Spike independent replication of human coronavirus in bat cells

Martin Linster,^{1*†} Marcus G Mah,^{1*} Dolyce HW Low,¹ Zhuang Yan,¹ Jayanthi Jayakumar,¹
Firdaus Samsudin,² Foong Ying Wong,¹ Peter J Bond,^{2,3} Ian H Mendenhall,¹ Yvonne CF Su,¹
Gavin JD Smith^{1,4,5†}

¹ Programme in Emerging Infectious Diseases, Duke-NUS Medical School, Singapore

² Bioinformatics Institute, Agency for Science, Technology, and Research, Singapore

³ Department of Biological Sciences, National University of Singapore, Singapore

⁴ SingHealth Duke-NUS Global Health Institute, SingHealth Duke-NUS Academic Medical
Centre, Singapore

⁵ Duke Global Health Institute, Duke University, United States

*The authors contributed equally.

†Corresponding authors.

Keywords: evolution, pandemic, receptor usage, zoonotic

Abstract

Bats are a likely zoonotic reservoir for a range of human pathogens including endemic human coronaviruses and SARS-CoV-2. Despite the high burden caused by these viruses, the factors required for the establishment and ongoing transmission in humans are not well understood, hampering efforts for pandemic preparedness. To help understand those adaptations required to cross the species barrier, we serially passaged endemic human coronavirus 229E isolates in a newly established *Rhinolophus* (horseshoe bat) kidney cell line. Here we report extensive mutations, including deletions, in the virus genome that result in the loss of spike protein expression, while maintaining the capability to infect bat cells. While we observed a loss of infectivity of human cells for all viruses with spike deletions, one isolate (2613) with an insertion that results in an early stop codon, was recovered from human cells. Deep sequencing of isolate 2613 showed that the majority population had acquired additional nucleotide insertions in the spike resulting in an additional codon that restores spike function. Spike-independent replication of coronaviruses provides an alternative route for infection of host species that don't share common cell-entry receptors.

Introduction

Coronaviruses (CoVs) are positive-sense RNA viruses with genomes ranging from 27kb to 32kb. CoVs are members of the *Coronaviridae* family, *Orthocoronavirinae* subfamily and are divided into four genera, *Alphacoronavirus*, *Betacoronavirus*, *Deltacoronavirus* and *Gammacoronavirus*¹. There are four seasonal CoVs, 229E, NL63, OC43 and HKU1, that together account for 10% to 30% of upper respiratory tract infections in adults² and circulate globally³. To date, three zoonotic coronaviruses have caused epidemics in human populations - SARS-CoV^{4,5}, MERS-CoV⁶ and SARS-CoV-2⁷. Based on genetic analyses, all seasonal and zoonotic CoVs have an ancestral origin in bats⁸. Recently, 229E-related bat coronaviruses

have been isolated from bats across central Africa⁹. Temporal analyses of 229E sequences from bats and humans have estimated that 229E diverged from bat ancestors more than 130 years ago¹⁰. The detection of 229E-related viruses in captive alpaca and dromedary suggests that 229E might have transmitted from bats to humans via camelids as intermediate hosts^{11,12}.

Bats harbor a variety of CoVs without displaying obvious disease symptoms¹³.

Compared to other mammals, the innate ability of bats to tolerate infection from pathogens has been linked to a loss of genes that trigger inflammation¹⁴. For CoVs to emerge, they need to overcome the host-species barrier by balancing between conserving and modulating genes while maintaining virus infectivity, replication and spread¹⁵. One key interaction that enables CoV entry into host cells is the binding of the spike protein to host receptors. CoVs recognize various cellular receptors – in humans these comprise: angiotensin-converting enzyme 2 (ACE2) in the case of SARS-CoV¹⁶, SARS-CoV-2¹⁷, and NL63¹⁸; dipeptidyl peptidase-4 for MERS-CoV¹⁹; and aminopeptidase N for 229E²⁰. It has been hypothesized that alphacoronaviruses have a higher propensity for zoonotic spillover compared to betacoronaviruses based on their broader bat host species range and higher frequency of virus jumps between bat genera²¹. To better understand the biology of CoVs in their ancestral bat host, we passaged 229E virus in a kidney cell line derived from *Rhinolophus lepidus* (Blyth's horseshoe bat).

Results

Bat cells are susceptible and permissive to 229E

We first established a *Rhinolophus lepidus* kidney (Rhileki) cell line to serially passage six 229E viruses. Stock viruses were derived from nasopharyngeal swabs, plus a reference strain passaged in human colon adenocarcinoma (Caco2) cells that are denoted C2 viruses (see Supplementary Fig. 1. for details on passaging and virus nomenclature). During virus

passaging in Rhileki cells, an increase in viral genomic copies from inoculum to 6 days post inoculation (dpi) was observed, with the exception of viruses at passage one and isolate SG/1197/2010 at all passages (Fig. 1a). The inoculation of Caco2 and Rhileki cells with C2 viruses resulted in infection as detected by nucleocapsid immunofluorescence staining (Supplementary Fig. 2). After ten passages of C2 viruses in Rhileki cells, these C2R10 viruses were inoculated on Caco2 cells, but no signs of infection were observed, with the exception of SG/2613/2011. When titrated on Caco2 cells, C2 virus titers ranged from 6.8×10^4 to 3.2×10^7 TCID₅₀/ml but C2R10 virus titers were below the limit of detection (Fig. 1B). Interestingly, when C2R10 viruses were once more inoculated in Caco2 cells (C2R10C1) only SG/2613/2011 was detectable at a virus titer of 1.8×10^6 TCID₅₀/ml, whereas all remaining C2R10 isolates were unable to infect Caco2 cells (Fig. 1b). Moreover, when C2 and C2R10 viruses were titrated in Rhileki cells (Fig. 1b), all isolates except SG/1197/2010 yielded a detectable virus titer, although infection of Rhileki cells resulted in low titers throughout. As an additional proof of virus genomic replication, double-stranded RNA was detected at 1 dpi in an immunofluorescent assay (two C2 and C2R10 viruses are shown in Supplementary Fig. 3).

Deletions in the spike and ORF4 genes

We performed next-generation sequencing (NGS) to determine the patterns of genomic changes that stem from adaptation of 229E viruses to Rhileki cells. Full genomes were recovered for Rhileki passage 1 (C2R1), passage 5 (C2R5) and passage 10 (C2R10) of the six viruses. Remarkably, spike/ORF4 deletions of varying lengths were observed as early as passage 1 in all Rhileki-passaged 229E isolates except SG/2613/2011 (Fig. 2 and Supplementary Fig. 4). These deletions were confirmed by Sanger sequencing with isolate-specific flanking primers (Supplementary Table 1). Critically, the spike deletions either

remained of constant size or enlarged during passaging. At passage 10 SG/1197/2010, SG/1340/2010 and TZ/4033K/2017 showed major deletions in the spike and the ORF4. Notably, the spike receptor binding domains (RBDs) were deleted in SG/1197/2010, SG/1340/2010, TZ/4033K/2017, and UK/VR740/1973 C2R10 viruses. Although no deletions were observed for SG/2613/2011 there was a single nucleotide insertion at position 21,069 that resulted in a premature stop codon at positions 21,097–21,099. Strikingly, these major genomic changes did not prevent reinfection during serial passage in Rhileki cells, indicating that virus replication was occurring independently of the spike protein.

To understand the potential consequence of these deletions, remaining amino acid positions in C2R10 viruses were mapped to the 3D structural models of spike and ORF4 proteins built using structures of their analogues from SARS-CoV-2 to estimate the positions of deletions and whether protein functions are possibly retained (Supplementary Fig. 5). No remaining function of the spike is expected for all C2R10 viruses with the exception of isolate SG/2326/2011 that retains an intact spike ectodomain. However, the lack of transmembrane (TM) and heptad repeat 2 (HR2) regions that are involved in membrane fusion may impair its function. The function of the ORF4 protein as a viroporin²² is expected to be retained in all C2R10 viruses except for SG/1197/2010 which lacks the TM domain and is a slowly growing virus in our cell-based assays.

We next compared the frequency of single nucleotide polymorphisms (SNPs) between passages, with SG/1197/2010 excluded due to insufficient coverage (Supplementary Table 2). The majority of SNPs (13/21) occurred in the non-structural proteins. With the exception of the deleted regions, bat adapted C2R10 viruses are almost identical to C2 viruses across the remaining genome. There were 17 SNPs that resulted in a non-synonymous mutation, of which four were fixed as the major variant at passage 1, twelve at passage 5 and all 17 at passage 10. Interestingly, two isolates each independently acquired mutations A4854G

(D621G in NSP3) and C25177T (L54F in the membrane protein) and SG/2613/2011 acquired mutation C24200T that resulted in an early stop codon (Q30* in ORF4). NSP3 has been implicated in the formation of molecular pores that span double membrane vesicles as part of coronavirus replication organelle²³.

Spike protein expression during replication in bat cells

To understand whether the spike/ORF4 deletions affect subgenomic RNA (sgmRNA) production, we probed for the presence of sgmRNA 2 and 4 specific sequences in C2 and C2R10 viruses (primers shown in Supplementary Table 1), including the leader-body junctions (LBJ), associated with the production of spike and ORF4 (Fig. 3a and Supplementary Fig. 6a). All C2 viruses produced PCR products of the expected 162 bp or 507 bp that corresponded to sgmRNA 2 and 4, respectively (Fig. 3b). In contrast, we observed variation in band sizes among C2R10 viruses, corresponding to the varying sized deletions present in gRNA. Interestingly, a non-canonical LBJ was detected for SG/2326/2011 that begins at nucleotide 23,600 of the spike open-reading frame that encodes sgmRNA X and a related putative ORF (Supplementary Fig. 6b). Analysis of the LBJs and sequences downstream of sgmRNA2 and 4 further confirms the presence of the described deletions and suggests that translation of canonical spike and ORF4 proteins is impaired.

To further test the expression of spike protein during passaging of isolates in Rhileki cells, we generated a post-immunization polyclonal rabbit serum directed against the spike protein of 229E. Culture supernatants of C2R1 generally contained little N protein (Fig. 3c), while higher levels of N were detected in all C2R5 and C2R10 viruses except SG/1197/2010, consistent with viral loads observed during passaging (Fig. 1a). Expression of spike was not detected in all passages in Rhileki cells, except for a faint band observed for SG/2326/2011 at passage 5.

Virus expressing the full spike protein was recovered in human cells

To determine if spike independent replication of 229E viruses was specific to Rhileki cells, we inoculated Caco2 cells with C2R10 viruses, termed C2R10C1. Quantitative PCR results were negative except for SG/2613/2011 (data not shown). The culture supernatant of SG/2613/2011 was clearly positive for spike expression in Western blot analysis (Fig. 3c). Detailed examination of the sequencing reads for SG/2613/2011spike, which did not display any deletions, showed virus subpopulations with insertions of one to three nucleotides at position 21,069 across its passage history (Fig. 4a) that resulted in loss and reinstatement of the spike reading frame. The C2R5 virus population had 3 variants, the wild type (GNFY–NE), the majority variant (GNFL–Q*) with a 1 nt insertion resulting in a stop at positions 21,097–21,099, and a final variant (GNFFIAL*) with 2 nt insertions and a stop at position 21,078–21,080. The wild type was not detected in C2R10, possibly due to low sequencing coverage. In C2R10C1, passaged in Caco2 cells, both the wild type (GNFY–NE) and a new variant (GNFFY–NE) with a 3 nt insertion that recovers the spike reading frame were present, consistent with the observed expression of spike (Fig. 3c), reiterating that spike is essential for efficient virus infection of human cells.

Discussion

To our knowledge this is the first report of active replication of coronaviruses without the required spike receptor binding. The spike gene, especially the RBD is highly variable with multiple insertions and deletions observed²⁴. Spike deletion variants have been documented in fatal human cases of MERS-CoV and SARS-CoV-2 and were attributed to immune driven antibody escape^{25,26}. While some bat sarbecoviruses bind human ACE2, most are unable to engage this receptor due to variation or deletions within the RBD, suggesting alternative

receptors or cellular entry pathways^{27–29}. Recent studies have revealed detail of coronavirus replication including the description of large virus-containing vesicles (LVCVs) containing intact^{30,31} or spike-less virions³², that might help to explain our observed spike-independent virus maintenance in Rhileki cells. Alternative pathways for egress of virions, possibly as part of LVCVs, subsequent attachment and internalization without a strict receptor requirement have also been elucidated^{33,34}.

Combined with the results presented here, this suggests that some coronaviruses may utilize receptor independent membrane fusion. This mechanism may not be found in all bat tissues or species but could help explain features of infection in bats such as low levels of viraemia³⁵ and the dampened immune response to highly pathogenic viruses³⁶. However, it is unlikely that the full spike gene deletions would be present during natural infection as spike binding would obviously increase virus fitness in the presence of the appropriate receptor, and the vast majority of natural spike proteins are full length. Rather, the infection dynamics in Rhileki cells likely favored replication of a shorter viral genome in the absence of other selection pressures present in a natural setting.

Given that alphacoronaviruses can infect a wide range of hosts³⁷, the ability to replicate without requiring the spike protein could provide an alternative route for infection between different bat species, and possibly other hosts, which may not share common receptors. In this scenario, upon transmission to a new host species, infection could be initiated by macropinocytosis of shed virions within LVCVs in urine or stool, but would be significantly increased by acquiring a matching receptor binding protein either from recombination with the host or other coronaviruses. It is therefore critical that we gain a better understanding of the infection biology of host species, particularly the role of LVCVs, involved in the emergence of zoonotic coronaviruses.

Methods

Establishment of the Rhileki cell line

Kidney tissue of a *Rhinolophus lepidus* bat (NUS-IACUC B01/12) were processed into cellular mixtures by mechanical disruption through a 100 µm cell strainer. One-hundred thousand cells were plated in a T-25 Corning CellBIND flask containing maintenance media (RPMI with L-glutamine, 10% FBS, 2.5% HEPES, 1x antibiotics-antimycotics) at 37 °C, 5% CO₂ for expansion. Cells were then serially diluted in 384-well plates. Wells containing single cell patches were selected, trypsinized and serially diluted additional two times before expansion. A spontaneously immortalized clonal cell line was selected and named Rhileki for *Rhinolophus lepidus* kidney which is currently maintained until passage 30.

Viruses

Isolates SG/1197/2010, SG/1340/2010, SG/2326/2011, SG/2613/2011 were obtained from a study in Singapore³⁸. Isolate TZ/4033K/2017 was obtained from a study conducted in Tanzania (unpublished). UK/VR740/1973 was procured from the American Type Culture Collection (ATCC). All viruses were cultured for 3 days in Caco2 cells (ATCC, HTB-37) at 33 °C in Dulbecco's Modified Eagle Medium GlutaMAX containing 3% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Viruses were titrated for 5 days in a 96-well format, plates were fixed with 100% methanol, blocked with 3% bovine serum albumin (BSA), stained with 1E7 primary monoclonal antibody (Eurofins Technologies Ingenasa) binding to the 229E nucleocapsid protein and a goat anti-mouse secondary antibody conjugated with fluorescein isothiocyanate (Abcam, ab6785). TCID₅₀ titers were calculated according to the method of Reed and Muench³⁹.

Serial virus passage

Rhileki cells were seeded at a density of 2×10^6 in T25 flasks. The virus inoculum at MOI 0.01 was incubated at 33 °C for 2 hours. The inoculum was removed, cells washed once with phosphate buffered saline (PBS) and medium was replenished (DMEM, 1% sodium pyruvate, 1% non-essential amino acids, and 0.5% BSA). Supernatant was collected at time points 0, 3, 6, 9 dpi. On 9 dpi, the cells were scraped, pelleted and stored at -80 °C. For virus passages, viral genome copies in the supernatant of 6 dpi were adjusted to contain 10^5 to 10^6 genome equivalents in the subsequent inoculum.

Double-stranded RNA staining

Caco2 and Rhileki cells were seeded into 2-well chambered coverglass on the day before infection. Caco2 and Rhileki cells were infected with C2 or C2R10 viruses respectively for 2 hours at 33 °C. Cells were fixed and permeabilized at 1 dpi and stained with J2 antibody that detects for double-stranded RNA complexes.

RNA extraction and viral genome copy analysis

Briefly, total RNA was extracted from 150 µl of supernatant collected at all time points using the Direct-zol RNA MiniPrep kit (Zymo Research) according to the manufacturer's instructions. Samples were analyzed by real-time quantitative reverse-transcription-PCR (RT-qPCR) using primers specific for 229E nucleocapsid as described previously⁴⁰. Nucleocapsid sequences were cloned and cycle threshold values were converted into viral genome copies per microliter based on regression analysis of plasmid dilutions.

Whole genome sequencing and genetic analysis

Complementary DNA libraries of passage 1, 5 and 10 were constructed using TruSeq RNA Library Prep Kit (Illumina) according to the manufacturer's instruction. The quality of the

libraries was verified using the RNA bioanalyzer (Agilent Technologies) and normalized using the KAPA Library Prep Kit (Roche). The libraries were sequenced using a 250 bp flow cell on the Illumina MiSeq System. Raw NGS reads were trimmed by Trimmomatic v0.39 to remove adaptors and low-quality reads⁴¹. In order to obtain consensus sequences, C2 virus consensus of each isolate was used as reference to assemble trimmed and raw reads using the BWA-MEM algorithm in UGENE v.34⁴². Coverage, depth and statistics of the assembled reads were visualized in Geneious R9.1.8 and Integrative Genomics Viewer⁴³. Consensus sequences were aligned using MAFFT v7.222 and annotated in Geneious R9.1.8.

Sanger deletion verification

Trimmed and untrimmed reads were compared to flag deleted regions in the genome. To confirm the deletions, flanking primers were designed (Supplementary Table 1). Using isolate specific primers, cDNA synthesis was performed using Superscript III First-Strand Synthesis System (ThermoFisher Scientific). This was followed by PCR, of which a 50 µl mixture consisted cDNA, sense/anti-sense primers (10 µM), 10x Pfu reaction buffer and DNA polymerase (Promega) and 10 mM dNTP mix (ThermoFisher Scientific). The PCR was conducted at 95 °C for 2 mins, 35 cycles at 95 °C for 1 min, 47 °C for 30 secs, 72 °C for 2 mins and final extension at 72 °C for 5 mins. PCR products were visualized using a 1.5% gel and sequences confirmed by Sanger sequencing (Macrogen).

Subgenomic mRNA leader-body junction for sgRNA 2 and 4

As part of the replication cycle of CoVs, translation of structural proteins is preceded by discontinuous transcription of subgenomic RNA (sgRNA)⁴⁵, whereby nested species of subgenomic messenger RNA (sgmRNA) are produced. Structural proteins of 229E are translated from sgRNA 2, 4, 5, 6, 7, while sgRNA 3 is truncated and was described to be

non-functional⁴⁶⁻⁴⁸. The leader-body junction consists of nucleotides within the transcription-regulating sequence of the leader (TRS-L) and the TRS of the body (TRS-B). The hybridization process is mediated by long range RNA-RNA interaction that leads to the formation of sgRNA which can be distinguished based on their sequence from the genomic RNA (gRNA). Reverse primers specific for the spike (226R) and ORF4 (617R) genes were designed to synthesize cDNA from total RNA extracted from infected cell lysates. Using a semi-nested PCR approach, the 2F primer targeting the start of the leader RNA was used in combination with 158R, and subsequently with 87R to generate PCR products that contain the leader RNA, leader-body junction and a portion of sgRNA 2. Similarly, to test the presence of sgRNA 4, 2F was employed together with primers 520R and 397R. PCR products were visualized on a 1.5% agarose gel.

Western blot

Supernatant harvested from infected Rhileki cells were centrifuged at 3,500 rpm and precipitated with 10% polyethylene glycol 8000 overnight in 4 °C. The solution was centrifuged at 12,000 rpm for 1 hour at 4 °C and pellet resuspended in NTE buffer (1M NaCl, 1 M Tris-HCl pH 8.0, 0.5 M EDTA pH 8.0). The suspension was subjected to a 20% sucrose cushion via ultracentrifugation using a SW41 rotor at 32,000 rpm for 2 hours at 4 °C. The pellet was resuspended in NTE buffer and reduced with 5x lane marker reducing sample buffer (ThermoFisher Scientific) at 95 °C for 10 minutes. Purified virions were separated down a 4 - 20% Mini-PROTEAN TGX stain-free protein gel (Bio-Rad Laboratories) at 90 volts for 90 minutes. The gel was then transferred onto a polyvinylidene fluoride membrane and blocked with 5% BSA at room temperature for 1 hour. This was followed by overnight probing with primary antibody at 4 °C and 1 hour with either anti-mouse or -rabbit horseradish peroxidase-linked IgG secondary antibodies. Blots were visualized using a CCD

imager detector (Bio-Rad Laboratories) after ECL detection (Amersham Biosciences). The primary antibodies include mouse monoclonal antibody 1E7 which is anti-229E nucleocapsid protein and polyclonal rabbit sera derived from immunization of rabbits with 229E spike protein (GenScript).

Protein modelling

The full genomic sequences of human coronavirus 229E C2R10 isolates were translated to amino acid sequences using the Expasy Translate webserver (<https://web.expasy.org/translate/>)⁴⁹ in all three forward 5' to 3' reading frames. These amino acid sequences were then aligned to the full-length sequences of 229E S and ORF4 proteins obtained from GenBank (accession number: NC_002645) using Clustal Omega webserver (<https://www.ebi.ac.uk/Tools/msa/clustalo/>)⁵⁰ to determine reading frames corresponding to each protein and deleted residues. The full-length structural models of human coronavirus 229E S protein was built using the SARS-CoV-2 S protein model as the template⁵¹. Modeller version 9.21⁵² was used to generate ten homology models and the best model was selected based on having the lowest discreet optimized protein energy⁵³, while minimizing Ramachandran outliers⁵⁴. Using similar protocols, the ORF4 protein model was built using the cryo-EM structure of SARS-CoV-2 ORF3a ion channel (PDB: 7KJR)⁵⁵ as the template. The N- and C-terminal regions were unresolved and therefore modelled as unstructured loops. Deleted regions of the C2R10 isolates were mapped to these structures and visualized with VMD⁵⁶.

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this paper.

318

319 **Data availability**

320 Data that support the findings of this study have been deposited in GenBank with accession
321 numbers XXXX and the SRA database under BioProject accession number XXXX.

322

323 **References**

- 324 1. Gorbalenya, A. E. *et al.* The species Severe acute respiratory syndrome-related
325 coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2. *Nat. Microbiol.* 5, 536–
326 544. *Link: <https://go.nature.com/3cW9qJR>* (2020).
- 327 2. Paules, C. I., Marston, H. D. & Fauci, A. S. Coronavirus Infections-More Than Just the
328 Common Cold. *JAMA* **323**, 707–708 (2020).
- 329 3. Nickbakhsh, S. *et al.* Epidemiology of Seasonal Coronaviruses: Establishing the Context
330 for the Emergence of Coronavirus Disease 2019. *J Infect Dis* **222**, 17–25 (2020).
- 331 4. Peiris, J. S. M. *et al.* Coronavirus as a possible cause of severe acute respiratory syndrome.
332 *Lancet* **361**, 1319–1325 (2003).
- 333 5. Ksiazek, T. G. *et al.* A novel coronavirus associated with severe acute respiratory
334 syndrome. *N Engl J Med* **348**, 1953–1966 (2003).
- 335 6. Zaki, A. M., van Boheemen, S., Bestebroer, T. M., Osterhaus, A. D. M. E. & Fouchier, R.
336 A. M. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N*
337 *Engl J Med* **367**, 1814–1820 (2012).
- 338 7. Zhu, N. *et al.* A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N Engl*
339 *J Med* **382**, 727–733 (2020).
- 340 8. Forni, D., Cagliani, R., Clerici, M. & Sironi, M. Molecular Evolution of Human
341 Coronavirus Genomes. *Trends Microbiol* **25**, 35–48 (2017).

9. Pfefferle, S. *et al.* Distant Relatives of Severe Acute Respiratory Syndrome Coronavirus and Close Relatives of Human Coronavirus 229E in Bats, Ghana. *Emerg Infect Dis* **15**, 1377–1384 (2009).
10. Lau, S. K. P. *et al.* Molecular Evolution of Human Coronavirus 229E in Hong Kong and a Fatal COVID-19 Case Involving Coinfection with a Novel Human Coronavirus 229E Genogroup. *mSphere* **6**, e00819-20 (2021).
11. Crossley, B. M., Mock, R. E., Callison, S. A. & Hietala, S. K. Identification and Characterization of a Novel Alpaca Respiratory Coronavirus Most Closely Related to the Human Coronavirus 229E. *Viruses* **4**, 3689–3700 (2012).
12. Sabir, J. S. M. *et al.* Co-circulation of three camel coronavirus species and recombination of MERS-CoVs in Saudi Arabia. *Science* **351**, 81–84 (2016).
13. Vijaykrishna, D. *et al.* Evolutionary insights into the ecology of coronaviruses. *J Virol* **81**, 4012–4020 (2007).
14. Ahn, M. *et al.* Dampened NLRP3-mediated inflammation in bats and implications for a special viral reservoir host. *Nat Microbiol* **4**, 789–799 (2019).
15. Menachery, V. D., Graham, R. L. & Baric, R. S. Jumping species—a mechanism for coronavirus persistence and survival. *Curr Opin Virol* **23**, 1–7 (2017).
16. Li, W. *et al.* Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* **426**, 450–454 (2003).
17. Hoffmann, M. *et al.* SARS-CoV-2 Cell Entry Depends on ACE2 and TMPRSS2 and Is Blocked by a Clinically Proven Protease Inhibitor. *Cell* **181**, 271-280.e8 (2020).
18. Hofmann, H. *et al.* Human coronavirus NL63 employs the severe acute respiratory syndrome coronavirus receptor for cellular entry. *Proc Natl Acad Sci U S A* **102**, 7988–7993 (2005).

- 366 19. Raj, V. S. *et al.* Dipeptidyl peptidase 4 is a functional receptor for the emerging
367 human coronavirus-EMC. *Nature* **495**, 251–254 (2013).
- 368 20. Yeager, C. L. *et al.* Human aminopeptidase N is a receptor for human coronavirus
369 229E. *Nature* **357**, 420–422 (1992).
- 370 21. Latinne, A. *et al.* Origin and cross-species transmission of bat coronaviruses in China.
371 *Nat Commun* **11**, 4235 (2020).
- 372 22. Zhang, R. *et al.* The ORF4a protein of human coronavirus 229E functions as a
373 viroporin that regulates viral production. *Biochim Biophys Acta* **1838**, 1088–1095 (2014).
- 374 23. Wolff, G. *et al.* A molecular pore spans the double membrane of the coronavirus
375 replication organelle. *Science* **369**, 1395–1398 (2020).
- 376 24. Li, Z. *et al.* The human coronavirus HCoV-229E S-protein structure and receptor
377 binding. *Elife* **8**, e51230 (2019).
- 378 25. Lu, X. *et al.* Spike gene deletion quasispecies in serum of patient with acute MERS-
379 CoV infection. *J Med Virol* **89**, 542–545 (2017).
- 380 26. McCarthy, K. R. *et al.* Recurrent deletions in the SARS-CoV-2 spike glycoprotein
381 drive antibody escape. *Science* **371**, 1139–1142 (2021).
- 382 27. Letko, M., Marzi, A. & Munster, V. Functional assessment of cell entry and receptor
383 usage for SARS-CoV-2 and other lineage B betacoronaviruses. *Nat Microbiol* **5**, 562–569
384 (2020).
- 385 28. Wells, H. L. *et al.* The evolutionary history of ACE2 usage within the coronavirus
386 subgenus Sarbecovirus. *Virus Evol* **7**, veab007 (2021).
- 387 29. Ren, W. *et al.* Difference in receptor usage between severe acute respiratory
388 syndrome (SARS) coronavirus and SARS-like coronavirus of bat origin. *J Virol* **82**, 1899–
389 1907 (2008).

30. Snijder, E. J. *et al.* A unifying structural and functional model of the coronavirus replication organelle: Tracking down RNA synthesis. *PLoS Biol* **18**, e3000715 (2020).
31. Klein, S. *et al.* SARS-CoV-2 structure and replication characterized by in situ cryo-electron tomography. *Nat Commun* **11**, 5885 (2020).
32. Mendonça, L. *et al.* Correlative multi-scale cryo-imaging unveils SARS-CoV-2 assembly and egress. *Nat Commun* **12**, 4629 (2021).
33. Ghosh, S. *et al.* β -Coronaviruses Use Lysosomes for Egress Instead of the Biosynthetic Secretory Pathway. *Cell* **183**, 1520-1535.e14 (2020).
34. Freeman, M. C., Peek, C. T., Becker, M. M., Smith, E. C. & Denison, M. R. Coronaviruses induce entry-independent, continuous macropinocytosis. *mBio* **5**, e01340-01314 (2014).
35. Plowright, R. K. *et al.* Ecological dynamics of emerging bat virus spillover. *Proc Biol Sci* **282**, 20142124 (2015).
36. Goh, G. *et al.* Complementary regulation of caspase-1 and IL-1 β reveals additional mechanisms of dampened inflammation in bats. *Proc Natl Acad Sci U S A* **117**, 28939–28949 (2020).
37. Zhou, P. *et al.* Fatal swine acute diarrhoea syndrome caused by an HKU2-related coronavirus of bat origin. *Nature* **556**, 255–258 (2018).
38. Low, J. G. H. *et al.* Early Dengue infection and outcome study (EDEN) - study design and preliminary findings. *Ann Acad Med Singap* **35**, 783–789 (2006).
39. Reed, L. J. & Muench, H. A Simple Method of Estimating Fifty Per Cent Endpoints. *American Journal of Epidemiology* **27**, 493–497 (1938).
40. Hoek, R. A. S. *et al.* Incidence of viral respiratory pathogens causing exacerbations in adult cystic fibrosis patients. *Scand J Infect Dis* **45**, 65–69 (2013).

41. Bolger, A. M., Lohse, M. & Usadel, B. Trimmomatic: a flexible trimmer for Illumina
sequence data. *Bioinformatics* **30**, 2114–2120 (2014).
42. Okonechnikov, K., Golosova, O., Fursov, M., & UGENE team. Unipro UGENE: a
unified bioinformatics toolkit. *Bioinformatics* **28**, 1166–1167 (2012).
43. Robinson, J. T. *et al.* Integrative Genomics Viewer. *Nat Biotechnol* **29**, 24–26 (2011).
44. Katoh, K., Misawa, K., Kuma, K. & Miyata, T. MAFFT: a novel method for rapid
multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res* **30**, 3059–
3066 (2002).
45. Pasternak, A. O., Spaan, W. J. M. & Snijder, E. J. Nidovirus transcription: how to
make sense...? *J Gen Virol* **87**, 1403–1421 (2006).
46. Viehweger, A. *et al.* Direct RNA nanopore sequencing of full-length coronavirus
genomes provides novel insights into structural variants and enables modification analysis.
Genome Res **29**, 1545–1554 (2019).
47. Schreiber, S. S., Kamahora, T. & Lai, M. M. Sequence analysis of the nucleocapsid
protein gene of human coronavirus 229E. *Virology* **169**, 142–151 (1989).
48. Raabe, T., Schelle-Prinz, B. & Siddell, S. G. Nucleotide sequence of the gene
encoding the spike glycoprotein of human coronavirus HCV 229E. *J Gen Virol* **71** (Pt 5),
1065–1073 (1990).
49. Gasteiger, E. *et al.* ExPASy: The proteomics server for in-depth protein knowledge
and analysis. *Nucleic Acids Res* **31**, 3784–3788 (2003).
50. Sievers, F. *et al.* Fast, scalable generation of high-quality protein multiple sequence
alignments using Clustal Omega. *Mol Syst Biol* **7**, 539 (2011).
51. Raghuvasi, P. V. *et al.* SARS-CoV-2 S protein:ACE2 interaction reveals novel
allosteric targets. *Elife* **10**, e63646 (2021).

- 438 52. Sali, A. & Blundell, T. L. Comparative protein modelling by satisfaction of spatial
439 restraints. *J Mol Biol* **234**, 779–815 (1993).
- 440 53. Eramian, D. *et al.* A composite score for predicting errors in protein structure models.
441 *Protein Sci* **15**, 1653–1666 (2006).
- 442 54. Ramachandran, G. N., Ramakrishnan, C. & Sasisekharan, V. Stereochemistry of
443 polypeptide chain configurations. *J Mol Biol* **7**, 95–99 (1963).
- 444 55. Kern, D. M. *et al.* Cryo-EM structure of SARS-CoV-2 ORF3a in lipid nanodiscs. *Nat*
445 *Struct Mol Biol* **28**, 573–582 (2021).
- 446 56. Humphrey, W., Dalke, A. & Schulten, K. VMD: visual molecular dynamics. *J Mol*
447 *Graph* **14**, 33–38, 27–28 (1996).
- 448

Acknowledgments

We acknowledge John A. Crump, Jenny G.H. Low, Venance P. Maro, Eng Eong Ooi, and Matt P. Rubach, for contributing patient specimens from which 229E viruses were isolated. We thank Edward C. Holmes and Raoul J de Groot for helpful comments and discussions.

Funding

This study was supported by the Duke-NUS Signature Research Programme funded by the Ministry of Health, Singapore, and grant NMRC/BNIG/2005/2013 from the National Medical Research Council. M.G.M. and D.H.W.L. are supported by the Integrative Sciences & Engineering Programme, NUS Graduate School, Singapore. F.S. and P.J.B were supported by BII (A*STAR) core funds.

Author contributions

ML and GJDS designed and supervised the research. ML, MGM, DHWL and ZY conducted experiments and performed genome sequencing. MGM, JJ and FYW performed genome assembly and annotation. ML, MGM, JJ, FYW, IHM, YCFS and GJDS performed the genome analysis and interpretation. FS and PJB conducted molecular modelling. ML, MGM and GJDS wrote the paper. All authors took part in data interpretation and edited the paper.

Competing interests

The authors declare no conflicts of interest.

Fig. 1. Rhileki cells are susceptible and permissive to 229E. **a**, Rhileki cells were inoculated with six 229E isolates at MOI = 0.01 and incubated for 6 days. Viral genome copies of the inoculum (circles) and at 6 days post inoculation (square) are plotted. The inoculum for subsequent passages was standardized based on copy number. **b**, Viral titers (TCID₅₀/ml) of C2, C2R10 and C2R10C1 viruses titrated in Caco2 cells. **c**, Viral titers of C2 and C2R10 viruses titrated in Rhileki cells. Black dashed lines represent the limit of detection.

Fig. 2. Schematic of nucleotide sequences within the spike and ORF 4 gene regions of 229E isolates at passages 1, 5, and 10 in Rhileki cells. Set of filled rectangles atop the figure is a genome schematic representing nucleotides within the spike and ORF 4 genes. RBD indicates the receptor-binding domain. Subsequent filled rectangles represent the genomes of different isolates present at the indicated passages, horizontal black lines indicate deleted parts of the genome with the respective size indicated by a Δ sign, and starts and ends of the deletions by nucleotide positions situated on top of the rectangles.

490 **Fig. 3. Sanger sequencing analysis of leader-body junction of sgmRNA 2 and 4. a,**
 491 Schematic of the leader-body junction of sgmRNA 2 and 4 layered with primer design
 492 strategy, nucleotide positions and expected sizes of products C2 and C2R10 viruses. **b,** Gel
 493 image of PCR products after amplification of sgmRNA 2 with primers 2F, 87R and 226R
 494 shown in panel A. Sequencing of PCR products confirmed the presence or absence of the
 495 canonical leader-body junction of sgmRNA 2 (162 bp). Gel image of PCR products after
 496 amplification of sgmRNA 2 and 4 with primers 2F, 397R and 617R shown in panel A.
 497 Sequencing of PCR products confirmed the presence of either the sgmRNA 4 (507 bp) or
 498 sgmRNA 2 canonical leader-body junction. **c,** Western blots of culture supernatants from
 499 viruses at passage 1, 5 and 10 on Rhileki cells incubated with 229E anti-spike (S) and anti-
 500 nucleoprotein (N).

Fig. 4. Genetic diversity of SG/2613/2011 spike gene across passages. **a**, Nucleotide sequences from position 21,059–21,100 and the corresponding amino acid translation within the S1 domain of the spike gene. Within this region wild type amino acid translation (turquoise) is GNFY–NE. Depending on the passage of the virus, the diversity is comprised of one to three uracil insertions at nucleotide position 21,064 that alter the reading frame. With one (orange) or two (dark green) uracil insertions the reading frame shifts leading to an early stop codon GNFL–Q* (E177*) and GNFFIAL* (L171*), respectively. At three (mustard) uracil insertions, the reading frame remains in-frame but with an additional phenylalanine GNFFY–NE. Double forward slash (//) indicates 8 amino acid residues not shown for clarity of presentation. **b**, Using next-generation sequencing data, pie charts describes the composition of uracil insertions for C2 (100% no insertions, n = 8,268), C2R5 (11.1% no insertions; 70.9% single insertion; 18% double insertion, n = 323) C2R10 (75.8% single insertion; 24.2% double insertion, n = 33), and C2R10C1 (11.2% no insertions; 88.8% triple insertion, n = 347) viruses.

Supplementary Figure 1. Schematic of virus passage experiments. Succession of the virus cultures in our study, nomenclature of viruses, and tests performed on individual batches.

Supplementary Figure 2. Microscopy and immunofluorescence of Caco2 and Rhileki cells infected with C2 or C2R10 viruses. a, Bright light and immunofluorescence microscopy of Caco2 cells infected with mock, C2, and C2R10 virus. **b,** Bright light and immunofluorescence microscopy of Rhileki cells infected with mock, C2, and C2R10 virus. 229E viral nucleocapsid staining (green) and nuclear staining (blue). White scale bar corresponds to 100 μm .

Supplementary Figure 3. Double-stranded RNA staining of Caco2 and Rhileki cells.

Representative images of Caco2 cells infected with C2 viruses and Rhileki cells infected with C2R10 viruses. Double-stranded RNA intermediates (green) and nuclear staining (blue) visualized at 1 day post inoculation. White scale bar corresponds to 20 μ m.

Supplementary Figure 4. Next-generation sequencing. Schematic on top of figure correspond to the location of the reads situated on the full genome. Coverage spanning the full genome for all six viruses passaged in Rhileki cells, quantified at C2R1, C2R5, and C2R10.

Supplementary Figure 5. Modelling of deleted regions of the spike and ORF4 proteins

of C2R10. a, Full-length model of 229E S protein built using SARS-CoV-2 S protein as a structural template. The model is shown in cartoon representation, with the deleted regions coloured in grey, and remaining S protein in cyan. **b**, Full-length model of ORF4 protein, built using the cryo-EM structure of the SARS-CoV-2 ORF3a ion channel (PDB: 7KJR).

Deleted regions are shown in grey and the remaining protein shown in purple. Domains are labelled as ECD (ectodomain), HR2 (heptad repeat 2 domain), TM (transmembrane domain), and CD (cytoplasmic domain).

Supplementary Figure 6. Strategy to detect 229E subgenomic mRNA 2 and 4. a,

Confirmation of the gel bands presented in Fig. 3a by Sanger sequencing show three leader-body junctions (LBJ) present, canonical LBJ of sgmRNA 2 (spike), canonical LBJ of sgmRNA 4 (ORF4), and non-canonical LBJ (putatively named sgmRNA X) observed only for SG/2326/2011. **b,** Schematic that explains the primer strategy and size explanation of the bands seen in Fig. 3a.

Supplementary Table 1. Primers used in this study.

Name	Directionality	Sequence	Usage
1197 P5 Del F	Forward	CGGCTGTGTTGGTCATTCG	Deletion verification
1197 P5 Del R	Reverse	GGCTGAGGTCTTTATCAATACG	Deletion verification
1340 P5 Del F	Forward	CGTATTCTTACCATAGC	Deletion verification
1340 P5 Del R	Reverse	AGATCTGCCACGGTGTGATC	Deletion verification
2326 P5 Del F	Forward	GCTCTGAGTTGCAAACCTATTGTGC	Deletion verification
2326 P5 Del R	Reverse	CGTGAAACTTCAGCACTAAC	Deletion verification
T140 P5 Del F	Forward	CTTGTTAGGAGTGGTAAGTTGC	Deletion verification
T140 P5 Del R	Reverse	GCAAGTTGAAGGTAACAGTGCC	Deletion verification
T140 P10 Del F	Forward	GCATATGCCTTGTTCATATTGC	Deletion verification
T140 P10 Del R	Reverse	CGCTAGCAAGTTGAAGGTAACAGTGC	Deletion verification
T140 Del 1 R	Reverse	GCACAACCCAGACCACG	Deletion verification
T4033 P5 Del F	Forward	CTCCATACTGTCTTGCTGC	Deletion verification
T4033 P5 Del R	Reverse	GCAGATCTGCCACGGTGTGAT	Deletion verification
T4033 P10 Del F	Forward	GGTTATATACCCTCCAACCTTGC	Deletion verification
T4033 P1-1Del F	Forward	AGACTGAGATGTGACCAGC	Deletion verification
T4033 P1-2Del F	Forward	TTCGTGCTTCCAGACAGCTTGC	Deletion verification
T4033 P1-1Del R	Reverse	GCATGGCGCTATTTCTTAAGGC	Deletion verification
T4033 P10Del2 F	Forward	CGCATAATGTTTGAACCACG	Deletion verification
2F	Forward	CTTAAGTACCTTATCTATCTACAGATA	sgmRNA 2/4 PCR 1/2
226R	Reverse	GCTGAAAACCTCCTCACAACACC	sgmRNA 2 cDNA
158R	Reverse	GCAAAGTTGGAGGGTATATAACC	sgmRNA 2 PCR 1
87R	Reverse	GCAAACAGAGTGACTAGTGTCGTC	sgmRNA 2 PCR 2
617R	Reverse	GCTGGGTGTTACAAAACAAGTATA	sgmRNA 4 cDNA
520R	Reverse	CGTACAAATCGTTAGTTGAGAG	sgmRNA 4 PCR 1
397R	Reverse	GCACATAGCAAAGTGTGGTTAC	sgmRNA 4 PCR 2

Supplementary Table 2. Synonymous and non-synonymous mutations in virus from passages 1, 5, and 10.

Isolate	Gene	Nucleotide mutation	Amino acid mutation	Mutation	C2	C2-R1	C2-R5	C2R10
SG/1340/2010	NSP3	A4854G	D621G	Non-synonymous	D	100% D	65.5% G	100% G
	NSP10	C12249T	T46I	Non-synonymous	T	47% T	64.0% I	100% I
SG/2326/2011	NSP3	A4854G	D621G	Non-synonymous	D	100% D	100% D	100% G
	NSP15	T19179G	-	Synonymous	T	100% G	95.0% G	100% G
	Membrane	C25177T	L54F	Non-synonymous	L	100% L	100% F	100% F
	Membrane	T25258C	F81L	Non-synonymous	F	100% F	100% F	100% L
SG/2613/2011	NSP8	C11462T	H88Y	Non-synonymous	H	100% Y	100% Y	100% Y
	Spike	C20603T	L5F	Non-synonymous	L	100% L	100% F	100% F
	ORF4	C24200T	Q30*	Non-synonymous	Q	100% *	100% *	100% *
	ORF4	G24423T	G104V	Non-synonymous	G	100% G	96.0% G	100% V
TZ/4033K/2017	NSP2	C2954T	-	Synonymous	C	100% T	100% T	100% T
	NSP3	C7074T	S1361F	Non-synonymous	S	100% F	100% F	100% F
	Membrane	C25177T	L54F	Non-synonymous	L	100% L	100% L	100% F
	Nucleocapsid	T26118C	V137A	Non-synonymous	V	100% V	97.1% V	100% A
UK/VR740/1973	NSP2	C712T	-	Synonymous	C	100% T	72.4% T	100% T
	NSP2	C1083A	S153Y	Non-synonymous	S	100% S	100% S	86.9% Y
	NSP3	A4907G	I634V	Non-synonymous	I	62.05% I	100% V	100% V
	NSP4	T7848C	M28T	Non-synonymous	M	51.7% T	100% T	100% T

*SG/1197/2010 was excluded due to low coverage