL3 facility following approved protocols and standard procedures. Experiments with live hantaviruses were performed under BSL3 conditions at the University of New Mexico. Experiments with replication defective VSV pseudotypes and replication competent rVSVs were performed at BSL2+ conditions at Washington State University and LSU Health Sciences Center-Shreveport, respectively.

Figures and depictions

All labware representations in figures were produced by the authors using PowerPoint. The Phyllostomid bat silhouette image in Fig 1A was generated by Roberto Díaz Sibaja and downloaded from PyloPic (<https://www.phylopic.org>).

Isolation of primary cells

Tissues were harvested from a male *C. perspicillata* bat at approximately 20 months of age, 17.1 g, from a disease-free colony. Tissue samples were washed and finely minced in sterile phosphate-buffered saline (PBS), transferred to fetal bovine serum (FBS) supplemented with

10% dimethyl sulfoxide, and frozen in liquid nitrogen. Primary cells were isolated as we have previously reported for other bat species [13,14]. Briefly, samples were thawed at 37 °C and transferred to sterile 10 cm plates. FBS/DMSO solution was removed with a pipette and tissues were washed with PBS. Tissues were incubated in trypsin for 10 min, with gentle agitation through a 1000 µL pipette to help loosen cells from the tissue matrix. Trypsin was neutralized with primary cell culture medium consisting of high glucose Dulbecco's modified Eagle's medium (DMEM; Corning), 12% FBS, penicillin/streptomycin, amphotericin B, L-glutamine, and minimum essential medium non-essential amino acids. The slurry of cells, tissue debris, and media were transferred to 75 cm² flasks, labeled "passage 0" and maintained at 37 °C with 5% CO₂. Flasks were monitored daily for cell growth and media was refreshed every 2 days. Flasks that achieved 70%–90% confluency with primary cell cultures were expanded into larger 150 cm² flasks for banking stocks of early passage cells and future experiments. Primary cells were used up to passage 10 for viral infection assays in Fig 4.

Immortalization of primary cells

Immortalization of bat cells with the large T-antigen from SV-40 virus and the human telomerase gene (hTERT) have been previously described [7,13]. Briefly, lentiviruses encoding each of these proteins and puromycin N-acetyltransferase were obtained commercially and used following manufacturers' instructions (GenTarget). Lentiviruses were generated using the second-generation system, where 500 ng of psPAX and pMDG2 and 1 µg of either construct were transfected into HEK293T cells. Following 48-h post-transfection, medium was collected, centrifuged, and stored at –80 °C. *Cp* cells were transduced with lentiviruses encoding either SV40 T-antigen or hTERT and then maintained in a primary cell culture media (see above) with puromycin at between 0.5 and 1.5 µg/mL depending on the cell source for two passages. After bulk, untransduced cell die-off, the media was changed to a standard maintenance media consisting of high-glucose DMEM supplemented with 10% FBS, L-glutamine, and penicillin streptomycin. MyPVT is an ortholog of the SV40 T-antigen, but from a bat polyomavirus and has been previously described [14]. MyPV T-antigen was amplified from the *M. polyomavirus* whole genome (NCBI Accession number NC 0113101.1) and cloned into a lentiviral plasmid. Cells transduced with MyPVT lentivirus were maintained in standard 10% FBS DMEM. TP53 KO was inspired by previous studies that used human, mouse, and canine cells [21–23]. At the time of designing this study, the sequenced genome for *C. perspicillata* was still in development. Therefore, we used the *R. aegyptiacus* (GenBank: (NW_023416309.1:73376916-73388351) and *A. jamaicensis* (GenBank: (NW_026521968.1:7682714-7693614) genomes to design a series of Cas9-guide RNAs directed toward the exon-intron junctions of exons 1 and 5 of the bat TP53 tumor suppressor gene. Four resulting gRNAs were designed:

RA TP53 – EXON 1 – FORWARD: 5'-CTTGTGGAACTGTGAGTAG-3'

RA TP53 – EXON 5 – REVERSE: 5'-TCCACCCGGATAAGATGCTG-3'

AJ TP53 – EXON 1 – REVERSE: 5'-TGACTCATTGGTGGCTCCAC-3'

AJ TP53 – EXON 5 – FORWARD: 5'-GGTGCCCTACGAAACACCTG-3'

Guide RNAs were cloned into a lentiviral Cas9/CRISPR vector (addgene #52961) [48] and used to produce VSV-g pseudotyped lentiviral particles in 293T cells. Cas9:bat TP53 lentiviruses were collected 48 h later, filtered through 0.45 µm mixed cellulose ester filters, and stored at –80°C until use. Cells were transduced with a combination of all four lentiviruses and maintained in standard media consisting of high-glucose DMEM supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin. All transduced cells were expanded in large culture flasks for stock banking shortly after transduction and, when applicable, puromycin selection.

Clonal cell lines

Early passage stocks of *C. perspicillata* kidney cells transduced with Cas9:bat TP53 lentivirus were thawed and maintained for one passage before seeding at low density of 0.8 cells/well in 96 well plates. Wells were monitored over two weeks for single-cell isolation and growth. Twenty wells that reached 100% confluency the fastest were selected for expansion into 6-wells. Five of the 20 clones maintained continual growth to be expanded into larger culture flasks. Clonal cells were maintained in standard media consisting of high-glucose DMEM supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin. DMEM with high glucose (DMEM; Gibco, #12491015) supplemented with 10% FBS (Sigma-Aldrich, #12107C) and 1× antibiotic antimycotic (Gibco, #15240062).

Plasmids

Glycoproteins from MERS-CoV/EMC12, SARS-CoV-2/Wuh1, HCoV-229E, and Lassavirus/Josiah were codon optimized and cloned into pcDNA3.1 as previously described [13,49,50]. Marburg/Musoke glycoprotein in pCAGGS vector was generously provided by Andrea Marzi (NIH/NIAID). Orthohantavirus M-segments from plasmids generously provided by Jay Hooper (USAMRIID) were subcloned into pcDNA3.1 using standard PCR approaches. Plasmids used for the expression of His-tagged version of sEC1–2 of human PCDH1 and mAb-3305, a human IgG targeting EC1 domain of human PCDH1, were described previously [9,10,15].

Pseudotype entry assay

Pseudotype entry assays were performed as described previously [13,49,50]. Briefly, 293Ts were seeded in poly-lysine treated 6-well plates and transfected with plasmids encoding viral glycoproteins. Twenty-four hours later, cells were infected with single cycle vesicular stomatitis luciferase-GFP dual reporter virus pseudotyped with VSV-G (VSVΔG-luc/GFP) for 1 h and washed three times with PBS to remove residual input virus. Culture supernatants containing viral pseudotypes were collected 48 h later, briefly centrifuged to remove debris, aliquoted, and stored at −80 °C. For entry assays, cells were seeded in 96-well plates and infected with equal volumes of viral pseudotypes diluted in DMEM supplemented with 2% FBS, L-glutamine, and penicillin/streptomycin. Inoculated cells were centrifuged at 1,200g, 4 °C for 1 h and then transferred to 37 °C incubators with 5% CO₂. Luciferase was measured as a readout for cell entry (BrightGlo; Promega), and data were normalized by comparing raw luciferase signal from pseudotyped viruses against luciferase signal from non-pseudotyped negative control particles [13,17,51,52].

rVSV infection assays

Titers of the stocks of rVSVs expressing Andes virus (ANDV) Gn/Gc, Hantaan virus (HTNV) Gn/Gc, or VSV G glycoproteins as their sole entry proteins were determined on Vero cells to determine multiplicity of infection (MOI) for infection experiments on *Cp* cells. Vero cells plated in 96-well plates (8,000 cells per well) were infected with serial log dilutions of rVSVs. At 8-h post-infection, the cells were fixed using 2% formaldehyde for 15 min at room temperature and nuclei were counter-stained with Hoechst 33342. Infected (eGFP-positive) cells were imaged on a Cytation-5 (Agilent) and automatically enumerated using the onboard Gen5.0 software to calculate titers of the stocks. For comparison of infectivity of these rVSVs on *Cp* cells, immortalized kidney cells plated in 96-well plates (8,000 cells per well) were infected with 2-fold serial dilutions of viruses (an MOI range of 1.5–374 for rVSV Andes virus

and 0.8–207 for rVSV Hantaan virus and VSV G). At 16-h post-infection, cells were fixed and scored for infection as described above. The results were expressed as percent infection.

For neutralization of rVSV infection with sEC1–2, rVSV-ANDV Gn/Gc and HTNV Gn/Gc viruses were titrated to determine the amount of virus required to obtain 20%–30% infection as 16-h post-infection in immortalized *Cp* kidney cells and human osteosarcoma U2OS cells. These pre-titrated amounts were incubated with 3-fold serial dilutions of sEC1–2 starting at 3 μ M for 1 h at 37° C. These rVSV:sEC1–2 mixtures were then applied to monolayers of immortalized *Cp* kidney cells in 96-well plate and infection was scored at 16-h post-infection as described above. The results were expressed as relative percent infection as compared to that in well without sEC1–2 protein (infection in no sEC1–2 well was set at 100%).

The rVSV neutralization experiments of rVSV infection with an anti-PCDH1 antibody, mAb-3305 were performed similarly except that the cells were incubated with mAb-3305 or a human IgG control (Ctrl) at 11 to 680 nM concentration in 4-fold serial dilutions for 1 h at 37 °C or on ice before infection with pre-titrated amounts of rVSV-ANDV Gn/Gc or HTNV Gn/Gc. For on ice experiments, cell monolayers were prechilled on ice for 10 min before the addition of antibodies. At 16-h post-infection, the percent relative infection was estimated as described above as compared to that in the well without antibody (infection in no antibody wells was set at 100%). Primary human pulmonary microvascular cells (HPMECs) were used as positive controls for these studies.

Viruses

Genetically engineered VSV encoding a green fluorescent protein (VSV-GFP) cassette was propagated in Vero76 cells in DMEM and stored at –80 °C. MERS-CoV (isolate EMC/2012) and ancestral SARS-CoV-2 (isolate SB3) were propagated in Vero76 cells [53]. All work with infectious MERS-CoV and SARS-CoV-2 was completed at VIDO in a containment level 3 laboratory and was approved by the institutional biosafety committee. Standard operating procedures approved by the institutional biosafety committee were followed for sample inactivation. Seoul virus strain SR11 and Andes virus strain CHI-9717869 were propagated on Vero E6 cells (ATCC, CRL-1586) for 12 days. Infectious virus was isolated by harvesting supernatant and centrifuging at 1000rcf for 10 min to remove cellular debris. The rVSVs expressing Andes virus and Hantaan virus glycoproteins have been described previously [54].

Transfection

Cells were seeded at a density of 1.5×10^5 cells/well for 24 h followed by transfection with 10 μ g of poly(I:C) chemically labeled with rhodamine (Invivogen, #tlrl-picr) for 16h, or with 100 ng of poly(I:C) (Invivogen, #tlrl-pic) for 8h. Cells transfected with poly(I:C) chemically labeled with rhodamine were fixed in methanol and stained for GAPDH (EMD Millipore, #MAB374) and DAPI (Thermo Scientific, #62248) and visualized using confocal microscopy.

Whole-virus infection

Carollia kidney cells were seeded at a seeding density of 1.5×10^5 cells/well. Following 24h, cells were transfected with 100 ng of poly(I:C) (Invivogen, #tlrl-pic) for 8h prior to infection with VSV-GFP (MOI 1) or MERS-CoV (MOI 0.1) for 16h and 48h, respectively. Protein lysate was harvested and assessed by immunoblotting, while supernatant for MERS-CoV infected cells was harvested to assess viral titer by tissue culture infectious dose assay (TCID₅₀). Kidney cells were similarly seeded for VSV-GFP (MOI 1), MERS-CoV (MOI 0.1), and SARS-CoV-2 (MOI 0.01) growth curves. Supernatant was harvested following 1, 24, 48, and 72 h of infection and titered on Vero76 cells using a TCID₅₀ assay. For Seoul and Andes virus infections, cells were seeded in

cell culture vessels 18–24 h prior to infection at a target density of 70%. Virus stock was diluted to the target, cell-specific MOI using serum-free DMEM (VWR 4000-304) supplemented with 1× pen/strep, 1% nonessential amino acids, 2.5% HEPES, and cells were infected for 1 h at 37 °C. Cells were washed twice with sterile PBS solution (FisherScientific, SH30264FS), and appropriate culture medium was added for the duration of the experiment.

Tissue culture infectious dose 50 assay

Supernatants from VSV-GFP, MERS-CoV, and SARS-CoV-2 infected cells were titrated in triplicate on Vero76 cells using TCID₅₀ assay. Briefly, 3×10^4 cells were seeded per well of a 96-well plate. Following 24 h of seeding, media was removed from the cells and 50 µL of serially diluted virus-containing supernatant was added to each well for 1 h at 37 °C. Following inoculation, the supernatant was removed and replaced with DMEM containing 2% FBS. The plates were incubated for 1 (VSV-GFP) to 5 days (MERS-CoV, SARS-CoV-2), and cytopathic effect was observed under a light microscope. Tissue culture infectious dose₅₀/mL was calculated using the Spearman and Karber algorithm.

Immunofluorescence assays

SEOV-infected cells were incubated at 37 °C for 24–96 h. At each time point, cell monolayers were washed twice with sterile PBS solution and fixed with ice-cold 95% ethanol:5% acetic acid solution for 10 min on ice as previously described [55]. Cells were then washed twice with PBS and blocked with 3% fetal calf serum in PBS for 1 h. Cells were probed overnight at 4 °C for SEOV N protein (custom, Genscript) at dilution 1:400 in 1×PBS and with secondary antibody AlexaFluor 555 goat α mouse (Thermo Fisher Scientific, A-551,31,570) at dilution 1:400 for 2 h at room temperature. Nuclear stain DAPI was used at 1:1,000 for 10 min at room temperature (SeraCare, 5930-0006). Representative images were acquired on the EVOS FL Auto imaging system (Thermo Fisher Scientific, AMF7000).

Immunoblots

Protein lysates were collected in 1× sample buffer and boiled for 10 min at 96 °C. Proteins were separated by SDS-PAGE using homemade 10% polyacrylamide gels and semi-dry transferred to a 0.2 µm nitrocellulose membrane using the Trans-Blot Turbo Transfer System (BioRad, #1704270). Membranes were blocked using intercept blocking solution (Licor Biosciences, #927-60001) and probed with primary antibodies diluted in 50% intercept blocking solution and 50% TBS overnight on a rocker. Primary antibodies include VSV-M (Kerafast, #EB0011), MERS-CoV N (Sino Biological, #40068-RP01-200), IFIT1 (Thermo Scientific, #PA531254), GAPDH (EMD Millipore, #MAB374), and/or ACTB (Abcam, #ab8227). The appropriate secondary antibody (Licor Biosciences, #926-6870 or #926-32213) diluted in 50% intercept blocking solution and 50% TBS was then added for 60 min. Membranes were imaged and analyzed using the Odyssey imager (Licor Biosciences) and Image Studio Software (Licor Biosciences).

RNA and qRT-PCR assays

Cells were lysed for RNA analysis using TRIzol Reagent (ThermoFisher Scientific, 15596026). RNA used for ANDV viral RNA quantification was purified using Zymo Research Direct-zol RNA Miniprep Plus kit (VWR 76211-340) according to manufacturer's instructions. RNA concentrations were quantified using a NanoDrop UV-Vis Spectrophotometer (ND-1000). cDNA was synthesized using Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (FisherScientific, 43-688-14) by adding 400ng total RNA to the reaction mixture containing random hexamers following manufacturer guidelines.

Quantitative real-time PCR was performed using the Taqman Fast Advanced Master Mix (Thermo Fisher, 4444556) and the following primer sets as described by Warner et al.: ANDV S129f – AAGGCAGTGGAGGTGGAC, ANDV S291r – CCCTGTTGGATCAACTGGTT, ANDV TM – 56-FAM/ACGGGCAGC/ZEN/TGTGTCTACATTGGA/3IABkFQ (PMID: 33627395). Standard curve was generated with plasmid DNA containing the coding region of the ANDV nucleoprotein.

RNA was extracted from cells which received 100 ng of nonlabeled poly(I:C) following the manufacturer's instructions for the RNeasy Plus Mini Kit (Qiagen, #74136). Four hundred nanograms of purified RNA was reverse transcribed into cDNA following the manufacturer's instructions for the iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad, #1725035). qRT-PCR was performed using the SsoAdvanced Universal SYBR Green Supermix (BioRad, #1725274) to assess *IFN β* , *IFIT1*, and *GAPDH* transcript levels. The following primer sets were designed for this analysis: CpIFNb_F – GCAGCCCTGGAGGAAATC, CpIFNb_R – CCAGGCA CATCTGCTGTAC, CpIFIT1_F – CTGTGATGCTTTGCCGAACC, CpIFT1_R – GGTTG GGCCTTGCTGAAATG, CpGAPDH_F – GGAGCGAGATCCCACCAACAT, and CpGAPDH_R – TGGAGTTGTCATACTTGTCTGA.

Data and Reagent availability

The following top-performing cell lines from this study are available through BEI Resources and distributed by ATCC: Bulk, non-clonal *C. perspicillata* kidney cells immortalized with SV-40 T-antigen (NR-59778) and clonal CKg9 cells (NR-59779). All other cell lines are available upon request. Please refer to [S3 Fig](#) for a bright field image that depicts the morphology of the kidney cells. Raw numerical values for all graphed data can be available for download as supplementary information.

Supporting information

S1 Table. Cell types established from different bat species. Databases include the American Type Culture Collection (ATCC; USA), Collection of Cell Lines in Veterinary Medicine (CCLV; Germany), and Kunming Cell Bank of Chinese Academy of Science (KCB; China). References are included for cell lines not available through a database. (DOCX)

S1 Fig. Susceptibility of select previously published bat cell lines. (A) Immortalized *Artibeus jamaicensis* (Aji) cells were infected with indicated pseudotypes at passages 8 and 25. (B) Immortalized *Eptesicus fuscus* (EfK3B) cells were infected with indicated pseudotypes. (C) Primary *Rousettus aegyptiacus* kidney and lung cells were infected at passage 1 with indicated pseudotypes. (D) *R. aegyptiacus* kidney cells transduced with SV40 T-antigen were infected with indicated pseudotypes. The data underlying this figure can be found in the [S1 Data](#) file available from the journal. (DOCX)

S2 Fig. Susceptibility of late-passage immortalized cultures. (A) Immortalized *Carollia perspicillata* cells were maintained in culture for multiple passages and infected with the indicated pseudotypes. (B) Late passage immortalized *Carollia* kidney cells were infected with indicated pseudotypes. All infections were performed in four replicates. The data underlying this figure can be found in the [S1 Data](#) file available from the journal. (DOCX)

S3 Fig. Bright field image of primary CaPsm cells. (A) Primary kidney, spleen, brain, liver, and lung cell cultures. (B) Heterogenous cell populations are visible in the primary

cell cultures. Images were acquired at 4× and 10× magnification on an EVOS M3000 microscope.

(DOCX)

S1 Raw images. PDF file containing un-cropped images of all western blots in this manuscript.

(PDF)

S1 Data. Excel file containing all plotted raw values from all graphs in this manuscript.

(XLSX)

Acknowledgments

We would like to thank Dr. Jay Hooper at USAMRIID for generously providing orthohantavirus M segment genes and B. Haagmans and R. Fouchier, Erasmus Medical Center, for providing MERS-CoV (isolate hCoV-EMC/2012).

Author contributions

Conceptualization: Arinjay Banerjee, Stephanie N Seifert, Michael Letko.

Data curation: Victoria Gonzalez, Rohit K. Jangra, Arinjay Banerjee, Michael Letko.

Formal analysis: Victoria Gonzalez, Cierra Word, Nahomi Guerra-Pilaquinga, Alison M. Kell, Rohit K. Jangra, Arinjay Banerjee, Michael Letko.

Funding acquisition: Christine Portfors, Alison M. Kell, Rohit K. Jangra, Stephanie N. Seifert, Arinjay Banerjee, Michael Letko.

Investigation: Victoria Gonzalez, Cierra Word, Nahomi Guerra-Pilaquinga, Mitra Mazinani, Stephen Fawcett, Alison M. Kell, Rohit K. Jangra, Arinjay Banerjee, Stephanie N Seifert, Michael Letko.

Methodology: Christine Portfors, Darryl Falzarano, Alison M. Kell, Rohit K. Jangra, Arinjay Banerjee, Stephanie N Seifert, Michael Letko.

Project administration: Arinjay Banerjee, Michael Letko .

Resources: Christine Portfors, Darryl Falzarano, Stephanie N. Seifert, Alison M. Kell, Rohit K. Jangra, Arinjay Banerjee, Michael Letko .

Supervision: Rohit K. Jangra, Arinjay Banerjee, Michael Letko.

Validation: Michael Letko.

Visualization: Victoria Gonzalez, Michael Letko.

Writing – original draft: Victoria Gonzalez, Christine Portfors, Alison M. Kell, Rohit K. Jangra, Arinjay Banerjee, Stephanie N Seifert, Michael Letko.

Writing – review & editing: Victoria Gonzalez, Cierra Word, Nahomi Guerra-Pilaquinga, Christine Portfors, Alison M. Kell, Rohit K. Jangra, Arinjay Banerjee, Stephanie N Seifert, Michael Letko.

References

1. Gonzalez V, Banerjee A. Molecular, ecological, and behavioral drivers of the bat-virus relationship. *iScience*. 2022;25(8):104779. <https://doi.org/10.1016/j.isci.2022.104779> PMID: [35875684](https://pubmed.ncbi.nlm.nih.gov/35875684/)
2. Witkowski PT, Drexler JF, Kallies R, Ličková M, Bokorová S, Maganga GD, et al. Phylogenetic analysis of a newfound bat-borne hantavirus supports a laurasiatherian host association for ancestral mammalian hantaviruses. *Infect Genet Evol*. 2016;41:113–9. <https://doi.org/10.1016/j.meegid.2016.03.036> PMID: [27051047](https://pubmed.ncbi.nlm.nih.gov/27051047/)

3. Simmons N, Cirranello A. Bat species of the world: a taxonomic and geographic database. Version 1.6. 2024.
4. Kniazeff A, Constantine D, Nelson-Rees W, Schmidt D, Owens R. Studies on chiropteran cell lines. Progress Report. 1967;CC–8:97–105.
5. Willoughby A, Phelps K, Olival K. A comparative analysis of viral richness and viral sharing in cave-roosting bats. *Diversity*. 2017;9(3):35. <https://doi.org/10.3390/d9030035>
6. Moreno Santillán DD, Lama TM, Gutierrez Guerrero YT, Brown AM, Donat P, Zhao H, et al. Large-scale genome sampling reveals unique immunity and metabolic adaptations in bats. *Mol Ecol*. 2021;30(23):6449–67. <https://doi.org/10.1111/mec.16027> PMID: 34146369
7. Crameri G, Todd S, Grimley S, McEachern JA, Marsh GA, Smith C, et al. Establishment, immortalisation and characterisation of pteropid bat cell lines. *PLoS One*. 2009;4(12):e8266. <https://doi.org/10.1371/journal.pone.0008266> PMID: 20011515
8. Van Tol S, Port J, Fischer R, Gallogly S, Bushmaker T, Griffin A, et al. Jamaican fruit bats (*Artibeus jamaicensis*) competence for Ebola virus but not Marburg virus is driven by intrinsic differences in viral entry and IFN-I signaling antagonism. *bioRxiv*. 2024. <https://doi.org/10.1101/2024.10.17.618736>
9. Sabino-Santos G Jr, Maia FGM, Vieira TM, de Lara Muylaert R, Lima SM, Gonçalves CB, et al. Evidence of hantavirus infection among bats in Brazil. *Am J Trop Med Hyg*. 2015;93(2):404–6. <https://doi.org/10.4269/ajtmh.15-0032> PMID: 26078322
10. Sabino-Santos G Jr, Maia FGM, Martins RB, Gagliardi TB, Souza WM de, Muylaert RL, et al. Natural infection of neotropical bats with hantavirus in Brazil. *Sci Rep*. 2018;8(1):9018. <https://doi.org/10.1038/s41598-018-27442-w> PMID: 29899544
11. Hoffmann M, Müller MA, Drexler JF, Glende J, Erdt M, Gützkow T, et al. Differential sensitivity of bat cells to infection by enveloped RNA viruses: coronaviruses, paramyxoviruses, filoviruses, and influenza viruses. *PLoS One*. 2013;8(8):e72942. <https://doi.org/10.1371/journal.pone.0072942> PMID: 24023659
12. Jordan I, Horn D, Oehmke S, Leendertz FH, Sandig V. Cell lines from the Egyptian fruit bat are permissive for modified vaccinia Ankara. *Virus Res*. 2009;145(1):54–62. <https://doi.org/10.1016/j.virusres.2009.06.007> PMID: 19540275
13. Letko M, Marzi A, Munster V. Functional assessment of cell entry and receptor usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nat Microbiol*. 2020;5(4):562–9. <https://doi.org/10.1038/s41564-020-0688-y> PMID: 32094589
14. Banerjee A, Rapin N, Miller M, Griebel P, Zhou Y, Munster V, et al. Generation and characterization of *Eptesicus fuscus* (Big brown bat) kidney cell lines immortalized using the *Myotis polyomavirus* large T-antigen. *J Virol Methods*. 2016;237:166–73. <https://doi.org/10.1016/j.jviromet.2016.09.008> PMID: 27639955
15. Barbosa Dos Santos M, Koide Albuquerque N, Patroca da Silva S, Silva da Silva F, Damous Dias D, Brito Mendes S, et al. A novel hantavirus identified in bats (*Carollia perspicillata*) in Brazil. *Sci Rep*. 2024;14(1):6346. <https://doi.org/10.1038/s41598-024-56808-6> PMID: 38491115
16. Shaw TI, Srivastava A, Chou W-C, Liu L, Hawkinson A, Glenn TC, et al. Transcriptome sequencing and annotation for the Jamaican fruit bat (*Artibeus jamaicensis*). *PLoS One*. 2012;7(11):e48472. <https://doi.org/10.1371/journal.pone.0048472> PMID: 23166587
17. Seifert SN, Letko MC, Bushmaker T, Laing ED, Saturday G, Meade-White K, et al. *Rousettus aegyptiacus* bats do not support productive Nipah virus replication. *J Infect Dis*. 2020;221(Suppl 4):S407–13. <https://doi.org/10.1093/infdis/jiz429> PMID: 31682727
18. Kim HS, Shin JY, Yun JY, Ahn DK, Le JY. Immortalization of human embryonic fibroblasts by overexpression of c-myc and simian virus 40 large T antigen. *Exp Mol Med*. 2001;33(4):293–8. <https://doi.org/10.1038/emmm.2001.47> PMID: 11795494
19. Kassem M, Abdallah BM, Yu Z, Ditzel N, Burns JS. The use of hTERT-immortalized cells in tissue engineering. *Cytotechnology*. 2004;45(1–2):39–46. <https://doi.org/10.1007/s10616-004-5124-2> PMID: 19003242
20. Jeong Y-J, Cho J, Kwak J, Sung YH, Kang B-C. Immortalization of primary marmoset skin fibroblasts by CRISPR-Cas9-mediated gene targeting. *Anim Cells Syst (Seoul)*. 2022;26(6):266–74. <https://doi.org/10.1080/19768354.2022.2151509> PMID: 36605591
21. Eun K, Park MG, Jeong YW, Jeong YI, Hyun S-H, Hwang WS, et al. Establishment of TP53-knockout canine cells using optimized CRISPR/Cas9 vector system for canine cancer research. *BMC Biotechnol*. 2019;19(1):1. <https://doi.org/10.1186/s12896-018-0491-5> PMID: 30606176

22. Harvey DM, Levine AJ. p53 alteration is a common event in the spontaneous immortalization of primary BALB/c murine embryo fibroblasts. *Genes Dev.* 1991;5(12B):2375–85. <https://doi.org/10.1101/gad.5.12b.2375> PMID: 1752433
23. Weiss MB, Vitolo MI, Mohseni M, Rosen DM, Denmeade SR, Park BH, et al. Deletion of p53 in human mammary epithelial cells causes chromosomal instability and altered therapeutic response. *Oncogene.* 2010;29(33):4715–24. <https://doi.org/10.1038/onc.2010.220> PMID: 20562907
24. Jangra RK, Herbert AS, Li R, Jae LT, Kleinfelter LM, Slough MM, et al. Protocadherin-1 is essential for cell entry by New World hantaviruses. *Nature.* 2018;563(7732):559–63. <https://doi.org/10.1038/s41586-018-0702-1> PMID: 30464266
25. Mosca JD, Pitha PM. Transcriptional and posttranscriptional regulation of exogenous human beta interferon gene in simian cells defective in interferon synthesis. *Mol Cell Biol.* 1986;6(6):2279–83. <https://doi.org/10.1128/mcb.6.6.2279-2283.1986> PMID: 3785197
26. Hurst E. An outbreak of rabies in trinidad without history of bites, and with the symptoms of acute ascending myelitis. *The Lancet.* 1931;218(5638):622–8. [https://doi.org/10.1016/S0140-6736\(01\)07332-9](https://doi.org/10.1016/S0140-6736(01)07332-9)
27. Pawan JL. The transmission of paralytic rabies in Trinidad by the vampire bat (*Desmodus rotundus murinus* Wagner, 1840). *Ann Trop Med Parasitol.* 1936;30(1):101–30. <https://doi.org/10.1080/00034983.1936.11684921>
28. Temmam S, Vongphayloth K, Baquero E, Munier S, Bonomi M, Regnault B, et al. Bat coronaviruses related to SARS-CoV-2 and infectious for human cells. *Nature.* 2022;604(7905):330–6. <https://doi.org/10.1038/s41586-022-04532-4> PMID: 35172323
29. Zhou P, Yang X-L, Wang X-G, Hu B, Zhang L, Zhang W, et al. A pneumonia outbreak associated with a new coronavirus of probable bat origin. *Nature.* 2020;579(7798):270–3. <https://doi.org/10.1038/s41586-020-2012-7> PMID: 32015507
30. Baid K, Irving A, Jouvenet N, Banerjee A. The translational potential of studying bat immunity. *Trends in Immunology.* 2024.
31. Aicher S-M, Streicher F, Chazal M, Planas D, Luo D, Buchrieser J, et al. Species-specific molecular barriers to SARS-CoV-2 replication in bat cells. *J Virol.* 2022;96(14):e0060822. <https://doi.org/10.1128/jvi.00608-22> PMID: 35862713
32. Yang Y, Du L, Liu C, Wang L, Ma C, Tang J, et al. Receptor usage and cell entry of bat coronavirus HKU4 provide insight into bat-to-human transmission of MERS coronavirus. *Proc Natl Acad Sci U S A.* 2014;111(34):12516–21. <https://doi.org/10.1073/pnas.1405889111> PMID: 25114257
33. Kuzmin IV, Schwarz TM, Ilinykh PA, Jordan I, Ksiazek TG, Sachidanandam R, et al. Innate immune responses of bat and human cells to filoviruses: commonalities and distinctions. *J Virol.* 2017;91(8):e02471-16. <https://doi.org/10.1128/JVI.02471-16> PMID: 28122983
34. !!! INVALID CITATION!!! [35].
35. !!! INVALID CITATION!!! [26, 36].
36. !!! INVALID CITATION!!! [32].
37. Hernández LHA, da Paz TYB, da Silva SP, de Barros B de CV, Coelho TFSB, Cruz ACR. First description of a mobatvirus (Hantaviridae) in the Amazon region. *Virus Res.* 2024;350:199494. <https://doi.org/10.1016/j.virusres.2024.199494> PMID: 39521252
38. Otter CJ, Renner DM, Fausto A, Tan LH, Cohen NA, Weiss SR. Interferon signaling in the nasal epithelium distinguishes among lethal and common cold coronaviruses and mediates viral clearance. *Proc Natl Acad Sci U S A.* 2024;121(21):e2402540121. <https://doi.org/10.1073/pnas.2402540121> PMID: 38758698
39. Towner JS, Pourrut X, Albaniño CG, Nkogue CN, Bird BH, Grard G, et al. Marburg virus infection detected in a common African bat. *PLoS One.* 2007;2(8):e764. <https://doi.org/10.1371/journal.pone.0000764> PMID: 17712412
40. Towner JS, Amman BR, Sealy TK, Carroll SAR, Comer JA, Kemp A, et al. Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. *PLoS Pathog.* 2009;5(7):e1000536. <https://doi.org/10.1371/journal.ppat.1000536> PMID: 19649327
41. Ng M, Ndungo E, Kaczmarek ME, Herbert AS, Binger T, Kuehne AI, et al. Filovirus receptor NPC1 contributes to species-specific patterns of ebolavirus susceptibility in bats. *Elife.* 2015;4:e11785. <https://doi.org/10.7554/eLife.11785> PMID: 26698106
42. Carette JE, Raaben M, Wong AC, Herbert AS, Obernosterer G, Mulherkar N, et al. Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. *Nature.* 2011;477(7364):340–3. <https://doi.org/10.1038/nature10348> PMID: 21866103

43. Amman BR, Bird BH, Bakarr IA, Bangura J, Schuh AJ, Johnny J, et al. Isolation of Angola-like Marburg virus from Egyptian rousette bats from West Africa. *Nat Commun*. 2020;11(1):510. <https://doi.org/10.1038/s41467-020-14327-8> PMID: 31980636
44. Bentim Góes LG, Fischer C, Almeida Campos AC, de Carvalho C, Moreira-Soto A, Ambar G, et al. Highly diverse arenaviruses in neotropical bats, Brazil. *Emerg Infect Dis*. 2022;28(12):2528–33. <https://doi.org/10.3201/eid2812.220980> PMID: 36417964
45. Cogswell-Hawkinson A, Bowen R, James S, Gardiner D, Calisher CH, Adams R, et al. Tacaribe virus causes fatal infection of an ostensible reservoir host, the Jamaican fruit bat. *J Virol*. 2012;86(10):5791–9. <https://doi.org/10.1128/JVI.00201-12> PMID: 22379103
46. Almeida MF, Martorelli LFA, Aires CC, Sallum PC, Durigon EL, Massad E. Experimental rabies infection in haematophagous bats *Desmodus rotundus*. *Epidemiol Infect*. 2005;133(3):523–7. <https://doi.org/10.1017/s0950268804003656> PMID: 15962559
47. Negredo A, Palacios G, Vázquez-Morón S, González F, Dopazo H, Molero F, et al. Discovery of an ebolavirus-like filovirus in europe. *PLoS Pathog*. 2011;7(10):e1002304. <https://doi.org/10.1371/journal.ppat.1002304> PMID: 22039362
48. Sanjana NE, Shalem O, Zhang F. Improved vectors and genome-wide libraries for CRISPR screening. *Nat Methods*. 2014;11(8):783–4. <https://doi.org/10.1038/nmeth.3047> PMID: 25075903
49. Letko M, Miazgowicz K, McMinn R, Seifert SN, Sola I, Enjuanes L, et al. Adaptive evolution of MERS-CoV to species variation in DPP4. *Cell Rep*. 2018;24(7):1730–7. <https://doi.org/10.1016/j.celrep.2018.07.045> PMID: 30110630
50. Fischer RJ, Purushotham JN, van Doremalen N, Sebastian S, Meade-White K, Cordova K, et al. ChAdOx1-vectored Lassa fever vaccine elicits a robust cellular and humoral immune response and protects guinea pigs against lethal Lassa virus challenge. *NPJ Vaccines*. 2021;6(1):32. <https://doi.org/10.1038/s41541-021-00291-x> PMID: 33654106
51. Khaledian E, Ulasan S, Erickson J, Fawcett S, Letko MC, Broschat SL. Sequence determinants of human-cell entry identified in ACE2-independent bat sarbecoviruses: a combined laboratory and computational network science approach. *EBioMedicine*. 2022;79:103990. <https://doi.org/10.1016/j.ebiom.2022.103990> PMID: 35405384
52. Seifert SN, Bai S, Fawcett S, Norton EB, Zvezdaryk KJ, Robinson J, et al. An ACE2-dependent sarbecovirus in Russian bats is resistant to SARS-CoV-2 vaccines. *PLoS Pathog*. 2022;18(9):e1010828. <https://doi.org/10.1371/journal.ppat.1010828> PMID: 36136995
53. Banerjee A, Nasir JA, Budykowski P, Yip L, Aftanas P, Christie N, et al. Isolation, sequence, infectivity, and replication kinetics of severe acute respiratory syndrome coronavirus 2. *Emerg Infect Dis*. 2020;26(9):2054–63. <https://doi.org/10.3201/eid2609.201495> PMID: 32558639
54. Kleinfelter LM, Jangra RK, Jae LT, Herbert AS, Mittler E, Stiles KM, et al. Haploid genetic screen reveals a profound and direct dependence on cholesterol for hantavirus membrane fusion. *mBio*. 2015;6(4):e00801. <https://doi.org/10.1128/mBio.00801-15> PMID: 26126854
55. Kell AM, Hemann EA, Turnbull JB, Gale Jr M. RIG-I-like receptor activation drives type I IFN and antiviral signaling to limit Hantaan orthohantavirus replication. *PLoS Pathog*. 2020;16(4):e1008483.